The dynamics of Th17 and Th1 cells during anti-TNF therapy in patients with inflammatory arthritis and relationship with treatment response

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Abstract

Anti-TNF agents have revolutionised the treatment of rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA), however a significant proportion of patients respond inadequately. Studies in murine and human arthritis have paradoxically shown that anti-TNF treatment can increase circulating Th17 and Th1 cells but the relationship of these changes to treatment response remains unclear. The aim of the work in this thesis was to conduct a prospective, longitudinal investigation of patients with inflammatory arthritis during anti-TNF treatment and using clinical, ultrasound and immunological assessments to gain an understanding of the immune correlates of treatment response. Patients with RA (n=25), AS (n=15) and PsA (n=8) were recruited and followed over the first 12 weeks of treatment. Improvement in validated disease activity scores defined treatment responders and non-responders. Power Doppler ultrasound (PDUS) provided a quantitative assessment of changes in synovial thickening and vascularity during treatment, with synovial vascularity showing faster and greater reduction with treatment than synovial thickening. PBMCs testing using IL17 and IFNγ ELISpot assays and flow cytometry consistently showed increased frequencies of circulating Th1 and Th17 cells in all three disease groups during anti-TNF therapy. Multiplex cytokine testing demonstrated a decrease in serum levels of proinflammatory cytokines and chemokines. Analyses of relationships between clinical, ultrasonographic and T-cell immunological changes revealed significant negative correlations between the increased frequency of Th1 and Th17 cells and reduction in synovial thickening and vascularity from baseline to 12 weeks on treatment in the RA group. Higher numbers of circulating Th17 cells at baseline in the RA group were associated with poorer anti-TNFα treatment response as defined by DAS28 score and ultrasonographic measures. This is the first study to link changes in T-cell immunopathology evaluated by cellular assays with morphological changes in arthritis assessed by PDUS during anti-TNFα treatment.
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Statement of Contribution

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# Abbreviations

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<td>ACPA</td>
<td>Anti-Citrullinated Peptide Antibody</td>
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<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>AE</td>
<td>AlloPhycocyanin</td>
</tr>
<tr>
<td>APC</td>
<td>A Proliferation Inducing Ligand</td>
</tr>
<tr>
<td>APRIL</td>
<td>Adverse Event</td>
</tr>
<tr>
<td>AS</td>
<td>Ankylosing Spondylitis</td>
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<tr>
<td>ASAS</td>
<td>Assessment of Spondyloarthropathy International Society</td>
</tr>
<tr>
<td>BASDAI</td>
<td>Bath Ankylosing Spondylitis Disease Activity Index</td>
</tr>
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<td>BASFI</td>
<td>Bath Ankylosing Spondylitis Functional Index</td>
</tr>
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<td>BASMI</td>
<td>Bath Ankylosing Spondylitis Metrology Index</td>
</tr>
<tr>
<td>BLyS</td>
<td>B Lymphocyte Stimulator</td>
</tr>
<tr>
<td>BSR</td>
<td>British Society of Rheumatology</td>
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<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
</tr>
<tr>
<td>CBA</td>
<td>Cyclic Citrullinated Peptide</td>
</tr>
<tr>
<td>CCP</td>
<td>complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CD</td>
<td>Cytometric Bead Array</td>
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<tr>
<td>cDNA</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CIA</td>
<td>Collagen-Induced Arthritis</td>
</tr>
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<td>CRP</td>
<td>C-Reactive Protein</td>
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<td>DAS</td>
<td>Disease Activity Score</td>
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<td>DKK-1</td>
<td>Dickkopf-related protein-1</td>
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<tr>
<td>DMARD</td>
<td>Disease Modifying Anti-Rheumatic Drug</td>
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<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>ELISpot</td>
<td>Enzyme-Linked Immunospot Assay</td>
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<tr>
<td>ERAP</td>
<td>Endoplasmic Reticulum Aminopeptidase</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
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<td>EULAR</td>
<td>European League Against Rheumatism</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FACIT-F</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<tr>
<td>FBC</td>
<td>Full Blood Count</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein IsoThioCyanate</td>
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<tr>
<td>FMO</td>
<td>Fluorescence Minus One</td>
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<tr>
<td>GUESS</td>
<td>Glasgow Ultrasound Enthesitis Scoring System</td>
</tr>
<tr>
<td>HAQ</td>
<td>Health Assessment Questionnaire</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-Inducible Factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>ICAM-1</td>
<td>Inter-Cellular Adhesion Molecule 1</td>
</tr>
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<td>IFNγ</td>
<td>Interferon gamma</td>
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<td>Interleukin</td>
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<td>IP-10</td>
<td>Interferon-Inducible Protein-10</td>
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<td>KIR</td>
<td>Killer cell Immunoglobulin like Receptor</td>
</tr>
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<td>LFT</td>
<td>Liver Function Test</td>
</tr>
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<td>MCP</td>
<td>Metacarpo-Phalangeal</td>
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<td>MCP-1</td>
<td>Monocyte Chemotactic Protein-1</td>
</tr>
<tr>
<td>MCP-4</td>
<td>Monocyte Chemotactic Protein-4</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP1β</td>
<td>Macrophage Inflammatory Protein 1β</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MSD</td>
<td>MesoScale Discovery</td>
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<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegrin</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDA</td>
<td>Power Doppler Area</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PDUS</td>
<td>Power Doppler Ultrasound</td>
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<tr>
<td>PE</td>
<td>PhycoErythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridin-Chlorophyll-Protein-Complex</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-Myristate Acetate</td>
</tr>
<tr>
<td>PsA</td>
<td>Psoriatic Arthritis</td>
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<tr>
<td>PsARC</td>
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<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>RANKL</td>
<td>Receptor Activator of NF-κB Ligand</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid Factor</td>
</tr>
<tr>
<td>RORC</td>
<td>Retinoic Acid-Related Orphan Nuclear Hormone Receptor C</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute cell media</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>SF-36</td>
<td>Short Form survey-36</td>
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<tr>
<td>SIJ</td>
<td>Sacroiliac Joint</td>
</tr>
<tr>
<td>SJC</td>
<td>Swollen Joint Count</td>
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<tr>
<td>SpA</td>
<td>Spondyloarthropy</td>
</tr>
<tr>
<td>spSFC</td>
<td>Specific Spot Forming Cells</td>
</tr>
<tr>
<td>STA</td>
<td>Synovial Thickness Area</td>
</tr>
<tr>
<td>STi</td>
<td>Synovial Thickness Index</td>
</tr>
<tr>
<td>STIR</td>
<td>Short Tau Inversion Recovery</td>
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<tr>
<td>TARC</td>
<td>Thymus-And Activation-Regulated Cytokine</td>
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<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TJC</td>
<td>Tender Joint Count</td>
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<td>TLR</td>
<td>Toll-like Receptor</td>
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<tr>
<td>TNFRI</td>
<td>Tumour Necrosis Factor alpha Receptor I (p55)</td>
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<td>TNFRII</td>
<td>Tumour Necrosis Factor alpha Receptor II (p75)</td>
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<td>TNFα</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>---------</td>
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<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
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<tr>
<td>VAS</td>
<td>Visual Analogue Scale</td>
</tr>
<tr>
<td>VASCi</td>
<td>Vascularity Index</td>
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<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Protein 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>Wnt</td>
<td>Wingless Family of Glycoproteins</td>
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Chapter 1. Introduction

1.1 Clinical features and aetiology of rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis

1.1.1 Rheumatoid arthritis (RA)

Chronic inflammatory arthritis is an umbrella term used to cover a wide range of joint diseases including rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA), which are characterised by uncontrolled immune responses that result in joint inflammation. RA is considered the prototype chronic inflammatory arthritis with features of autoimmunity, which targets the synovial lining of joints, bursae and tendon sheaths and also has extra-articular and systemic effects (Cope, 2008a). RA affects 0.5-1% of the population, it is three to four times more common in women and its prevalence rises with age (Silman and Hochberg, 1993). Synovial inflammation causes cartilage destruction and bone erosions leading to joint pain, deformity and progressive disability. Classically, RA tends to affect joints symmetrically and the small joints of the hands and feet, as well as the wrists and shoulders are most commonly involved. Patients often present with swelling, in addition to pain and tenderness of the affected joints. Swelling may arise as a consequence of the disease process affecting intra-articular structures, typically presenting with soft tissue swelling and joint fluid, or be due to swelling of the periarticular structures, including the joint capsule. However, RA is a heterogeneous disease and it can exhibit marked variations in clinical expression and in addition to the classical symmetrical polyarthritis, there are also oligoarticular presentations and those that predominantly affect large joints (Cope, 2008a). In addition to the generalised polyarthralgia in RA, common associated signs and symptoms can include systemic features, such as anorexia, weight loss and fatigue. The extra-articular manifestations of RA can affect almost any system in the body, although with the change in the treatment paradigm of RA to early and intensive suppression of disease activity, these are now less commonly seen (Cope, 2008a). However, other long term effects, such as cardiovascular disease, osteoporosis and lymphoma remain significant effects of RA (del Rincon I, 2003; Klareskog et al., 2009).

Diagnosis of RA is largely clinical, aided by routine laboratory haematological and biochemical tests, as well as immunological profiling for the presence of autoantibodies,
including IgM rheumatoid factors (RF) and anti-citrullinated protein antibodies (ACPA). RF are autoantibodies that recognise the Fc portion of IgG and distinguish between seropositive and seronegative RA (Shmerling and Delbanco, 1991). The presence of RF identifies those patients who are more likely to progress to erosive disease and who may have extra-articular manifestations (Shmerling and Delbanco, 1991). However, RF can be detected in up to 5% of healthy individuals and in 10-20% of the aging population, as well as in other rheumatic conditions (Cope, 2008a). ACPA have increased specificity compared to RF and can be present very early in the disease course of RA. ACPA positivity is associated with radiographic progression (van Venrooij WJ, 2004). Radiological investigations, such as plain radiographs showing erosive changes, high frequency ultrasound and MRI which have increased sensitivity compared to X-rays and clinical examination in detecting early as well as active synovitis can also aid the diagnosis of RA (Backhaus et al., 1999).

Classification criteria, such as the 1987 revised American College of Rheumatology criteria (Arnett et al., 1988) are a set of clinical and laboratory parameters which were established largely for epidemiological purposes and for use in clinical trials (Table 1.1). However, they are limited by poor sensitivity and specificity for the classification of patients with early inflammatory arthritis as having RA. The current effective and intensive treatment regimens for RA also can delay or avert a large proportion of patients fulfilling these criteria. As a result of these limitations, the American College of Rheumatology (ACR) and the European League Against Rheumatic Diseases (EULAR) devised new classification criteria for RA in 2010, focusing on features at earlier stages of RA, rather than defining the disease by its late features (Aletaha et al., 2010). These new classification criteria assess joint involvement, autoantibody status, acute phase response and symptom duration (Table 1.2).

The aetiology of RA is multi-faceted, with a longitudinal course and different phases. In genetically susceptible individuals, specific environmental and hormonal factors are thought to induce an immune response to a hitherto unidentified antigen and activate pathogenic immune reactions, including antibody formation. Years later, additional events such as infection or trauma have been proposed to contribute to disease development by directing immune reactions to the joints, causing joint inflammation and leading to clinical presentation of RA (Klareskog et al., 2009).
Table 1.1  The 1987 American College of Rheumatology classification criteria for rheumatoid arthritis

<table>
<thead>
<tr>
<th>The 1987 American College of Rheumatology classification criteria for rheumatoid arthritis*</th>
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<tbody>
<tr>
<td>1. Morning stiffness (at least 30 minutes)</td>
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<tr>
<td>2. Arthritis of three or more joint areas</td>
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<tr>
<td>3. Arthritis of hand joints (≥1 swollen joints)</td>
</tr>
<tr>
<td>4. Symmetrical arthritis</td>
</tr>
<tr>
<td>5. Rheumatoid nodules</td>
</tr>
<tr>
<td>6. Serum rheumatoid factor</td>
</tr>
<tr>
<td>7. Radiographic changes (erosions)</td>
</tr>
</tbody>
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*Four of these seven criteria must be present and criteria 1-4 must be present for ≥ 6 weeks for a diagnosis to be made
Table 1.2 The ACR/EULAR 2010 Classification Criteria for rheumatoid arthritis

ACR/EULAR 2010 criteria

*Diagnosis requires ≥6 out of 10 points*

1. Joint involvement (0-5)
   - One medium-to-large joint (0)
   - Two to ten medium-to-large joints (1)
   - One to three small joints (large joints not counted) (2)
   - Four to ten small joints (large joints not counted) (3)
   - More than ten joints (at least one small joint) (5)

2. Serology (0-3)
   - Negative RF and negative ACPA (0)
   - Low positive RF or low positive ACPA (2)
   - High positive RF or high positive ACPA (3)

3. Acute-phase reactants (0-1)
   - Normal CRP and normal ESR (0)
   - Abnormal CRP or abnormal ESR (1)

4. Duration of symptoms (0-1)
   - Less than 6 weeks (0)
   - 6 weeks or more (1)

Points are shown in brackets. Cutpoint for rheumatoid arthritis is 6 points or more.
Patients can also be classified as having rheumatoid arthritis if they have:
   i) typical erosions;
   ii) long-standing disease previously satisfying the classification criteria.
Findings of twin studies have estimated the relative contribution of genetic factors to be about 50% in RA (MacGregor et al., 2000). However, due to the clinically heterogeneous nature of RA, it has proven difficult to identify disease susceptibility genes. The most well characterised link is an association with major histocompatibility complex (MHC) Class II, specifically \( HLA-DRB1 \) alleles. These alleles were found to share a common amino acid sequence, termed the ‘shared epitope’ in the \( \beta \) chain of the HLA-DR molecule (Cope, 2008a). Theories linking the shared epitope to disease pathogenesis include that the shared epitope determines specific pathogenic peptide binding, or that the shared epitope influences T cell receptor recognition by binding and selecting autoreactive T cells during thymic maturation (Gregersen PK, 1987). \( PTPN22 \) is another confirmed susceptibility gene coding for a tyrosine phosphatase and thus having a role in T and B cell signalling (Gregersen, 2005). Other risk alleles for RA have been identified in gene regions containing TNF Receptor Associated Factor 1 (\( TRAF1, \) C5 locus), Signal Transducer and Activator of Transcription 4 (\( STAT4 \)), Cytotoxic T Lymphocyte Antigen 4 (\( CTLA4 \)), Peptidyl Arginine Deiminase type IV (\( PADI4 \)) and various cytokine and cytokine-receptor loci have also been implicated in disease association, but all these are thought to make only a small contribution to genetic susceptibility (Kurkó et al., 2013).

This leaves a substantial contribution for disease susceptibility to environmental and other factors. The most established environmental risk factor for RA is cigarette smoking. Smoking was shown in several studies to be a risk factor for the RF positive or ACPA-positive subset of RA and to have no or a very minor effect on the seronegative subset of patients (Harel-Meir et al., 2007). A major environmental interaction was noted between HLA-\( DR \) risk alleles and smoking in patients who were positive for RF or ACPA in 3 European (Klareskog et al., 2006; Linn-Rasker et al., 2006; Pedersen et al., 2007) and 1 North American study (Lee et al., 2007). Smoking promotes citrullination of self-proteins and may thus be directly linked to pathogenic auto-antigen driven immune responses (Klareskog et al., 2009).

For years there has been a presumed link between infection and autoimmunity through several proposed mechanisms, including an overactive host innate inflammatory response, host failure to terminate such a response, or through molecular mimicry between infective pathogens and self-peptides. However, no single pathogen or group of pathogens has been identified and linked to RA development (Carty et al., 2004).
Age and gender can also contribute to disease occurrence. Age-related changes may be due to immune senescence, resulting in the increased susceptibility to infection and increased reactivity to self-antigens (Cope, 2008a). The female preponderance of RA implies that hormonal factors may also play a role in its aetiology (Klareskog et al., 2009).

1.1.2 Ankylosing spondylitis (AS)

The spondyloarthropathies (SpA) are a group of chronic inflammatory arthritides that can affect the axial skeleton and peripheral joints and are characterised by the absence of rheumatoid factor. The group of diseases comprises AS (the prototypic form), psoriatic arthritis, reactive arthritis and arthritis associated with inflammatory bowel disease. The other links between these conditions are the association with HLA-B27 and the same pattern of peripheral and axial joint involvement (Sieper et al., 2006).

AS commonly affects the axial skeleton, resulting in inflammatory-type back pain, but it can also affect the peripheral joints and entheses, as well as have extra-articular manifestations, such as inflammatory bowel disease, uveitis and psoriasis (Tam et al., 2010). AS has a prevalence of 0.5-1% worldwide, it is more common in men and usually presents in the third to fourth decade of life (Sieper et al., 2006). AS is thought to affect the cartilage/bone interface, where inflammation, bone erosion and new bone formation all play a part in disease pathogenesis (Tam et al., 2010). Clinically, a patient with AS describes inflammatory-type spinal pain, characterised by pain and early morning stiffness, which improves with motion. In the majority of cases, the disease starts with a sacroiliitis. Further in the disease course, the whole spine can be affected with spondylitis, spondylodiscitis and arthritis of the small intervertebral joints. Outgrowths of bone, called syndesmophytes can develop in later stages of the disease, which can fuse together with the adjacent vertebral bodies, leading to a ‘bamboo spine’ severely restricting spinal motion. Involvement of the costovertebral joints initially leads to chest pain and later to restriction of chest expansion (Sieper, 2009). The pain and ankylosis in AS can therefore cause considerable disability. Peripheral arthritis, tending to affect medium-to-large joints, as well as enthesitis commonly of the lower limbs, can also occur in 40-60% of AS patients (Sieper, 2009).
Diagnosing AS is mainly a clinically driven process, based on the observation of clinical signs, aided by imaging modalities (de Vlam, 2010). Unlike RA, where synovitis of the joints and joint deformities can be evident clinically, spinal inflammation in the early stages of AS may not be present on plain radiographs, but be detectable by MRI (Sieper et al., 2009). Hence, imaging plays an important role in diagnosis and assessing treatment response in AS. MRI can assess acute inflammation presenting as bone oedema, whereas X-rays can assess the presence of long-term damage in the form of new bone formation, syndesmophytes and ankylosis (Maksymowych and Landewé, 2006). Classification criteria, including the modified New York criteria have been proposed to aid diagnosis (Table 1.3) (Linden et al., 1984). These have good sensitivity and specificity, however radiographic sacroiliitis is a requirement for making the diagnosis of AS, but these changes are the consequences of inflammation, rather than reflecting the presence of active inflammation. Since AS is a slowly progressing disease, definite sacroiliitis can take several years to develop, thus reaching a diagnosis of AS using the modified New York criteria may only capture established forms of the disease (Sieper, 2009). In addition, these criteria focus exclusively on axial features, excluding other clinically relevant features of the disease process (van Tubergen and Weber, 2012). Thus, a new set of criteria were developed in 2009 to facilitate the diagnosis of AS early, the Assessment of Spondyloarthitis International Society (ASAS) criteria (Sieper et al., 2009). These criteria include the presence of active inflammation as defined by MRI and combine several clinical and imaging parameters (Rudwaleit et al., 2009) (Table 1.4).

Susceptibility to AS has been estimated to be greater than 90% genetically determined (Brown, 2010). The association with HLA-B27 is the strongest; however despite intense research, its functional role in the pathogenetic process has not been clearly defined (Colbert et al., 2010). Although in most populations more than 80% of AS patients carry HLA-B27, only a small proportion of HLA-B27 carriers develop AS, hence additional genes must also be involved (Brown, 2010). Other loci have been identified through genome-wide association studies, including the endoplasmic reticulum aminopeptidase 1 (ERAP1) and interleukin-23 receptor (IL23R) amongst others (Brown, 2010).

Other contributing factors to AS aetiology have been proposed to be environmental factors, including infectious agents and mechanical stress (Tam et al., 2010). The association with
Table 1.3  The 1984 modified New York criteria for classification of ankylosing spondylitis

<table>
<thead>
<tr>
<th><strong>1984 Modified New York criteria for classification of ankylosing spondylitis</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Low back pain present for at least 3 months, improved by exercise and not relieved by rest</td>
</tr>
<tr>
<td>2. Limitation of the lumbar spine in sagittal and frontal planes</td>
</tr>
<tr>
<td>3. Chest expansion decreased relative to normal values for age and sex</td>
</tr>
<tr>
<td>4. Bilateral sacroiliitis grades 2 to 4</td>
</tr>
<tr>
<td>5. Unilateral sacroiliitis grades 3 to 4</td>
</tr>
</tbody>
</table>

*A definitive diagnosis of ankylosing spondylitis requires any of the first 3 criteria to be present plus either criterion 4 or 5*
### Table 1.4  The ASAS criteria for axial spondyloarthritis

#### The ASAS criteria for axial spondyloarthritis (SpA)

*In patients with ≥3 months back pain and age of onset <45 years*

**Sacroiliitis on imaging and ≥1 SpA feature OR HLA-B27 positive and ≥ 2 SpA features**

<table>
<thead>
<tr>
<th><strong>SpA features:</strong></th>
<th><strong>Sacroiliitis on imaging:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory back pain</td>
<td>Active (Acute) inflammation on MRI highly suggestive of sacroiliitis associated with SpA</td>
</tr>
<tr>
<td>Arthritis</td>
<td></td>
</tr>
<tr>
<td>Enthesitis (heel)</td>
<td>Definite radiographic sacroiliitis according to New York criteria</td>
</tr>
<tr>
<td>Uveitis</td>
<td></td>
</tr>
<tr>
<td>Dactylitis</td>
<td></td>
</tr>
<tr>
<td>Psoriasis</td>
<td></td>
</tr>
<tr>
<td>Crohn's/colitis</td>
<td></td>
</tr>
<tr>
<td>Good response to NSAIDs</td>
<td></td>
</tr>
<tr>
<td>Family history of SpA</td>
<td></td>
</tr>
<tr>
<td>HLA-B27</td>
<td></td>
</tr>
<tr>
<td>Elevated CRP</td>
<td></td>
</tr>
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</table>
infectious agents is due to the known trigger between bacteria and reactive arthritis and the association of AS with inflammatory bowel disease, in which the immune system can interact with local gut bacteria due to mucosal damage (Girschick HJ, 2008). Inflammation is a key feature in AS and the involved sites are synovium, entheses and bone. Synovitis alone does not explain all the features of the disease. With advances in MRI imaging, the concept of ‘the enthesitis organ’ as the main target tissue in AS has been proposed, which may explain many of the disease features, such as spinal disease and tendinitis, which occur in non-synovial areas (Benjamin et al., 2004). Biomechanical stress has been proposed to act as a trigger to enthesal inflammation, which in susceptible individuals could then lead to chronic arthritis (Benjamin et al., 2004; Benjamin et al., 2007). In addition, MRI also shows osteitis (bone marrow involvement) as a prominent feature in AS. Thus synovitis and osteitis could occur secondarily to chronic enthesal inflammation due the close anatomical relationship between these structures (Lories and Baeten, 2009).

1.1.3 **Psoriatic arthritis (PsA)**

PsA, another type of spondyloarthritis is a chronic inflammatory arthritis of unknown aetiology which is associated with psoriasis. It can affect the peripheral joints, where the synovium is the primary site of inflammation, as well as the spine and entheses. It has distinctive clinical features which differentiate it from RA, including the involvement of the distal interphalangeal (DIP) joints, an asymmetric distribution of the inflamed joints, the presence of dactylitis, enthesitis, sacroiliitis, spinal involvement and psoriasis (Gladman, 2009). Patients experience joint pain, swelling, restriction of mobility and fatigue. Joint destruction is often progressive, with almost 50% of PsA patients from an early arthritis clinic showing evidence of joint damage 2 years after first presentation, leading to significant disability (Kane et al., 2003). Radiological changes can present as erosive lesions or osteolysis, as well as new bone formation (Kuijk and Tak, 2011). Five clinical subtypes of PsA were originally described based on phenotype, including polyarthritis, asymmetric oligoarthritis, spondylitis, predominantly DIP arthritis and arthritis mutilans (Moll and Wright, 1973). However, these distinctions can be misleading as the disease pattern in an individual patient can change over time. Extra-articular manifestations aside from psoriatic skin lesions include ocular involvement presenting as conjunctivitis or iritis, aortic incompetence, as well as increased risk of cardiovascular disease. PsA affects men and
women almost equally and has a prevalence of 0.5% in the general population (Gladman, 2009). Up to 30% of patients with psoriasis also develop PsA, although it can also precede the skin condition in 15% of patients (Anandarajah and Ritchlin, 2009). The ClAssification Criteria for Psoriatic Arthritis (CASPAR) were developed in 2006 for use in groups of patients in the context of clinical research, although these criteria have also been used diagnostically (Table 1.5) (Taylor et al., 2006).

There is a clear genetic predisposition in PsA and similarly to AS, it is the class I MHC genes, that are involved (FitzGerald and Winchester, 2009). The HLA-C allele, \textit{Cw*0602} (psors 1) is a major determinant of psoriasis and PsA susceptibility, it is present in 60% of most series and is associated with earlier psoriasis onset and a later onset of PsA (Nograles et al., 2009). Of the HLA-B alleles, \textit{B*27} is associated with spinal involvement in PsA and \textit{B*38} and \textit{B*39} are associated with earlier onset of PsA more synchronous with the skin manifestations (Nograles et al., 2009). The presence of the RA shared epitope \textit{HLA-DRB1} in the HLA Class II region has been associated with the development of radiological erosions in PsA (Gladman, 2009). Recently the \textit{IL23R} has also been found to be associated with PsA and the same alleles associated with increased risk in PsA are also risk factors for AS and Crohn’s disease. In addition, the allele of the \textit{IL12p40} (\textit{IL12B}) gene has been implicated in PsA and this together with the \textit{IL23R} supports a role for Th17 cells in PsA pathogenesis (Nograles et al., 2009).

Environmental factors may also play a role in PsA aetiology. It has been proposed that infectious agents may trigger the psoriatic process and that the immunological response in patients with psoriasis and PsA may be the result of molecular mimicry between infectious antigens and epidermal autoantigens (Prinz, 2001). The exacerbation of psoriasis seen in the context of acquired immunodeficiency virus infection raises the possibility that HIV may play a role (Gladman, 2009). The observation that the activating killer immunoglobulin-like receptors 2DS1 and 2DS2 in the absence of the appropriate HLA ligand are associated with PsA, supports the notion that viruses may play a role in PsA development through lowering the threshold for natural killer cell and T cell activation (Martin et al., 2002). Trauma has also been proposed to play a role in PsA pathogenesis by initiating inflammation at the enthesis and the associated bone and adjacent soft tissue. Microtrauma at enthesal sites has been postulated to initiate aberrant repair processes and expose enthesal antigens.
Table 1.5  The CASPAR criteria for classification of psoriatic arthritis

The CASPAR criteria for classification of psoriatic arthritis

Inflammatory joint disease (joint, spine or entheséal) with ≥3 points from the following 5 categories:

1. Evidence of current psoriasis, a personal history of psoriasis or a family history of psoriasis
   Current psoriasis defined by skin or scalp disease
   Personal history of psoriasis obtained from patient or physician
   Family history of psoriasis in first or second degree relative

2. Typical psoriatic nail dystrophy including onycholysis, pitting and hyperkeratosis on current physical examination

3. A negative test for the presence of rheumatoid factor

4. Either current dactylitis, defined as swelling of entire digit, or a history of dactylitis recorded by a rheumatologist

5. Radiographic evidence of juxta-articular new bone formation on plain radiographs of hand or foot
that subsequently, in a genetically susceptible individual, can trigger downstream immune pathways (McGonagle et al., 2007).

1.2 Pathogenesis of rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis

1.2.1 Rheumatoid arthritis

The target tissue in RA is the synovium. Healthy synovium lines non-cartilaginous surfaces and provides essential nutrients to avascular structures, such as cartilage, tendons and bursae (Cope, 2008a). The normal synovium consists of a lining layer and a sublining layer. The lining layer is only a few cells thick and comprises 2 main cell populations, macrophages and fibroblast-like synoviocytes. The sublining layer, consisting of loose connective tissue, fat and blood vessels has considerable potential to undergo angiogenesis and accrue an inflammatory cell infiltrate. The autoimmune processes in RA are predicted to occur up to years prior to the clinical onset of the disease (Figure 1.1). Clinical onset is thought to be preceded by a pre-articular phase, with breach of self-tolerance, as exemplified by the presence of RF and ACPA (Majka et al., 2008). It is not known if a transitional event must then occur to lead to the development of clinically evident synovitis and features of RA disease. The molecular nature of such a transitional process is at present unclear, but it may be combination of immune permissive microvascular, neurological or biomechanical factors (McInnes and O'Dell, 2010). Clinically evident disease manifests within the joint with synovitis and in cartilage and bone with joint space narrowing and erosive changes respectively. After onset of clinical disease, the normally hypocellular synovial membrane becomes hyperplastic, comprising a superficial lining layer of synovial fibroblasts and macrophages, overlying an interstitial zone containing a dense cellular infiltrate, including synovial fibroblasts, macrophages, mast cells, CD4+T cells, CD8+T cells, natural killer (NK) cells, B cells and plasma cells (McInnes and Schett, 2007). The osteoclast-rich portion of the synovial membrane with villous-like projections is called pannus and can destroy bone, whereas enzymes secreted by neutrophils, synoviocytes and chondrocytes can degrade cartilage (Cope, 2008a) (Figure 1.2).

The initial onset of inflammation in the synovium has two major consequences. Firstly, it leads to the increased vascularity of the synovium and the development of perivascular
Figure 1.1  A conceptual framework for the events leading to the clinical presentation of rheumatoid arthritis

Autoimmune processes have been postulated to take place up to years before the clinical onset of rheumatoid arthritis, representing a ‘pre-articular phase’ of the disease. The trigger for the transition to the ‘articular phase’ corresponding to the development of clinical symptoms and signs is poorly defined, but may include infection or biomechanical events. After this, inflammation-driven pathogenesis occurs leading to joint destruction and systemic complications including cardiovascular disease and osteoporosis. CCP, cyclic citrullinated peptide; CTLA4, cytotoxic T lymphocyte antigen-4; GP39, cartilage glycoprotein 39; PADI4; peptidyl arginine deaminase type IV; PTPN22, protein tyrosine phosphatase non-receptor type 22. Reprinted by permission from Macmillan Publishers Ltd: McInnes IB, Schett G. Nat Rev Immunol 2007; 7: 449-44, Copyright 2007.
Figure 1.2  Adaptive and innate immune pathogenic processes occurring in the rheumatoid arthritis joint

Co-stimulation dependent interactions between T cells, dendritic cells and B cells are shown as occurring primarily in the lymph node; as a result of these processes an autoimmune response is generated including to citrulline-containing self-proteins. In the synovial membrane and adjacent bone marrow, adaptive and innate immune pathways integrate to promote tissue remodelling and damage. The chronic phase is driven by feedback loops mediated by interactions between leukocytes, synovial fibroblasts, chondrocytes, osteoclasts, together with the molecular products of damage.

ADAMTS, a disintegrin and metalloprotease with thrombospondin-1-like domains; DAMP, damage associated molecular pattern; Dkk-1, dickkopf-1; FcR Fc receptor; FGF, fibroblast
growth factor; GM-CSF, granulocyte macrophage colony stimulating factor; HA, hyaluronan; HSP, heat shock protein; IFN α/β, interferon α/β; MMP, matrix metalloproteinase, NLR, nucleotide-binding oligomerisation domain like receptor; PAMP, pathogen associated molecular pattern; PAR2, protease-activated receptor 2; PDGF, platelet derived growth factor; RANKL, receptor activator of nuclear factor κB ligand; TGF-β, transforming growth factor β; Th0; T helper type 0 cells; Th1, type 1 helper cell; Th17 cell, type 17 helper cell; TLR, toll-like receptor, TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor.

inflammatory infiltrates (Bresnihan B, 1999). Sustained activation of the microvascular endothelium also leads to upregulation of adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) (Haskard, 1995). With the production of chemoattractants from the synovial stromal cells, the second major consequence is that these changes lead to the adhesion and transmigration of mononuclear cells, contributing to the synovial hypertrophy and hyperplasia (Cope, 2008a). Synovial fibroblasts express a range of toll-like receptors (TLRs) and are very sensitive to inflammatory cytokines including TNFα, IL1 and IL6 and thus play a role in the acute phase of the initial innate immune response, which precedes the progression to a chronic inflammatory phase. Other key cell types involved in the innate immune response include macrophages, neutrophils and mast cells (McInnes and Schett, 2011). Macrophages produce pro-inflammatory cytokines, such as TNFα, IL6 and IL1, reactive oxygen species, matrix degrading enzymes and are also involved in antigen presentation (McInnes and Schett, 2011). Neutrophils contribute to synovitis by synthesising prostaglandins, proteases and reactive oxygen species, while mast cells produce vasoactive amines, cytokines, chemokines and proteases (McInnes and Schett, 2011).

These events all lead to a further cellular infiltrate of inflammatory leukocytes, including polymorphs and immature and undifferentiated monocytes, which secrete further inflammatory cytokines, contributing to the inflammatory milieu (Cope, 2008a; Gorman and Cope, 2008b). Further neovascularisation of the synovium promotes the influx of more inflammatory cells and angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and hypoxia-inducible factors (HIF) also play important roles in this process (Taylor PC, 2005). In established disease, the organisation of the synovial tissue appears to be suited to supporting differing patterns of cellular infiltrates, as well as facilitating the interactions between them (Cope, 2008a). The commonest form of synovitis comprises diffuse lymphocytic infiltrates. In about 40-50% of patients, more organised follicular structures may exist and in about 25% of patients, these follicular structures may include organised germinal centres, consisting of zones of proliferating B cells in addition to a distinct T cell zone. These structures can support B cell maturation and class switching thus promoting autoantibody production (Cope, 2008a).

There is good evidence to support a major role for T cells in the pathogenesis of RA. Flow cytometric analysis of dissociated synovial cell cultures indicates that infiltrating T lymphocytes can make up to 10-35% of the cells in inflamed tissue, with the ratio of CD4+ to
CD8+ T cells skewed in favour of the CD4+ subset. Similar enrichment of these T cell subsets is also seen in peripheral blood mononuclear cells (Cope, 2008b). The synovial tissue T cells have been shown to express phenotypic markers of antigen-experienced, terminally-differentiated T cells with an enhanced migratory potential (Cope, 2008b; Cush, 1988). The findings of reproducible levels of IFNγ and more recently, IL17 by subsets of synovial T helper cells has suggested the involvement of Th1 and Th17 cells in RA pathogenesis (Cope, 2008b; Miossec et al., 2009).

TNFα production in RA synovia has been shown to be T-cell dependent as synovial T cells from patients with RA have been demonstrated to stimulate TNFα production from PBMCs (Brennan et al., 2002). Thus, synovial T cells could contribute to synovitis directly through the production of proinflammatory cytokines, as well as via interactions with neighbouring macrophages and synovial fibroblasts, promoting their activation. In support of this, freshly isolated synovial T cells have been shown to induce TNFα and IL1β release from syngeneic macrophages in a cell-contact dependent manner (McInnes, 1997).

The association of RA with the shared epitope, as well as with PTPN22 and CTLA4 further supports the notion for a T cell contribution. PTPN22 codes for a protein tyrosine phosphatase, which regulates lymphocyte activation, whereas CTLA4 is implicated in the costimulation of T cells (McInnes and O'Dell, 2010).

Other supporting evidence for a role for T cells in RA pathogenesis comes from animal models of the disease. The SKG mouse model of RA has a loss of function mutation in the SH2 domain of the ZAP70 gene, encoding a protein tyrosine kinase essential for T cell receptor signalling. This leads to a failure in deletion of autoreactive T cells in the thymus and the mice develop a symmetrical, destructive polyarthritis. Adoptive transfer of SKG lymph node and splenic cells leads to arthritis in severe combined immunodeficiency disease (SCID) mice, supporting a role for T cells in initiating arthritis (Sakaguchi et al., 2003).

More recently, the beneficial effects of abatacept in RA, which is a recombinant CTLA-4Ig fusion protein inhibiting T cell activation by interrupting CD28-CD80/86 interaction and thus costimulation, lends further support to the role of T cells in RA disease pathogenesis as it supports a role for T cell costimulation and effector T cell activation in RA (Schiff, 2011).

T regulatory cells (Tregs) have also been implicated in RA pathogenesis and have been detected in the synovium of patients with active disease. However, Tregs derived from patients with active RA have been shown to be defective in their ability to suppress cytokine production and convey a suppressive phenotype to CD4+ effector T cells (Ehrenstein et al.,
The implication of this cell type in RA thus provides a crucial link between inflammation and the failure of immune regulation.

The precise site of differentiation of T cells into pathogenic effector cells in RA is not known. The inflammatory synovial milieu containing a variety of macrophage-derived and synovial fibroblast-derived cytokines including IL1β, IL6, IL12 and TNFα can support the expansion and differentiation of T cells. Dendritic cells may also contribute to T cell differentiation in the synovium through the production of IL12p70, IL23p19, IL15 and IL18 (Cope, 2008a).

The lack of therapeutic efficacy of anti-CD4 monoclonal antibodies in RA which were among the first specific therapeutic agents to target T cells (Choy et al., 1996) and the efficacy of the B-cell depleting therapy rituximab, has renewed interest in the role of B cells in RA pathogenesis (Edwards et al., 2004). The characteristics of RA which suggest an important role for B cells in its pathogenesis include the production of autoantibodies, the presence of B cells in the synovial microarchitecture, their ability to act as antigen-presenting cells, their ability to produce chemokines and inflammatory cytokines such as IL6 and that levels of B cell activators such as APRIL (a proliferation inducing ligand) or BLyS (B lymphocyte stimulator) have been shown to correlate with disease activity in RA (Gorman and Cope, 2008a). However, synovial T cell oligoclonality, germinal centre interactions and the production of autoantibodies suggest ongoing antigen-specific T-cell mediated B cell help and implicates a role for both cell types in RA (McInnes and Schett, 2011).

The enhanced expression of inflammatory cytokines is one of the hallmarks of RA and cytokines are implicated in each phase of the pathogenesis of RA by promoting autoimmunity, by maintaining chronic inflammatory synovitis and by driving the destruction of adjacent cartilage and bone. Explants of synovial mononuclear cells constitutively express inflammatory cytokines including IL1, IL6, IL8 and a vast array of chemokines and growth factors (Brennan and McInnes, 2008; Butler DM, 1995). Although there are anti-inflammatory and immune-regulatory cytokines including IL10, TGFβ and soluble TNF-receptors as well, studies have suggested that the overriding milieu is inflammatory (McInnes and Schett, 2007). For example, TNF bioactivity persists in spite of the upregulation of soluble TNF receptors in synovial fluid in patients with RA, suggesting that attempts to suppress the cytokine activity in the disease are insufficient (Cope, 2008a). The central roles of TNFα and IL6 in RA pathogenesis have been confirmed by the successful therapeutic
blockade of TNF\(\alpha\) and IL6 receptor in patients with RA (Feldmann and Maini, 2003; Nishimoto and Kishimoto, 2006). TNF\(\alpha\) plays a central role in RA pathogenesis through the activation of cytokine and chemokine expression, expression of endothelial-cell adhesion molecules, promotion of angiogenesis, suppression of Tregs and induction of pain, fever and cachexia (Feldmann et al., 1996). IL6 promotes T cell recruitment by regulating chemokine secretion, it drives autoantibody production, promotes angiogenesis and mediates systemic effects including anaemia and dysregulation of lipid metabolism (Nishimoto and Kishimoto, 2006).

Destruction of bone and cartilage in RA manifests as erosions and joint space narrowing respectively. Cartilage destruction in RA occurs when TNF\(\alpha\), IL1, IL6 and IL17 act synergistically to activate synoviocytes, resulting in the secretion of matrix metalloproteinases (MMP) from fibroblasts and macrophages into the synovial fluid. Cytokines also activate chondrocytes, leading to the direct release of additional MMPs and causing cartilage damage (Klareskog et al., 2009).

The development of bone erosions can occur through the activation of osteoclasts from macrophage-like precursors after stimulation by receptor activator of nuclear factor-\(\kappa\)B ligand (RANKL) and the inflammatory cytokines TNF\(\alpha\), IL1 and IL17 can also drive RANKL expression and its release from fibroblasts and osteoblasts. Activated T cells can also act directly on osteoclasts to activate them and all these processes lead to osteopenia of bone and the development of local bony erosions (Klareskog et al., 2009).

### 1.2.2 Ankylosing spondylitis

MRI and histopathological studies suggest that the primary target of the immune response in AS is at the cartilage/bone interface, including the insertion of tendons and ligaments into bone (the enthesis). The pathological mechanisms in AS include inflammation, bone destruction and new bone formation (Sieper, 2009). Studies on histological samples from the zygapophyseal, hip and sacroiliac joints from patients with AS have shown the presence of T cells, B cells, bone-marrow derived macrophages and osteoclasts (Poddubnyy et al., 2010).
As HLA-B27 transgenic rats develop a disease resembling spondyloarthritis with gut inflammation, skin lesions and ankylosed tails, it has been proposed that HLA-B27 itself may play a direct role in the disease pathogenesis (Hammer et al., 1990). There are 3 different hypotheses that propose a role for HLA-B27 in AS disease pathogenesis (Figure 1.3). The structure of HLA-B27 has 3 components: an HLA heavy chain that is associated with β2 microglobulin and a short peptide, called the antigenic peptide. The antigenic peptides are generated in the cytosol by proteasomes and are then transported to the endoplasmic reticulum, where they are trimmed by aminopeptidases to form a complex with the HLA heavy chain and β2 microglobulin (Colbert et al., 2010). One of these aminopeptidases is encoded by ERAP1, suggesting this step may play a role in the development of AS. A defective ERAP1 may lead to a slower rate of folding of HLA molecules, which in turn may trigger an intracellular signalling response in the endoplasmic reticulum, called the unfolded protein response (UPR). In macrophages, the UPR may lead to generation of IL23, which then leads to Th17 cell activation (Colbert et al., 2010). Occurrence of the UPR in vivo is supported by studies showing high UPR-marker expression in synovial biopsies (Tam et al., 2010).

An alternative hypothesis for the role of HLA-B27 in disease pathogenesis is termed the free-heavy chain hypothesis. On the cell surface, β2 microglobulin may dissociate from the HLA heavy chain, which is then termed the free heavy chain and these free heavy chains can interact to form homodimers. These homodimers can activate cell surface receptors on natural killer (NK) cells and T lymphocytes, termed the killer cell-Ig-like receptors, such as KIR3DL2 and activation of cell types bearing these receptors could trigger arthritis (Tam et al., 2010). KIR3DL2 has been shown to bind to homodimers expressed on the leukocytes of AS patients but to not to be able to recognise the normal HLA-B27/β2 microglobulin/peptide heterotrimeric complexes (Kollnberger et al., 2002). An expansion of B27 free heavy chain responsive-CD4+KIR3DL2+ cells has been reported in the peripheral blood and synovial fluid of AS patients. Moreover, antigen-presenting cells expressing B27 homodimers have been shown to promote the survival and stimulate the proliferation of superantigen-activated KIR3DL2+-expressing CD4+T cells producing IL17 (Bowness et al., 2011; Chan et al., 2005).

The third hypothesis of AS pathogenesis is the molecular mimicry hypothesis. The HLA class I complex engages CD8+ T cell receptors on reaching the cell surface. Self-tolerance may be lost in this interaction if an infectious agent activates the immune system through a peptide
Figure 1.3  HLA-B27 structures and hypotheses linking them to the pathogenesis of ankylosing spondylitis

HLA molecules are first generated as free heavy chains which are associated with β2 microglobulin and an antigenic peptide and then expressed on the cell surface as a trimer complex. These complexes interact with CD8+ T cells to initiate an immune response. A pathogen-derived peptide that mimics a self peptide could lead to an autoimmune response (the molecular mimicry hypothesis). HLA-B27 can also be expressed on the cell surface as homodimers of heavy chains only without β2 microglobulin. These heavy chains can activate NK cells, T and B cells (the heavy chain hypothesis). Incompletely assembled components of the HLA-B27 can lead to an unfolded protein response in the endoplasmic reticulum, activating Th17 cells and initiating IL23 production (the unfolded protein response hypothesis).
β2M, β2 microglobulin; IL, interleukin; KIRs, killer cell immunoglobulin-like receptors; LILRs, leukocyte immunoglobulin-like receptors; NK, natural killer; Th17, type 17 T helper cells.

that mimics a self-peptide. The activated T cells can then migrate to the joints, reactivate on encountering similar peptides and cause joint inflammation (Tam et al., 2010).

In biopsy samples from sacroiliac, facet and hip joints in patients with AS, dense cellular infiltrates of CD4+ and CD8+ T cells, B cells, macrophages, polymorphs and mast cells have been found, showing that both the innate and adaptive immune systems play a role in AS pathogenesis (Appel et al., 2006b; Braun et al., 1995; Noordenbos et al., 2012; Poddubnyy et al., 2010). Further information on the cell types involved in AS pathogenesis is available from animal models of disease, especially the HLA-B27 transgenic rat model. These rats develop arthritis and spondylitis responding to treatment by TNF blockade. T cells have been shown to play a role in AS pathogenesis as arthritis is absent in athymic animals, which lack these cells. It is also notable that arthritis in HLA-B27 transgenic rat models is transferrable by CD4+ T cells, but not by CD8+ T cells (Inman and El-Gabalawy, 2009). Dendritic cells have also been found to be abnormal in the HLA-B27 model, suggesting that CD4+T cells and dendritic cells are responsible for the generation of TNFα and other inflammatory cytokines in this animal model (Tam et al., 2010). Active inflammatory lesions in sacroiliac joints seen on MRI have been shown to correspond to a morphological substrate in the form of T cells and macrophages in the biopsy samples from AS patients. The numbers of these inflammatory cells were significantly higher in patients with active sacroiliitis on MRI in comparison with patients with less active disease or those with chronic advanced changes (Bollow et al., 2000).

Neoangiogenesis has also been shown to be a significant process in AS immunopathology. Microvessel density has been demonstrated to be significantly higher in AS patients, especially at sites with active inflammation, both at the bone/cartilage interface and in subchondral bone marrow in comparison with non-AS controls, indicating activation of neoangiogenesis as a result of chronic inflammation (Appel et al., 2006a; Appel et al., 2006b).

Histological studies have also shown increased expression of TNFα, IL6 and IL10 in AS (Poddubnyy et al., 2010). The efficacy of anti-TNF agents in AS and the presence of TNFα in the sacroiliac joints show that TNFα is one of the key cytokines responsible for inflammation in AS, however the stimulus to TNFα production and its role as a mediator in AS are still unclear (Tam et al., 2010).
Synovitis is less common in AS compared to RA, but many of the pathological features of inflamed synovia in AS have been shown to be similar to RA. A distinguishing feature however is the number of CD163+ macrophages (M2 regulatory phenotype) in AS, whereas in RA, the synovium is rich in M1 proinflammatory macrophages (Baeten et al., 2004a; Baeten et al., 2004b).

Other key processes in AS pathogenesis are bone destruction and bone formation. Unlike in RA, it seems that in AS bone erosion and syndesmophyte formation may not be completely coupled to inflammation, but the new bone formation may also be part of a physiological repair mechanism (Sieper, 2009). This is evidenced by the fact that anti-TNF, while effectively suppressing inflammation in AS still allows syndesmophyte formation to persist (Schett et al., 2007; van der Heijde et al., 2008). TNF\(\alpha\) causes bone resorption by inducing the expression of Dickkopf-related protein 1 (DKK-1), which leads to the suppression of the wingless (Wnt) pathway, which communicates signals for osteoblastogenesis and new bone formation (Diarra et al., 2007). Thus TNF blockers do not inhibit osteoproliferation, but stimulate new bone formation by removing the inhibitory effect of TNF\(\alpha\) on osteoblasts (Sieper, 2009). However, what is unclear is whether new bone formation can be prevented if anti-TNF is started early, before the occurrence of erosive structural damage.

AS has more of a remodelling phenotype compared to the destructive features in RA. Cathepsin-K and MMP-1 are among some of the bone destructive factors involved in AS, whereas RANKL or MMP-3 which are prominent in RA are not commonly expressed in AS-affected vertebrae. The mechanisms underlying bone formation in AS remain incompletely understood but may involve the Wnt signalling pathway which may be triggered by entheséal stress or microdamage (Lories and Baeten, 2009). In addition, skeletal expression of sclerostin and serum DKK1 levels, two inhibitors of bone formation have been shown to be low in AS patients which may also contribute to syndesmophyte formation (Appel et al., 2009; Daoussis et al., 2010).

Because of the heavy presence of innate immune cells in AS tissues, as well as the lack of autoantibodies, the lack of female preponderance and the fact that AS could be triggered by biomechanical stress and microbial pathogens, AS does not appear to exhibit the typical features of an autoimmune disease (Ambarus C, 2012). A concept has been proposed that AS may in fact have more of an autoinflammatory, rather than an autoimmune basis, as the
chronic inflammation in this condition may not be primarily driven by autoreactive T or B lymphocytes (McGonagle and McDermott, 2006). The heavy chain hypothesis and UPR hypothesis of HLA-B27 are independent of antigen presentation but related to the intrinsic biochemical properties of HLA-B27 and this also supports an autoinflammatory role for HLA-B27 (Ambarus C, 2012).

1.2.3 Psoriatic arthritis

The majority of PsA patients present with peripheral synovitis of the oligoarticular or polyarticular type, thus the synovium is the primary site of inflammation in PsA. The presence of susceptibility genes identifies the preclinical stage of the development of PsA. Once triggered, the immune process results in the development of two main features of PsA, an inflammatory cell infiltrate into entheses and synovium and the response of these target tissues to the inflammatory cell infiltrate (FitzGerald and Winchester, 2009).

Inflammation is the central pathological process present in the skin and joint lesions in PsA and the histological pattern of synovial inflammation while sharing some similarities with the other spondyloarthropathies, also shares cytokine and osteoclast promoting pathways with RA. The synovial tissue in PsA consists of a sublining infiltrate of T and B cells, vascular proliferation and a relatively thin layer of proliferating intimal synoviocytes (FitzGerald and Winchester, 2009). While there are no unique pathological hallmarks to distinguish PsA from other inflammatory arthropathies, histological studies have shown that PsA synovitis is markedly vascular, with dilated and tortuous blood vessels, which is similar to findings in AS. Synovial T cell and plasma cell numbers in PsA were lower than in RA, whereas the numbers of fibroblast-like synoviocytes and macrophages were similar to RA. The expression of TNFα, IL1β, IL6, MMPs and vascular adhesion molecules were also found to be as high as in RA (van Kuijk et al., 2006).

T and B cells infiltrate the PsA synovium, with both CD4+ and CD8+ T cell subsets of a memory phenotype present (Nogales et al., 2009) There are expansions of CD8+ T cell clones in PsA joint fluid and tissue, but these lack common structural motifs which would imply activation by an autoantigen and antigen-driven T cell responses may thus have a role in promoting on-going inflammation (Tassiulas et al., 1999). The activated T cells in the PsA joints contribute to the enhanced production of pro-inflammatory cytokines, including IL1β,
IL17, IFNγ and TNFα (van Kuijk et al., 2006). The role of T cells in disease pathogenesis is further supported in psoriasis and PsA by the beneficial effects of therapies targeting T cells, such as cyclosporine and alefacept (Kuijk and Tak, 2011). Moreover, clinical improvement with anti-TNF treatment in PsA was found to strongly correlate with a decrease in CD3+ T cells and CD4+ T cells in the synovial tissue after initiation of anti-TNF treatment, underscoring the importance of T cells in PsA (Pontifex et al., 2011; van Kuijk et al., 2009).

PsA is characterised in some patients by bone resorption, whereas ankylosis can occur in other joints. Lymphocytes and fibroblasts from PsA joints can induce osteoclastogenesis, triggering bone resorption via RANKL and TNFα (Gladman, 2009). However, other mediators also play a role since expression of RANKL and its decoy receptor osteoprotegerin (OPG) is not different between patients with PsA, those with non-PsA spondyloarthritis and those with RA and it does not seem to be significantly related to the degree of inflammation, nor is it modulated by anti-TNF blockade (Vandooren et al., 2008). Monocytes may contribute to erosive changes in PsA through production of MMPs and MMP1 and 3 have been demonstrated in PsA synovial tissue (Gladman, 2009).

The link between PsA and HLA-B27 and the involvement of the entheses in the disease process have prompted the proposal of an alternative model of PsA pathogenesis, suggesting it may exhibit autoinflammatory in addition to autoimmune features (McGonagle and McDermott, 2006). In this hypothesis, the enthesitis complex is proposed to play a role in pathogenesis, where high mechanical stress may initiate inflammation in the associated bone and soft tissues. However, while some patients display prominent enthesal involvement, others do not and thus this hypothesis may not explain all the disease features. On the basis of pathogenetic models, a unifying explanation may be that microtrauma at the enthesal sites may expose enthesal antigens, which subsequently in a genetically predisposed individual may trigger antigen-specific T cell responses (Haroon M, 2012).

1.2.4 Similarities and differences in the pathophysiology of RA and SpA

RA, AS and PsA are immune-mediated inflammatory diseases, which target the joints causing chronic inflammation and have the potential to cause significant tissue damage. Although inflammation is common to all three diseases, there are distinct differences between
them (Lories and Baeten, 2009). While genetic factors define susceptibility, in RA there are strong association with MHC Class II and PTPN22, whereas in SpA, there are associations with HLA-B27 and IL23R. Age and sex distribution is different; RA affects females more often, AS is more common in males, whereas in PsA, the prevalence may be similar in men and women. Localisation and disease course also differ, with RA affecting the synovium leading to inflammation and having a chronic disease course, whereas AS targets the cartilage/bone interface, where inflammation, bone loss and new bone formation coexist and the disease typically waxes and wanes (Schett, 2009). Although AS can also cause synovitis, especially in the peripheral joints, many of the manifestations of AS occur in the spine in the absence of synovial joints. RA and SpA thus differ in the interphase between inflammation and repair, with RA characterised by absence of repair, whereas AS is characterised by exaggerated repair mechanisms leading to ankylosis (Schett, 2009). Autoantibodies, such as RF and ACPA can be present in RA, but these are typically absent AS or PsA. In contrast to RA, innate immune cells such as neutrophils, macrophages and mast cells play a role in the inflammatory process in SpA, leading to the proposal that SpA has more of an autoinflammatory, rather than an autoimmune basis (Lories and Baeten, 2009; McGonagle and McDermott, 2006).

Despite these differences, the downstream inflammatory processes and mediators share similarities, as exemplified by the efficacy of anti-TNF therapies in all three diseases (Inman and El-Gabalawy, 2009). T cells have been shown to play a role in all three diseases. In RA, the CD4/CD8 T cell ratio is skewed in favour of the CD4+ subset and CD4+ cells are the predominant lymphocyte population in the synovium. In AS, despite the association with MHC Class I, there are abundant CD4+ T cells in sacroiliac and hip joints and in the rat model of the disease, AS is transferrable by CD4+ T cells, not by CD8+ T cells. In PsA, CD4+ and CD8+ T cells are present in the synovium and therapies targeting T cells have been shown to be clinically effective. Th17 cells have been shown to be present and play a role in the disease pathogenesis in RA, AS and PsA as discussed in detail below (Inman and El-Gabalawy, 2009).

An inflammatory cytokine milieu, characterised by the high expression of IL1, IL6, TNFα, as well as IL17 is central to the pathogenesis of all three diseases (Schett, 2009). Thus, although the relative contributions of the innate and adaptive immune responses may differ between RA and SpA, both types of conditions result in downstream chronic inflammation with
common mediators, suggesting autoinflammation and autoimmunity rather than being a dichotomous classification may in fact be part of a continuum (Ambarus C, 2012).

1.3 Th17 cells and their roles in inflammatory arthritides

Until recently, CD4+ T cells were thought to comprise two distinct subsets, Th1 and Th2, which develop from naïve T cells depending on the master transcription factors and polarising cytokine signals present. Th1 cells were the subset implicated to play a major role in autoimmune diseases (Mosmann and Coffman, 1989; Romagnani, 1995). However, the Th1/Th2 paradigm was challenged with the characterisation of a highly pro-inflammatory T helper cell subset, secreting interleukin 17A, called Th17 cells. Th17 cells rapidly initiate an inflammatory response dominated by neutrophils and have been shown to play a role in clearing infections with pathogens, such as *Klebsiella pneumonia*, *Mycobacterium tuberculosis* and *Candida albicans*, as well as playing a role in chronic inflammation and autoimmunity (Korn et al., 2009; Miossec et al., 2009; Tesmer et al., 2008).

IL17A, referred from now on as IL17 is the founding member of the IL17 family, which consists of six ligands, IL17A-F and IL17A is the signature cytokine of Th17 cells (Miossec, 2007). The first evidence for an inflammatory role of IL17 came from the observation that IL17 added to primary cultures of human RA synovial fibroblasts induced the expression of IL6 and IL8 (Fossiez et al., 1996). These results linked IL17 to inflammation through the production of IL6 and to neutrophil biology through the production of IL8. Synovial explants from patients with RA were shown to produce functional IL17 and IL17-specific antibody added to synovial cell cultures reduced the high levels of IL6 production, showing IL17 had a role in chronic inflammation (Chabaud et al., 1999). Subsequently, a number of studies in animal models of autoimmune diseases have showed that IL17 and Th17 cells contribute to disease pathogenesis (Korn et al., 2009). The unexpected discovery that mice deficient in IFNγ or IFNγ receptor were not resistant to experimental autoimmune encephalomyelitis (EAE) but were in fact more susceptible to it also challenged the concept of a Th1 association with autoimmune diseases (Annunziato and Romagnani, 2009; Willenborg et al., 1996). The link with IL12 in these diseases was further called into question by the discovery that IL23 shares the p40 subunit with IL12, as IL23 is composed of a p40 and a p19 subunit, whereas IL12 is composed of a p40 and a p35 subunit (Oppmann et al., 2000). IL12 and IL23
cytokines are involved in Th1 and Th17 cell differentiation respectively. Mice deficient in IL12p40 were shown not to exhibit arthritis and this was initially attributed to an ablation of Th1 cells. However, subsequent studies showed that collagen-induced arthritis (CIA) and EAE did not develop in mice deficient for the IL23p19 subunit, but the diseases developed in mice deficient for the IL12p35 subunit, suggesting IL23 and not IL12 is linked to autoimmunity (Cua et al., 2003; Murphy et al., 2003). Another study in a Th17-cell dependent model of RA, the IL1RA -/- mouse which develops spontaneous arthritis, showed development of more severe disease after application of IL23 (Ju et al., 2008). Subsequently, several studies have shown that blockade of the IL17 receptor or antibody neutralisation of IL17 reduces severity of CIA, whereas inflammation and autoimmunity are induced by injecting IL17-producing T cells (Langrish et al., 2005). These studies have established that Th17 cells are a distinct subset of T helper cells and have proposed a role for Th17 cells in immunopathology (Harrington et al., 2005; Korn et al., 2009).

The main features of human Th17 cells include surface expression of IL23R, CD161 and chemokine receptors CCR6 and CCR4, as well as expression of the transcription factor retinoic acid-related orphan nuclear hormone receptor C (RORC) and the secretion of cytokines IL17A, IL17F, IL21, IL22 and the chemokine CCL20 (Korn et al., 2009). The origin and factors involved in Th17 differentiation in humans have been somewhat contentious, especially with respect to the role of transforming growth factor β (TGFβ) (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007). Recent studies support a role for TGFβ in Th17 development, as an inflammatory cytokine milieu with macrophage-derived and dendritic-cell derived IL1β, IL6, IL21 and IL23 allows TGFβ together with additional cytokines such as IL6 to promote Th17 differentiation and inhibit Treg development (Manel et al., 2008; Volpe et al., 2008). Subsequently, activation of RORC occurs which together with other transcription factors allows surface expression of IL23R and induces transcription of the IL17 gene in naïve T cells. Th17 cells then become responsive to IL23 which is essential for stabilising and maintaining the Th17 phenotype and further enhances IL17 expression, as well as IL22 production. Mature Th17 cells also produce IL21, which together with TGFβ amplifies Th17 cell differentiation in an autocrine loop (Figure 1.4) (Korn et al., 2009; Miossec et al., 2009). TGFβ may also act indirectly to promote Th17 differentiation by inhibiting Tbet expression and therefore development of Th1 cells (Annunziato et al., 2009a).
Figure 1.4  Differentiation of human Th17 cells

The current concepts in Th17 differentiation in humans are shown- key cytokines are TGFβ, IL6, IL21 and IL23. In addition to the classic Th1 and Th17 phenotypes, a mixed Th1-Th17 phenotype has also been described.

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Most parenchymal cells express IL17 receptors and thus IL17 contributes to most of the pathophysiological features of inflammatory arthritis (Figure 1.5). IL17 has pleiotropic effects on effector cells of the immune system and induces production of pro-inflammatory cytokines, such as TNFα, IL1β, IL6 by macrophages and is strongly synergistic in its actions with these cytokines. It also contributes to cartilage and bone damage in inflammatory arthritis by acting on synovial fibroblasts to release matrix metalloproteinases to break down cartilage and it induces RANKL which increases osteoclast differentiation and leads to bony erosions (Annunziato et al., 2009b; Miossec et al., 2009; Tesmer et al., 2008). IL17 has also been shown to play a role in angiogenesis in inflammatory arthritis (Pickens et al., 2010).

In addition to Th17 cells, other cellular sources of IL17 have been shown to include neutrophils, mast cells, natural killer cells and γδT cells which produce IL17 in response to stress, injury or pathogens and can also initiate IL17-dependent immune responses (Cua and Tato, 2010).

### 1.3.1 Th17 cells in RA

Functional IL17 was firstly shown to be produced by synovium explants from patients with RA and to be able to synergise with TNFα to induce IL6 production (Chabaud et al., 1999). In human RA, IL17 is thought to contribute to neutrophil recruitment to the synovium through induction of IL6 and IL8 production by fibroblasts, endothelial and epithelial cells (Fossiez et al., 1996). CCL20 is the most abundantly expressed gene following IL17 activation of synoviocytes and it is further induced by TNFα. CCL20 then contributes to further synovitis by attracting immature dendritic cells and Th17 cells through its receptor CCR6 expressed on their cell surface (Hirota et al., 2007; Korn et al., 2009). CCL20 concentration in synovial fluid and tissue is markedly higher than serum levels and correlates with the degree of synovial intimal lining hyperplasia, supporting a chemotactic role for CCL20 (Hirota et al., 2007; Melis et al., 2010). Thus, Th17 cells may play an important role from the early stages of RA.

Increased frequencies of Th17 cells and IL17 levels have been found in the peripheral blood of RA patients compared to healthy controls or osteoarthritis patients and Th17 cells are further enriched in RA synovial fluid and synovial tissue, where their levels correlate with inflammatory markers (Chabaud et al., 1999; Gullick et al., 2010; Leipe et al., 2010; Shen et al., 2009). Increased Th17 cell frequencies have been shown both in RA patients with early disease who are treatment naïve and in patients with established disease on various treatment
Figure 1.5  Pleiotropic effects of Interleukin-17 on different cell functions and its role in pathophysiology of various diseases.

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regimens (Leipe et al., 2010). Histological studies have also shown the presence of IL17 in T cell rich areas of the synovium (Chabaud et al., 1999; Tesmer et al., 2008). Furthermore, in a 2 year prospective study of RA patients, the expression of TNFα, IL1 and IL17 by synovial cells directly correlated with joint damage progression, showing that IL17 may also contribute to arthritis chronicity. In contrast, IFNγ expression was negatively correlated with joint damage progression (Kirkham et al., 2006).

Clinical trials with anti-IL17 monoclonal antibodies in RA are underway and have demonstrated clinical efficacy in early phase studies, although the proportion of responders at ACR categorical response levels at the doses currently used in these studies has been relatively less than has been observed in historical trials of anti-TNF agents (Genovese et al., 2012; Genovese et al., 2010).

1.3.2 Th17 cells in PsA and psoriasis

A role for Th17 cells in psoriasis and psoriatic arthritis is also postulated as polymorphisms in the IL23R and IL12p40 genes are associated with psoriasis and PsA (Nograles et al., 2009). Significantly increased levels of Th17 cells have been found in the peripheral blood of patients with PsA, as well as in psoriatic plaques (Jandus et al., 2008; Lowes et al., 2008; Pene et al., 2008). IL23 is overproduced by keratinocytes and dendritic cells in psoriatic skin lesions and intra-dermal injection of IL23 induces hyperkeratosis in mice (Korn et al., 2009). The expression of CCR6 and CCL20 is upregulated in psoriatic plaques and synovial fluid from PsA patients (Melis et al., 2010; Tesmer et al., 2008). Mast cells and neutrophils represent additional sources of IL17 in skin affected by psoriasis (Lin et al., 2011).

The most distinct evidence for a role of the IL23/Th17 pathway comes from clinical studies. Anti-TNF treatment with etanercept has been shown to ameliorate psoriasis and this correlated with reduction in Th17 responses through reduced production of IL23, IL17 and CCL20, as well as a decrease in Th1 responses through a reduction in IFNγ in psoriatic plaques (Zaba et al., 2007). To further support the role of Th17 cells in psoriasis and PsA, treatment with ustekinumab, a monoclonal antibody against the p40 subunit shared between IL12 and IL23 has shown significant improvements in patients with moderate to severe psoriasis and in PsA (Griffiths et al., 2010; Mease, 2011b). Anti-IL17 monoclonal antibodies are in clinical trials for patients with PsA and early phase studies are showing promising results (McInnes et al., 2013).
1.3.3 Th17 cells in AS

The association of the IL23R with AS was the first evidence that linked the IL23/Th17 pathway to AS pathogenesis (Brown, 2010). Increased frequencies of Th17 cells have been found in the peripheral blood of patients with AS compared to healthy controls (Jandus et al., 2008; Shen et al., 2009; Xueyi et al., 2012) and increased levels of IL17 have been detected in synovial fluid from AS patients (Melis and Elewaut, 2009; Wendling et al., 2007). Histological studies in bone biopsy samples from facet joints have shown positive staining for IL17 (Appel et al., 2011; Noordenbos et al., 2012). While CD3+ T cells were found to be positive for IL17, IL17-positive neutrophils and mast cells have also been shown to be major IL17-expressing cell populations in AS, suggesting that cells of the innate immune system make an important contribution to IL17 production and synovial inflammation in AS (Noordenbos et al., 2012).

In vitro studies have suggested that endoplasmic reticulum stress and the UPR polarize macrophages to produce more IL23, which could in turn increase Th17 responses, thus supporting a role for the Th17 pathway in AS pathogenesis (Colbert et al., 2010). HLA-B27 homodimers have also been shown to be able to interact with KIR3DL2-expressing CD4+ T cells which can produce IL17, stimulating their proliferation and expansion, thereby also linking HLA-B27 with IL17 production (Bowness et al., 2011).

Clinical trials with anti-IL17 monoclonal antibodies are in progress and early results appear to be very promising, showing a rapid and marked reduction in AS disease activity (Baeten et al., 2013).

1.4 Treatment of inflammatory arthritis

The therapeutic armamentarium available to treat inflammatory arthritis has expanded significantly in recent years and comprises synthetic and biological disease modifying anti-rheumatic drugs (DMARDs), together with analgesics and non-drug therapies. The treatment strategy for the management of inflammatory arthritis has also changed with the aim of early diagnosis and treatment according to a ‘treat to target‘ approach using combination drug treatments with the goal of achieving low disease activity or inducing remission and preventing joint damage (Smolen et al., 2010).
Synthetic DMARDs are a heterogeneous group of agents comprising methotrexate, sulfasalazine, leflunomide and hydroxychloroquine which form the mainstay of treatment in inflammatory arthritis, but whose diverse mechanisms of action remain incompletely understood (Donahue et al., 2008). They all act to reduce joint swelling and pain, limit progressive joint damage and improve function. The efficacy of these agents has been established in placebo-controlled clinical trials (Donahue et al., 2008; Tugwell et al., 2000). Methotrexate is the most commonly used DMARD in RA, either as monotherapy, or more frequently as combination therapy. Combination DMARDs are recommended early in the disease course of RA, preferably within the first 3 months of onset, to take advantage of the ‘window of opportunity’ to gain early control of disease activity and limit tissue damage (Smolen et al., 2010). Various DMARD combinations are used in RA clinical practice, commonly methotrexate and hydroxychloroquine or ‘triple therapy’ with methotrexate, hydroxychloroquine and sulfasalazine (Choy et al., 2005).

Methotrexate has also been used for the treatment of PsA for many years. Leflunomide is also clinically effective for both peripheral arthritis and psoriasis with response rates up to 59% and concurrent improvements in function and quality of life (Ash et al., 2012).

Non-steroidal anti-inflammatory drugs (NSAIDs) are the first choice of therapy in AS with proven efficacy on clinical symptoms, as well as a possible disease modifying effect through the inhibition of new bone formation (Wanders et al., 2005). In AS, the use of DMARDs (sulfasalazine or methotrexate) is mainly for the control of peripheral synovitis, as these agents have minimal effects on axial symptoms or signs (Braun and Baraliakos, 2009).

However, DMARDs are ineffective in their ability to adequately control disease activity and symptoms in a proportion of patients with inflammatory arthritis and these agents also have significant side effects, which can range from mild (nausea) to more severe (hepatotoxicity, blood dyscrasias and interstitial lung disease) (Gullick, 2012).

Corticosteroids have been used for many years in RA. In the short term they reduce synovitis and improve signs and symptoms of arthritis. They reduce joint damage progression in early RA and are recommended in combination with DMARDs in early disease (Scott et al., 2010). However, short term use is recommended due to their significant side effects. Their use in PsA is less frequent, due to the risk of flares of skin disease on withdrawal (Mease, 2011b).
Systemic corticosteroids are not used for long-term therapy in AS, although intra-articular injections may be helpful in patients with a mono or oligo peripheral arthritis (Braun and Baraliakos, 2009).

The improved understanding of the molecular mechanisms of inflammation and cytokine involvement in inflammatory arthritis pathogenesis has led to the development of biologic agents which have revolutionised the treatment of inflammatory arthritides. Anti-TNF therapies were the first biologic agents to enter routine use for RA and shortly after for AS and PsA (Brandt et al., 2000; Elliott et al., 1994; Feldmann and Maini, 2003; Maini et al., 1998). Anti-TNF therapies (infliximab, etanercept, adalimumab, certolizumab and golimumab) currently include monoclonal antibodies, a TNF receptor fusion protein and a pegylated Fab' fragment (Taylor and Feldmann, 2009). In the majority of patients with RA, these agents have a profound effect on symptoms and also slow radiographic progression (Singh JA, 2009). Similarly, in PsA anti-TNF treatment leads to improvement in signs and symptoms of arthritis, dactylitis, enthesitis, skin and nail disease and treatment has a significant impact on radiographic progression (Mease, 2011b; Schett et al., 2007; van der Heijde et al., 2007). AS patients experience benefits at any stage of the disease, both in axial and extra-spinal manifestations, although the effects seem to be greatest in early disease (Braun and Kalden, 2009).

Despite the success of anti-TNF therapy in the majority of patients, around 20-30% of patients do not respond to this treatment. In addition, some patients experience side effects, such as injection site reactions, reactivation of tuberculosis or increased susceptibility to infections, some of which may be severe (Ding et al., 2010; Singh JA, 2009).

The success of anti-TNF agents has also led to the development of other biologic agents, including B cell depleting therapy (rituximab), T cell costimulatory blockade (abatacept) and anti-IL6 receptor blockade (tocilizumab) which are all currently licensed for use in RA patients who have failed anti-TNF therapy (McInnes and O'Dell, 2010). In PsA and AS, anti-TNF is currently the only type of biological therapy licensed for use, although costimulatory blockade with abatacept, anti-IL17 antibody and anti-IL12/23 p40 antibodies are currently in clinical trials showing promising results (Baeten, 2010; Genovese et al., 2012; Griffiths et al., 2010; McInnes et al., 2013).
1.5  Anti-TNF therapy for inflammatory arthritis

1.5.1  Rationale for use of anti-TNF therapy in inflammatory arthritis

TNFα has been shown to be a ‘master regulator’ in the complex cytokine network underlying the inflammatory processes in inflammatory arthritides (Feldmann and Maini, 2003). It is a pleiotropic cytokine whose amplified and dysregulated production in inflammatory arthritis contributes to pathogenesis in a number of ways, including synovial proliferation, production of metalloproteinases, adhesion molecule expression, angiogenesis, as well as through the regulation of other proinflammatory cytokines. It also mediates some of the systemic effects of inflammatory arthritis, including fatigue, fever and cachexia (Feldmann and Maini, 2008).

TNFα and its two receptors are expressed within the synovial membrane and at the cartilage/pannus junction in patients with inflammatory arthritis (Chu et al., 1991). High concentrations of TNFα have been demonstrated in synovial fluid, synoviocytes and synovial macrophages of patients with RA (Cope et al., 1992; Saxne et al., 1988). In patients with PsA, high levels of TNFα have been found in the synovial fluid and tissue, as well as in psoriatic lesional skin (FitzGerald and Winchester, 2009). TNFα upregulation has also been demonstrated in biopsy samples from sacroiliac joints, zygapophyseal and hip joints of patients with AS (Appel et al., 2006a; Appel et al., 2006b).

In vitro experiments using an RA synovial cell culture system in which there is spontaneous production of many pro-inflammatory cytokines without external stimulation, showed that neutralisation of TNFα down-regulated the production of IL1, IL6, IL8 and GM-CSF. This was not observed in synovial cultures from patients with osteoarthritis. Moreover, blockade of IL1 resulted in reduced production of IL6 and IL8, but not TNFα, which suggested that TNFα plays a central role in the cytokine network (Brennan, 1989; Buchan, 1988; Butler DM, 1995). The first demonstration of the importance of TNFα in vivo was provided by animal studies. Injection of TNFα in animals resulted in synovitis, with infiltration of lymphocytes, monocytes and neutrophils in the articular cavity (Thorbecke et al., 1992). Systemic administration of antibodies blocking TNFα or soluble TNF receptor:Fc fusion proteins before or after disease onset in the collagen-induced arthritis model of RA led to amelioration of joint disease (Williams et al., 1992). In another mouse model of RA engineered to express human TNFα, the onset of destructive polyarthritis could be prevented...
by the administration of monoclonal antibodies to human TNFα (Keffer, 1991). These observations led to the conclusion that TNFα was a leading therapeutic target in RA and provided the rationale for clinical trials of TNF blocking agents, initially in patients in RA and subsequently in patients with AS and PsA.

1.5.2 Evidence from clinical studies for use of anti-TNF in inflammatory arthritis

The chimeric anti-TNF monoclonal antibody (infliximab) was first used to treat RA and the soluble TNF receptor fusion protein (etanercept) and human monoclonal antibody (adalimumab) were tested subsequently (Taylor and Feldmann, 2009). An initial open-label and later, a randomised placebo-controlled trial using infliximab as a single agent in RA demonstrated a significant and substantial improvement of signs and symptoms of the disease in a dose-dependent manner (Elliott M.J., 1993; Elliott et al., 1994). The clinical trials of etanercept and adalimumab demonstrated that TNF blockers are more effective when combined with methotrexate (Breedveld et al., 2006; Klarskog et al., 2004; Lipsky et al., 2000) and more recently, other studies demonstrated that other combinations of biologics with DMARDs are also effective in treating RA (Burmester et al., 2007). The two newest TNF blockers, certolizumab and golimumab have also shown comparable efficacy to the other anti-TNF agents in patients with RA (Weinblatt et al., 2013; Weinblatt et al., 2012). Efficacy in RA appears to be remarkably similar among these anti-TNF agents across multiple trials, often referred to as the ‘60-40-20’ rule. In other words, when using the ACR response criteria of ACR20, 50 and 70 responses, one can expect 60-70% of patients to achieve ACR20; 40% to achieve ACR50 and 20% to achieve ACR70 responses, regardless of the agent used (McInnes and O'Dell, 2010).

Anti-TNF agents have also been shown to be able to inhibit radiographic progression in RA patients (Maini et al., 2004; van der Heijde et al., 2006b). More recent studies using anti-TNF agents in RA have shown that the early use of anti-TNF agents in the RA disease course has particularly favourable outcomes, with a high proportion of patients achieving sustained low disease activity or remission (Emery et al., 2008; St. Clair et al., 2004).

In AS where DMARDs do not affect axial signs or symptoms, anti-TNF agents are used alone. Trials initially with infliximab (Brandt et al., 2000; Braun et al., 2002) and subsequently with etanercept and adalimumab (Davis et al., 2003; van der Heijde et al.,
2006a) and most recently with golimumab (Inman et al., 2008) have demonstrated significant improvement in disease activity, function, spinal mobility and quality of life. Despite the improvement in symptoms, it appears that anti-TNF agents do not affect radiographic progression in AS (Heijde et al., 2008; van der Heijde et al., 2008).

In PsA, anti-TNF agents control the signs and symptoms of articular disease, as well as dactylitis, enthesitis, skin and nail disease and also have a significant impact on radiographic progression (Ravindran et al., 2008; van der Heijde et al., 2007).

Despite these positive effects of anti-TNF agents, a number of shortcomings exist. A significant proportion of patients, 20-30% do not respond to treatment (primary non-response) and others experience secondary loss of efficacy over time (secondary non-response) (Singh JA, 2009). Currently, data on predictors of response to anti-TNF agents has emerged from registry studies, but no defined imaging, laboratory or immunological biomarkers of response to anti-TNF have been characterised. In RA, concurrent use of methotrexate was found to be associated with a better treatment response, whereas smoking and higher baseline disability predicted a worse outcome (Hyrich et al., 2006; Kristensen et al., 2008). Genetic and genomic predictors of anti-TNF response in RA have also been reported, but the majority of these have not been consistently replicated between studies (Prajapati et al., 2011). In AS patients, registry data, as well as data from clinical trials have identified that higher baseline CRP levels, higher disease activity levels, higher functional status, younger age and HLA-B27 positivity were independent baseline predictors of response (Arends, 2012; Rudwaleit et al., 2004). In PsA patients, older patients, females and patients on concomitant corticosteroids have been reported to be less likely to achieve clinical response to anti-TNF agents (Saad et al., 2010).

Anti-TNF agents also have a range of side effects and toxicities, including injection site reactions, reactivation of latent tuberculosis and increased incidence of infections (common or opportunistic). Other possible side effects include the exacerbation of congestive cardiac failure and multiple sclerosis (Taylor and Feldmann, 2009). Long term side effects studied by registry trials and meta-analyses have shown that these agents may also be associated with an increased risk of non-melanoma skin cancers (Ding et al., 2010; Singh JA, 2009).
In view of these shortcomings, improved understanding of the mechanisms of action of anti-TNF therapies, the immunological pathways underlying treatment response, as well as ways in which to enhance the currently obtainable clinical efficacy of these agents is needed.

### 1.5.3 Pharmacology and mechanism of action of anti-TNF agents

At low concentrations, TNFα is thought to have beneficial effects, such as the augmentation of host defence mechanisms against infections, but at high concentrations TNFα can lead to inflammation and target organ damage (Feldmann and Maini, 2008). TNFα is released from cells as a soluble cytokine (soluble TNF, sTNF) after being enzymatically cleaved from its cell-surface bound precursor, transmembrane TNF (tmTNF). Both sTNF and tmTNF are biologically active and mediate their effects by interaction with either of 2 distinct receptors, TNF receptor I (TNFRI or p55) and TNF receptor II (TNFRII or p75) expressed on a wide variety of cell types. TNFRI and TNFRII differ in their cellular expression profiles, cytoplasmic tail structures and signalling mechanisms (Tracey et al., 2008). TNFRI is constitutively expressed on virtually all cell types, except red blood cells, whereas TNFRII is inducible and expressed preferentially on endothelial and haematopoietic cells. Data from in vivo studies suggest that most of the biologic activities of sTNF are mediated through TNFRI (Ksontini R, 1998). Transmembrane TNF preferentially binds to TNFRII and has been shown to exert most of its inflammatory and proapoptotic activities through TNFRII (Grell et al., 1995). The majority of these studies however have been carried out in mouse and the situation in vivo in humans is less clear.

Although the anti-TNF agents share some properties in common, such as being able to neutralise sTNF, they also differ in their molecular structure, binding specificities and the ability to form complexes (Taylor, 2010). Five different anti-TNF agents are currently licensed for patient use. Infliximab, adalimumab and golimumab are TNF-specific monoclonal antibodies that bind TNFα, with infliximab being a chimeric mouse/human protein, whereas adalimumab and golimumab are fully human monoclonal antibodies. Certolizumab is a humanised TNF-specific PEGylated Fab’ antibody fragment. Etanercept is a TNF-receptor Fc-fusion protein that is unique among all the other TNF agents in its ability to bind TNFα as well as lymphotoxin family members. A possible consequence of this is that
not all TNFα may be fully neutralised if lymphotoxin concentrations are similar to or greater than the etanercept concentrations in the tissue (Taylor, 2010).

Differences have been reported in the size, composition and stability of complexes formed between sTNF or tmTNF and the different anti-TNF agents. As bivalent monoclonal antibodies, infliximab and adalimumab can bind two sTNF or tmTNF trimers simultaneously, allowing multimeric complexes to form. A bivalent monoclonal antibody can also bind two monomeric TNF subunits within the homotrimer or crosslink two TNF homotrimers which could be sTNF or tmTNF, or a combination of the two. In contrast, etanercept can bind only one sTNF or tmTNF trimer, resulting in small 1:1 complexes (Scallon et al., 2002). The bivalency of the monoclonal antibodies allows these molecules to bind tmTNF at much higher levels than is the case for etanercept (Scallon et al., 2002; Taylor, 2010). The clearance rate of these complexes also differs, with the etanercept-TNF complexes able to circulate for weeks, whereas the infliximab-TNF and adalimumab-TNF complexes being cleared quickly (Tracey et al., 2008). The TNF antagonist-TNF complexes are not static but constantly bind and release bioactive TNF and studies which have compared the in vitro stability of anti-TNF-TNF complexes, showed etanercept-sTNF complexes to be relatively unstable and to release bioactive sTNF more rapidly and in larger quantities than infliximab-sTNF complexes (Scallon et al., 2002).

Although all anti-TNF agents are able to neutralise sTNF as a major pharmacological mechanism of action, they differ with respect to the consequences following engagement of tmTNF. Transmembrane TNF can function as a ligand as well as a receptor. Binding to tmTNF by TNFRs or TNF antagonists can induce a process called ‘reverse signalling‘ through tmTNF, which triggers cell activation, cytokine suppression or apoptosis of the tmTNF-bearing cell (Tracey et al., 2008). All anti-TNF agents can act as antagonists by blocking the interaction between tmTNF and the TNFRI or TNFRII expressed on cells. Certolizumab, adalimumab and infliximab have been reported to be able to neutralise tmTNF-mediated signalling, whereas etanercept has been shown to be less potent (Taylor, 2010). The monoclonal antibodies are also able to induce reverse signalling, whereas etanercept cannot due to its inability to crosslink tmTNF as it can only bind a single TNF homotrimer. However, the relationship between crosslinking of tmTNF and clinical outcomes of anti-TNF therapy has not yet been established (Tracey et al., 2008).
The three monoclonal antibodies and etanercept have the ability to fix complement and bind to Fc receptors, whereas certolizumab lacks an Fc portion so cannot. The binding of Fc receptors can induce a number of cellular functions, including phagocytosis, antibody dependent cellular cytotoxicity, degranulation, cytokine release and regulation of antibody formation (Taylor, 2010). Infliximab, adalimumab and etanercept can all induce complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity of a cell line which highly expresses tmTNF (TNF6.5 cells), although etanercept has been shown to induce less antibody-dependent cellular cytotoxicity than the monoclonal antibodies (Taylor, 2010).

Other important differences between the different agents relate to their different pharmacokinetic properties. Infliximab, by virtue of the fact that it is administered in relatively large intravenous boluses, results in wide fluctuations in its serum concentrations. Adalimumab and etanercept are administered in smaller, subcutaneous doses and result in relatively constant serum drug concentrations. Etanercept also has a shorter half-life and greater clearance rate than infliximab or adalimumab (Tracey et al., 2008).

A common observation in the treatment of inflammatory arthritides with anti-TNF agents is the rapid reduction in cellularity at the site of inflammation, for which apoptosis, cytotoxicity, reduced cell influx into the joints and chemokine-mediated cell efflux out of the joints have been proposed as possible mechanisms. Anti-TNF agents may induce apoptosis through at least two mechanisms; neutralisation of sTNF may deprive a cell of survival signals mediated by the p55TNF receptor, or the bivalent monoclonal antibodies may induce apoptosis of TNF-producing cells by their ability to crosslink and signal through tmTNF (Taylor, 2010). However, studies which have addressed whether TNF antagonists induce apoptosis in vivo by measuring frequencies of apoptotic cells in peripheral blood and biopsy samples from patients with RA or psoriasis following anti-TNF treatment have yielded conflicting results. Decreased synovial cellularity has been reported 48 hours after infliximab administration, but without evidence of apoptosis in one study (Smeets et al., 2003). In contrast, another study reported apoptosis of synovial macrophages induced by etanercept and infliximab, with a corresponding increase in active caspase 3 expression, but without an increase in lymphocyte apoptosis (Catrina et al., 2005). In psoriasis, infliximab treatment has been shown to induce caspase-dependent apoptosis of lesional plaque keratinocytes, T cells and dendritic cells.
(Malaviya, 2006), whereas another study of skin and synovial biopsies taken 48 hours after infliximab infusion reported no increase in apoptosis at either site (Goedkoop et al., 2004b). Thus, the relevance of apoptosis to the efficacy and safety of anti-TNF agents remains unclear (Taylor, 2010).

Treatment with anti-TNF agents leads to the reduction of the many hallmarks of chronic inflammation, such as leukocyte recruitment, activation and proliferation, as well as a reduction in inflammatory mediators (Tracey et al., 2008). Many of the published studies on the effects of anti-TNF agents on inflammation have been conducted in RA patients treated with infliximab (Feldmann and Maini, 2008). Infliximab leads to reduction in RA synovial tissue expression and serum concentrations of IL6, IL8, granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage chemotactrant protein 1 (MCP1), IL1β and vascular endothelial growth factor (VEGF) (Tracey et al., 2008). Dramatic and rapid reductions in serum IL6 concentrations following infliximab infusion have been reported (Charles et al., 1999). In addition reductions in acute phase reactants, including CRP, serum amyloid A and fibrinogen have been demonstrated in infliximab-treated patients (Charles et al., 1999). Similar effects of adalimumab and etanercept with respect to reduction in inflammatory mediators have also been reported (Tracey et al., 2008).

There is also considerable evidence from studies in patients treated with anti-TNF agents that these drugs reduce inflammatory cellular infiltrates within the target tissues through an effect on cell trafficking. In patients with RA, infliximab causes a reduction in the cellularity of inflamed synovial tissue, which parallels the rapid reduction in swollen joints and significant reductions in the number of intimal and sublining macrophages, as well as plasma cells and T cells as early as 48 hours after infliximab infusion (Smeets et al., 2003). Similarly, after infliximab infusion in patients with PsA, reductions were seen in the frequency of synovial T cells and sublining macrophages (Goedkoop et al., 2004b). Etanercept has also been shown to induce a rapid and sustained reduction in cellular infiltrates, including macrophages and T cells in the peripheral joints of patients with SpA (Kruithof et al., 2005). Direct evidence for the reduction in cell trafficking by anti-TNF agents has been demonstrated in patients with RA using radiolabelled granulocytes, with a significant reduction in the migration and localisation of these cells in the joints (Taylor et al., 2000). In parallel, the serum concentrations of the chemokines IL8 and MCP1 were reduced. Other studies have also
shown reductions in the expression of adhesion molecules in RA synovial tissue following infliximab treatment (Tak et al., 1996).

Reduction in angiogenesis through the reduction in VEGF is an additional mechanism, which may lead to the reduction in cellularity with anti-TNF therapy by reducing cell trafficking in and out of inflamed tissues (Paleolog et al., 1998).

Another possible mechanism through which anti-TNF may ameliorate arthritis is by promoting immune regulation (Tracey et al., 2008). Regulatory T cells (Tregs) are thought to play a central role in the suppression of autoreactivity and in the regulation of immune responses. Their normal functions, including the suppression of proinflammatory cytokine secretion by activated T cells and monocytes, have been shown to be reduced in patients with RA compared to healthy controls (Ehrenstein et al., 2004). However, infliximab treatment of patients with RA was shown to induce a significant increase in the number of circulating Tregs and a normalisation of their phenotype. Moreover, infliximab was also demonstrated to restore the capacity of Tregs to inhibit cytokine production and convey a suppressive phenotype to conventional T cells (Ehrenstein et al., 2004).

The role of anti-TNF therapy in reducing cartilage and bone erosion in RA and PsA is likely to result from the dampening of TNF-driven production of matrix-degrading enzymes and osteoclastogenic factors (Tracey et al., 2008).

Thus, the potent neutralisation of sTNF and tmTNF by anti-TNF agents suggests that they achieve efficacy by preventing TNFα from inducing TNFR-mediated cellular functions. These functions include cell activation, cell proliferation, cytokine and chemokine production, as well as the downstream effects of these functions, including cell recruitment, inflammation, immune regulation, angiogenesis and bone and cartilage damage (Figure 1.6).

### 1.5.4 Effect of anti-TNF therapy on frequency of circulating Th1 and Th17 cells

Early studies on the mechanisms of action of infliximab in RA patients showed that treatment induced a reduction in leukocyte trafficking to inflamed joints due to reduced expression of
**Figure 1.6  Proposed mechanisms of action of anti-TNF agents**

Four categories of putative mechanisms of action of anti-TNF agents are illustrated. The large panel illustrates the primary mechanisms of action, resulting from the direct blocking of TNFR-mediated biologic activities. The anti-TNF agents bind to the ligands sTNF or tmTNF for all 5 TNF antagonists and additionally LTα3 and LTα2β1 for etanercept, blocking their ability to bind TNFRI or TNFRII. The right panel illustrates several mechanisms induced by the binding of TNF antagonists to tmTNF, which can include reverse signaling via tmTNF or cytotoxicity of the tmTNF-bearing cell by CDC or ADCC. The small panel on the lower left illustrates 2 LTαβ-mediated mechanisms thought to be blocked by etanercept. The lower center panel shows pharmacokinetic-related mechanisms that involve TNF antagonist binding to FcRn or forming complexes with sTNF.

ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CRP, C-reactive protein; ETN, etanercept; LT, leukotriene; sTNF, soluble TNF; tmTNF, transmembrane TNF; TNFRI, TNF receptor 1; TNFRII, TNF receptor II, TNF, tumour necrosis factor α. Reprinted from: Tracey, D., Klareskog, L., Sasso, E.H., Salfeld, J.G., and Tak, P.P. (2008). Tumor necrosis factor antagonist mechanisms of action: A comprehensive review. Pharmacology & Therapeutics 117, 244-279, Copyright 2008, with permission from Elsevier.
adhesion molecules and chemokines on synovial endothelium (Tak et al., 1996; Taylor et al., 2000). Consequently, it was shown that following treatment with infliximab, there was an increase in IFNγ-producing T cells and an increased Th1:Th2 cell ratio in the peripheral blood of RA patients, both in the short term (day 3 after infusion) and later on (6 weeks after infusion) (Maurice et al., 1999; Nissinen et al., 2004). Similarly, in AS patients, treatment with etanercept and infliximab has been shown to lead to an increase in circulating Th1 cells (Baeten et al., 2001; Zou et al., 2003).

Following the characterisation of IL17 and Th17 cells and the recognition of their involvement in inflammatory arthritis pathogenesis, it was of interest to investigate the effect of anti-TNF treatment on the balance of Th1 and Th17 cells. Collagen-induced arthritis is a well-established murine model of RA which was used in the pre-clinical studies of anti-TNF therapy (Williams et al., 1992). Recent work by our group demonstrated that anti-TNF therapy in mice with CIA ameliorated arthritis by decreasing numbers of Th1 and Th17 cells in arthritic joints, but paradoxically also caused an increase in Th1 and Th17 cells in the draining lymph nodes (Notley et al., 2008). These expanded populations of Th1 and Th17 cells were shown to be pathogenic and induce arthritis in adoptive transfer experiments where lymph node and spleen cells from CIA mice treated with anti-TNF were injected into SCID mice. By using knockout mice, the increase in these cells was shown to occur through signalling via the TNFp55 receptor, which increased expression of the p40 subunit that is shared between IL12 and IL23, the key cytokines involved in the differentiation of Th1 and Th17 cells respectively.

A number of small studies in patients with RA and in patients with other diseases treated using anti-TNF agents have also investigated changes in the frequency of circulating Th17 cells during anti-TNF therapy (Aerts et al., 2010; Alzabin et al., 2012; Bosè et al., 2011). In RA patients, increased IL17 and IFNγ production by cultured PBMCs has been reported after 12 weeks of anti-TNF treatment compared to pre-treatment levels (Aerts et al., 2010). In another study by our group in RA patients, an increased frequency of circulating Th17 cells was observed 4 weeks and 8-12 weeks after anti-TNF initiation (Alzabin et al., 2012). In a cross-sectional study, patients with psoriasis and inflammatory bowel disease were followed up 1 month after starting treatment with infliximab, adalimumab or etanercept. IL17 and IFNγ cytokine production by in vitro stimulated PBMCs was found to be increased 1 month after anti-TNF initiation (Bosè et al., 2011).
These preliminary observations suggest that anti-TNF treatment may have similar effects in human diseases as in the CIA model. However, longitudinal investigations of changes in the frequency of Th1 and Th17 cells at predefined time points during therapy are needed to elucidate the effects of anti-TNF treatment on these cell types and to determine whether these changes occur in all types of inflammatory arthritis treated with anti-TNF, whether the changes may be specific to particular types of anti-TNF agents used clinically and importantly, if there is any relationship with treatment response.
1.6 Aim and specific objectives of the PhD project

The aim of the work presented in this thesis was to conduct a prospective, longitudinal investigation of patients with inflammatory arthritis over the first 12 weeks of anti-TNF treatment using clinical, ultrasound and immunological assessments in order to gain further understanding of the immune correlates of treatment response. Based on results from studies in murine and human arthritis, the working hypothesis of this investigation was that anti-TNF treatment would increase the frequency of circulating Th1 and Th17 cells and/or induce phenotypic changes in these cell types in patients with RA, AS and PsA and that these changes would be associated with clinical and morphological improvements in disease activity.

In order to investigate this hypothesis, the specific objectives of the project were:

1. To prospectively follow a cohort of patients with RA, AS or PsA at pre-determined protocol visits to evaluate changes in clinical disease activity measures, as well as morphological changes in the target tissue using serial quantitative evaluations by power Doppler ultrasonography during the first 12 weeks of anti-TNF treatment, thus robustly characterising treatment responders and non-responders.

2. To determine the effect of anti-TNF treatment on the frequency and phenotype of circulating Th1 and Th17 cells in these patient groups using two different and complementary cell-based assays: enzyme-linked immunospot (ELISpot) and intracellular cytokine staining by flow cytometry. In addition, to determine changes in serum cytokines linked to the Th1 and Th17 cell pathways and serum chemokines serially during anti-TNF treatment at the same protocol time points.

3. To investigate the relationships between the clinical, ultrasonographic and immunological changes during anti-TNF treatment in the RA cohort in order to gain further understanding of the immunopathological correlates underlying treatment response.
1.7 Synopsis of the thesis

Chapters 3 and 4 detail the clinical study design and clinical methodology used in this thesis.

Chapter 3 describes the clinical study protocol design. Patients with RA, as well as with AS and PsA were recruited and followed prospectively over the first 12 weeks of anti-TNF treatment to determine if changes in the frequency of circulating Th1 and Th17 cells during treatment occurred in different types of inflammatory arthritis. The clinical study design allowed the effect of anti-TNF treatment to be determined on both validated clinical disease activity measures and patient-reported outcomes in order to allow robust phenotyping of the three patient cohorts. The clinical characteristics of each patient group at baseline, as well as the changes in disease activity and patient-reported outcome measures during anti-TNF treatment are described. Using validated treatment response criteria for each disease group, treatment responders and non-responders were identified and the changes in the disease activity measures were compared between responders and non-responders within each disease group.

Chapter 4 describes the use of grey scale and power Doppler ultrasonography (PDUS) to evaluate changes in synovial thickening and vascularity in the metacarpophalangeal joints (MCP) of RA and PsA patients and changes in entheseal pathology in patients with AS during anti-TNF treatment. The use of power Doppler ultrasound endpoints thus complements the information obtained from the changes in clinical outcome measures during anti-TNF treatment and allows better characterisation of anti-TNF responders and non-responders. This is the first longitudinal ultrasound study to incorporate comprehensive semi-quantitative and quantitative measures of synovial thickening and vascularity to characterise changes in the MCP joints of patients with RA and PsA during anti-TNF treatment, including shorter term (1 week and 4 weeks) and medium term (12 months) endpoints. This is also the first study to use grey scale and power Doppler ultrasound to characterise changes in lower limb entheseal pathology of AS patients longitudinally during anti-TNF treatment.

Chapters 5, 6 and 7 describe the laboratory-based investigations conducted as part of this thesis.
Chapters 5 and 6 describe the evaluation of changes in the frequency of circulating Th1 and Th17 cells over the first 12 weeks of anti-TNF therapy in patients with RA, AS and PsA using two different and complementary techniques, ELISpot and intra-cellular cytokine staining. The study compares these changes in patients with RA, AS and PsA and evaluates the magnitude and kinetics of change of these cell types during anti-TNF treatment across the three disease groups.

Chapter 5 details the investigation of the changes in the frequency of IL17 and IFNγ-producing cells in the peripheral blood of patients with RA, AS and PsA using IL17 and IFNγ ELISpot assays. This is the first study to use the ELISpot technique to characterise longitudinally the changes in IL17 and IFNγ-producing peripheral blood mononuclear cells during anti-TNF therapy.

Chapter 6 details the investigation of the changes in the frequency of peripheral blood CD4+IL17+ cells (Th17 cells) and CD4+IFNγ+ cells (Th1 cells) longitudinally during anti-TNF treatment in patients with RA, AS and PsA using intra-cellular cytokine staining and flow cytometry. Changes in the phenotype of Th17 cells during anti-TNF treatment were also investigated by flow cytometry.

Chapter 7 describes the use of multiplex assays and enzyme-linked immunosorbent assays (ELISAs) to quantitate the changes in soluble factors – cytokines and chemokines linked to the Th1 and Th17 cell pathways in order to allow further characterisation of the effects of anti-TNF therapy on these cell types. The focus was on IL12 and IL23 cytokines, which are involved in the differentiation of Th1 and Th17 cells respectively; IL17 and IFNγ as the signature cytokines produced by these cell types, as well as a range of other cytokines and chemokines with important roles in inflammation. In addition, the effects of anti-TNF therapy on systemic TNFα homeostasis were also determined by investigating changes in serum TNFα and soluble TNF receptors during treatment.

Chapter 8 explores the relationships between clinical, ultrasonographic and immunological changes during anti-TNF therapy in patients with RA in order to determine immune correlates of treatment response.
This is the first study to characterise simultaneously clinical, imaging and T cell immunological parameters longitudinally during anti-TNF therapy and to explore the relationships between them in RA patients. Specifically, relationships between clinical and immunological parameters were investigated to determine if a higher frequency of IL17 or IFNγ-producing cells at baseline is associated with poor anti-TNF treatment response. In addition, relationships between clinical and immunological parameters were investigated to test the hypothesis whether the changes in the frequency of Th17 or Th1 cells during anti-TNF treatment is related to clinical improvement in RA disease activity and morphological improvement of inflamed joints.

Chapter 9 presents the general discussion of the findings in the thesis. It brings together the major themes of the work and summarises the main findings from all the data chapters, as well as containing a discussion of the future areas of work arising in relation to this thesis.
Chapter 2. Materials and Methods

2.1 Stock Solutions

sRPMI RPMI 1640 tissue culture medium (Gibco, Paisley, UK) supplemented with 11.5 ml 1M HEPES (Fisher Scientific, Loughborough, UK), 3 ml 1M NaOH (VWR, Leicestershire, UK), 0.5 ml L-glutamine (Sigma, Gillingham, UK) and 5 ml penicillin/streptomycin (Sigma). This solution was prepared using a 0.22µM syringe filter (Millipore, Watford, UK), was stored at 4°C and used within 30 days to ensure sterility.

1M HEPES 119.15g HEPES powder (Fisher Scientific) added to 450ml deionised water. Stored at room temperature for up to 1 month.

1M NaOH 20g NaOH pellets (VWR) added to 450 ml deionised water. Stored at room temperature for up to 1 month.

PBS/1%BSA 10g BSA (Sigma) added to 1L PBS which was made by dissolving 10 PBS tablets (Sigma) in 1L deionised water.

PBS/0.05% Tween-20 10 PBS tablets (Sigma) added to 1L deionised water and allowed to dissolve completely. 500 µl Tween (Sigma) was then added and mixed thoroughly.

sRPMI/10% FCS 450 ml of sRPMI (Gibco) supplemented with 50 ml heat inactivated foetal calf serum (Gibco). Stored at 4°C and used within 30 days.

sRPMI/20% FCS 400 ml sRPMI (Gibco) supplemented with 100 ml heat inactivated foetal calf serum (Gibco). Stored at 4°C and used within 30 days.

sRPMI/10% AB serum 450 ml sRPMI (Gibco) supplemented with 50 ml human AB serum (Sigma) filtered through a 0.22 µM syringe filter (Millipore). Solution stored at 4°C for up to 1 month.
Counting solution for freshly isolated PBMCs

450 ml distilled water, 50 ml glacial acetic acid (VWR) and 500 μl trypan blue 0.4% (Sigma). Stored at room temperature for up to 6 months. This solution lysed red blood cells from a PBMC suspension allowing only lymphomonocytes to be seen and counted under the microscope.

Counting solution for frozen PBMCs

For one sample to be counted, 160 μl sRPMI were added to 40 μl filtered 0.4% trypan blue (Sigma) and mixed in an eppendorf tube.

Freezing mixture for PBMCs

3 ml sRPMI added to 2ml 10% dimethyl sulfoxide (Sigma) and solution was allowed to cool for at least 20 minutes at room temperature prior to use (exothermic reaction).

2.2 Clinical Study Design and Assessments

The study protocol was developed and study protocol approvals were gained from the Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (09/H0707/80), the Imperial College AHSC Joint Research Office (ABRS2001) and clinical trial authorisation was granted by the Medicines Healthcare Products Regulatory Agency (MHRA) (19174/0283/001-0001). The study was registered with EudraCT (2009-012424-87) and clinicaltrials.gov (NCT01060098). Written, informed consent was obtained from all study participants prior to study entry and the study was conducted in accordance with the Declaration of Helsinki.

The clinical component of the work in this thesis includes an exploratory, open label, longitudinal study comprising 3 groups of patients with inflammatory arthritis: patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA). The
patients were followed up for the first 12 weeks of treatment with anti-TNF agents, with repeat assessments at four predefined protocol time points.

2.2.1 Patients

The study included anti-TNF naïve male and female patients aged between 18 and 80 years who fulfilled either the 1987 revised American College of Rheumatology classification criteria for rheumatoid arthritis, (Arnett et al., 1988) or the Modified New York Criteria for ankylosing spondylitis (Linden et al., 1984) or the CASPAR criteria for psoriatic arthritis (Taylor et al., 2006) and were due to start treatment with either etanercept (Enbrel) or adalimumab (Humira). To be eligible for inclusion in the study and treatment with anti-TNF, patients with RA had to have a Disease Activity Score in 28 joints (DAS28) score ≥5.1 on two occasions at least 1 month apart; patients with AS had to have a Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score ≥4 on two occasions at least one month apart and patients with PsA had to have three or more tender and/or three or more swollen joints despite trial of disease-modifying anti-rheumatic drug (DMARD) treatment for RA and PsA or non-steroidal anti-inflammatory drug (NSAID) treatment for AS patients as per the guidance by the National Institute for Health and Care Excellence (NICE) at the time of the study design (TA130, NICE 2007; TA143, NICE 2008; TA199, NICE 2010). In addition, patients had to have been on a stable dose of concomitant DMARD or NSAID therapy for the preceding 1 month prior to study entry. Concomitant treatment with glucocorticoids was permitted if daily dose was ≤10 mg. Intramuscular or intra-articular steroid injections within the preceding 1 month prior to study entry were not permitted. Patients with psoriatic arthritis had to have evidence of active psoriasis skin lesions.

According to the main exclusion criteria in the protocol, patients who did not fulfil the diagnostic criteria for their type of arthritis, patients who had been treated with anti-TNF agents or other biologic agents in the past and patients with intercurrent active infection were not enrolled in the study.

Healthy controls were recruited from members of clinical and laboratory staff who also gave written, informed consent to participate.
2.2.1.1 Clinical Assessment of Study Patients

The decision to start anti-TNF treatment and which type of anti-TNF treatment was made by the patient’s Consultant Rheumatologist, independent of the study, and was made in accordance with the NICE guidance at the time of the study design (TA130, NICE 2007; TA143, NICE 2008; TA199, NICE 2010). Patients self–administered their treatment and drug dosing for etanercept was 50 mg subcutaneously every week and for adalimumab was 40 mg subcutaneously every fortnight.

All patients were screened for tuberculosis by a chest X-ray, Mantoux test and a TB Elispot prior to commencing anti-TNF therapy.

Patients were assessed at 4 time points: before starting anti-TNF treatment and at 1 week, 4 weeks and 12 weeks after starting treatment. To ensure consistency, the visits were scheduled to occur within 48 hours after the most recent injection with anti-TNF, except visit 2 which occurred exactly 1 week after the first injection.

2.2.1.2 Rheumatoid Arthritis Cohort Assessment (Table 2.1)

Clinical assessment of disease activity at each visit was made by clinical examination and determination of the DAS28 score, comprising swollen joint count, tender joint count, erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) measurement and patient global assessment of wellbeing on a 0-100 mm visual analogue scale. The 28 joint count comprised assessment of the bilateral glenohumeral, elbow, wrist, metacarpophalangeal (MCP), proximal interphalangeal (PIP) and knee joints. In addition, patients quantified the duration of early morning stiffness in minutes and rated their pain on a 0-100 mm visual analogue scale. The physician graded the disease activity on a 0-100 mm visual analogue scale. Further clinical assessment of disease activity was made by grey scale and power Doppler ultrasound of the MCP joints to determine presence and extent of synovial hypertrophy and synovial vascularity.

Functional status was determined by patients completing the Health Assessment Questionnaire (HAQ). Patients also completed the Short Form 36 (SF-36) questionnaire to determine the impact of the disease activity on their quality of life. Patients completed the Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-F) questionnaire to determine the effect of their disease on fatigue.
Table 2.1 Flow chart of the protocol assessments and procedures for rheumatoid arthritis patients at each of the 4 study visits.

<table>
<thead>
<tr>
<th>ASSESSMENT</th>
<th>Visit 1 Screening visit (prior to starting anti-TNF)</th>
<th>Visit 2 1 week after starting anti-TNF</th>
<th>Visit 3 4 weeks after starting anti-TNF</th>
<th>Visit 4 12 weeks after starting anti-TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obtain informed consent</td>
<td>●</td>
<td></td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Medical history</td>
<td>●</td>
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<tr>
<td>DAS28 score</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>HAQ questionnaire</td>
<td>●</td>
<td>●</td>
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<td>●</td>
</tr>
<tr>
<td>SF36 questionnaire</td>
<td>●</td>
<td></td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>FACIT-F questionnaire</td>
<td>●</td>
<td></td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Grey scale and power Doppler MCP joint ultrasound</td>
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<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Blood test for CRP/ESR, FBC, U&amp;E, LFTs</td>
<td>●</td>
<td></td>
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</tr>
<tr>
<td>Bloods for research investigations</td>
<td>●</td>
<td>●</td>
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<td>●</td>
</tr>
</tbody>
</table>

DAS28, disease activity score in 28 joints; HAQ, health assessment questionnaire; SF-36, short form 36 questionnaire; MCP, metacarpophalangeal joints; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; FBC, full blood count; U&Es, urea and electrolytes; LFTs, liver function tests.
2.2.1.3  **Ankylosing Spondylitis Cohort Assessment (Table 2.2)**

Clinical assessment of disease activity at each visit was made by clinical examination and patients were asked to complete the BASDAI, BASFI, FACIT-F and SF-36 questionnaires. Spinal movements were measured at each visit by a trained nurse and the BASMI score was completed. Joints were assessed for the presence of tenderness or swelling using 76/78 joint count.

2.2.1.4  **Psoriatic Arthritis Cohort Assessment (Table 2.3)**

Clinical assessment of disease activity was determined by clinical examination and using the Psoriatic Arthritis Response Criteria (PsARC) which incorporates: patient global assessment from 0-5, physician global assessment from 0-5, tender joint count and swollen joint count. Skin disease was assessed using the Psoriasis Area and Severity Index (PASI) if >3% of the patient’s body surface area was affected by psoriasis. Patients also completed the FACIT-F and SF-36 questionnaires.

2.2.2  **Grey Scale and Power Doppler Ultrasonography**

2.2.2.1  **Rheumatoid Arthritis and Psoriatic Arthritis Patients**

In accordance with the clinical study protocol, ultrasound assessments were performed at each study visit and included 2-dimensional scanning of the 10 MCP joints, scanned over the dorsal surface in the longitudinal and transverse planes using a GE Logiq 9 ultrasound machine and the M12L matrix array transducer (GE Healthcare, Buckinghamshire, UK). The patients were scanned by the same sonographer (D. Hull) at each visit to minimise image acquisition variability. To standardise image acquisition, the hands were scanned in a position of rest maintained by a splint and hands were scanned in the same sequence at each visit using coupling gel at 20°C. Patients were scanned in the same room in which temperatures were maintained at a constant level all year (20°C), with a delay of at least 10 minutes if patients arrived from outside, to avoid the confounding effects of temperature changes from differences in ambient temperatures. The time of day of the measurements at each visit were made within 1 hour of the time of the baseline visit. When scanning, care was taken to avoid undue pressure with the probe and a 1mm gap between the skin and ultrasound coupling gel on the screen image was maintained, to minimise alterations in blood flow within the joint.
Table 2.2  Flow chart of the protocol assessments and procedures for ankylosing spondylitis patients at each of the 4 study visits.

<table>
<thead>
<tr>
<th>ASSESSMENT</th>
<th>Visit 1 (Screening visit prior to starting anti-TNF)</th>
<th>Visit 2 (1 week after starting anti-TNF)</th>
<th>Visit 3 (4 weeks after starting anti-TNF)</th>
<th>Visit 4 (12 weeks after starting anti-TNF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obtain informed consent</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Medical history and clinical examination</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>BASDAI, BASFI, BASMI</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Joint assessment</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>SF-36 questionnaire</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>FACIT-F questionnaire</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Blood test for CRP/ESR, FBC, U&amp;Es, LFTs</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Bloods for research investigations</td>
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<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Grey scale and power Doppler ultrasound of entheses</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
</tbody>
</table>

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; SF-36, short form 36 questionnaire; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; FBC, full blood count; U&Es, urea and electrolytes; LFTs, liver function tests.
Table 2.3 Flow chart of the protocol assessments and procedures for psoriatic arthritis patients at each of the 4 study visits.

<table>
<thead>
<tr>
<th>ASSESSMENT</th>
<th>Visit 1 Screening visit (prior to starting anti-TNF)</th>
<th>Visit 2 1 week after starting anti-TNF</th>
<th>Visit 3 4 weeks after starting anti-TNF</th>
<th>Visit 4 12 weeks after starting anti-TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obtain informed consent</td>
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<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Medical history and clinical examination</td>
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<td></td>
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</tr>
<tr>
<td>PsARC</td>
<td>●</td>
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<td>●</td>
</tr>
<tr>
<td>PASI</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>SF36 questionnaire</td>
<td>●</td>
<td></td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>FACIT-F questionnaire</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
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<tr>
<td>Grey scale and power Doppler MCP joint ultrasound</td>
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<td>Blood test for CRP/ESR, FBC, U&amp;E, LFTs</td>
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<td>Bloods for research investigations</td>
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</tr>
</tbody>
</table>

PsARC, Psoriatic Arthritis Response Criteria; PASI, Psoriatic Arthritis Area and Severity Index; HAQ, health assessment questionnaire; SF-36, short form 36 questionnaire; MCP, metacarpophalangeal joints; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; FBC, full blood count; U&Es, urea and electrolytes; LFTs, liver function tests.
Longitudinal images were recorded by aligning the probe in the longitudinal axis as defined by the metacarpal head and proximal phalanx so that the probe bisected the joint. The probe was adjusted to place the maximum height of the dorsal triangular structure (an inverted triangular area formed by the metacarpal head, the phalangeal base and superiorly by the joint capsule) in the centre of the screen image. After recording the longitudinal image, the probe was rotated $90^0$ to achieve the transverse view.

Vascularity of the MCP joints was recorded using power Doppler ultrasonography in the transverse and longitudinal planes. A 5 second digital film clip was recorded for each MCP joint to allow for fluctuations of the power Doppler signal with the cardiac cycle and the maximum signal was evaluated. Pulse repetition frequency was adjusted to the lowest permissible value to maximise sensitivity and a low wall filter was used. Colour gain was set just below the level at which colour noise appeared underlying bone. The ultrasound settings were standardised and maintained the same at each scan and these are detailed in Table 2.4.

To assess parallel scan intra-reader variability in image acquisition of the grey scale and power Doppler images, 5 study patients selected at random were scanned twice using the above method, consecutively at the same visit and the images were stored and analysed to determine the intra-class correlation coefficient for each ultrasound endpoint.

2.2.2.2 Ultrasound Analysis of 2D Grey Scale and Power Doppler Imaging In Rheumatoid Arthritis and Psoriatic Arthritis Patients

The images obtained were anonymised and stored for analysis using a computerised image analysis system (Image J version 1.42q, NIH, Bethesda, USA) which has been used in previous work by our group (Seymour et al., 2012). The anonymised images were assessed semi-quantitatively and quantitatively by one assessor to minimise variability (D. Hull).

Synovium was defined as an anechoic or hypoechoic region over the dorsum of the joint, visible in the longitudinal and transverse planes (Figure 2.1). For each MCP joint, the degree of synovial thickening on grey-scale ultrasound both in the longitudinal and transverse planes
Table 2.4  Ultrasound settings used for grey scale and power Doppler ultrasound scanning of metacarpophalangeal joints in RA and PsA patients and lower limb entheses in AS patients

<table>
<thead>
<tr>
<th>Grey scale</th>
<th>Power Doppler Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency 14MHz</td>
<td>Frequency 7.5MHz</td>
</tr>
<tr>
<td>Gain 50</td>
<td>Gain 41</td>
</tr>
<tr>
<td>Depth 2.0cm</td>
<td>Pulse Repetition Frequency 1.4KHz</td>
</tr>
<tr>
<td>Frame Rate 24</td>
<td>Wall Filter 127Hz</td>
</tr>
</tbody>
</table>
Figure 2.1  **The anatomy of grey scale longitudinal and transverse ultrasound images of the left second metacarpophalangeal joint**

Grey scale ultrasound images of the left second metacarpophalangeal joint demonstrating the anatomy and areas of synovial thickening seen on a longitudinal image of the joint (A) and a transverse image of the joint (B). P= phalangeal base, T=triangular structure, white arrow=maximum height of the dorsal triangular structure, M= metacarpal head, N=metacarpal notch
was compared to a library of reference images and graded on a semi-quantitative scale of 0-4, where grade 0 is the absence of synovial thickening, grade 1 is minimal, grade 2 is mild, grade 3 is moderate and grade 4 is severe synovial thickening. This generated a semi-quantitative score, called the synovial thickness index, STi (Figures 2.2 and 2.3). The score of each of the MCP joints was then summed to give the 10 MCP longitudinal synovial thickness index (10 MCP Long STi) and the 10 MCP transverse synovial thickness index (10 MCP Trans STi).

To assess vascularity at each MCP joint recorded by power Doppler ultrasound in the longitudinal and transverse image, the frame from the recorded clip demonstrating maximal power Doppler activity was selected and was compared to a library of reference images and graded on a semi-quantitative scale of 0 to 4, where grade 0 is the absence of vascular signal, grade 1 is minimal signal, grade 2 is mild signal, grade 3 is moderate signal and grade 4 is severe power Doppler signal. This generated a semi-quantitative score, called the vascularity index, VASCi (Figure 2.4 and 2.5). The score of each of the MCP joints was then summed to generate the 10 MCP longitudinal vascularity index (10 MCP Long VASCi) and the 10 MCP transverse vascularity index (10 MCP Trans VASCi).

Quantitative assessment of the synovial thickness area (STA) and power Doppler area (PDA) was calculated as a count of the number of pixels with synovial thickening or power Doppler respectively, within a defined region of interest in a standardised 2D transverse and longitudinal image of each joint (Figure 2.6). For each longitudinal MCP joint image, the region of interest for synovial area analysis was drawn to envelop the synovium over the phalangeal base, triangular structure, metacarpal head and metacarpal notch to the joint capsule superiorly. The region of interest on the transverse image for synovial area enveloped the MCP joint synovium from the lower border of the triangular structure to the joint capsule superiorly. The computerised image analysis system Image J then automatically calculated the number of colour pixels within the selected region of interest. The synovial thickness area (STA) from the transverse and longitudinal scans of each of the 10 MCP joints was then summed to give the 10 MCP Trans STA and 10 MCP Long STA values respectively.
Figure 2.2  Semi-quantitative scale for assessment of metacarpophalangeal joint synovial thickness on grey scale longitudinal ultrasound images

Semi-quantitative (0–4) ultrasound scale for assessment of synovial thickness relating to hypoechoic areas: grade 0, normal; grade 1, minimal; grade 2, mild; grade 3, moderate; grade 4, severe on longitudinal images of metacarpophalangeal joints.
Figure 2.3  Semi-quantitative scale for assessment of metacarpophalangeal joint synovial thickness on grey scale transverse ultrasound images

Semi-quantitative (0-4) ultrasound scale for assessment of synovial thickness relating to hypoechoic areas: grade 0, normal; grade 1, minimal; grade 2, mild; grade 3, moderate; grade 4, severe on transverse images of metacarpophalangeal joints.
Figure 2.4 Semi-quantitative scale for assessment of metacarpophalangeal joint synovial vascularity on grey scale longitudinal ultrasound images

Semi-quantitative (0-4) ultrasound scale for assessment of vascularity relating to power Doppler signal: grade 0, normal; grade 1, minimal; grade 2, mild; grade 3, moderate; grade 4, severe on longitudinal images of metacarpophalangeal joints.
Figure 2.5  Semi-quantitative scale for assessment of metacarpophalangeal joint synovial vascularity on grey scale transverse ultrasound images

Semi-quantitative (0-4) ultrasound scale for assessment of vascularity relating to power Doppler signal: grade 0, normal; grade 1, minimal; grade 2, mild; grade 3, moderate; grade 4, severe on longitudinal (A) and transverse (B) images of metacarpophalangeal joints.
Figure 2.6  Quantitative assessment of the synovial thickness area (STA) and power Doppler area (PDA) on longitudinal and transverse ultrasound images

Synovial thickness area (STA) and power Doppler area (PDA) was calculated as a count of the number of pixels with synovial thickening or power Doppler respectively, within a defined region of interest in a standardised 2D transverse and longitudinal image of each joint. (A) Longitudinal grey scale ultrasound image of a metacarpophalangeal joint showing the region of interest drawn in black and analysis performed for synovial thickening area (STA) which is calculated to be 39544 pixels. (B) Transverse grey scale ultrasound image of a metacarpophalangeal joint showing the region of interest drawn in yellow and analysis performed for synovial thickening area (STA), which is calculated to be 39749 pixels. (C) Longitudinal power Doppler ultrasound image of a metacarpophalangeal joint, showing the region of interest drawn in black and analysis performed for power Doppler area (PDA) shows the Doppler pixel count to be 1938 pixels. (D) Transverse power Doppler ultrasound image of a metacarpophalangeal joint showing the region of interest drawn in yellow and analysis performed for power Doppler area (PDA), shows the Doppler pixel count to be 153 pixels.
To quantitatively analyse the number of pixels with power Doppler signal using Image J, the recorded 5 second clip was played and the frame displaying the most power Doppler activity was selected for further analysis. On longitudinal images, the region of interest was traced to envelop the entire synovium over the phalangeal base, triangular structure, metacarpal head and metacarpal notch to the joint capsule superiorly, making sure that any digital vessels and reflection artefacts were excluded. On the transverse images, the region of interest was drawn to envelop the entire MCP joint synovium from the lower border of the triangular structure to the joint capsule superiorly, excluding digital vessels and reflection artefacts. The Image J software then automatically calculated the number of colour pixels within the region of interest to generate the power Doppler area score (PDA). The PDA from the transverse and longitudinal scans for each of the 10 MCP joints was then summed to give the 10 MCP Trans PDA and 10 MCP Long PDA scores respectively.

Using these analysis methods, the following semi-quantitative and quantitative endpoints for synovial thickening and synovial vascularity were generated which have been described and used by our group in previous work (Seymour et al., 2012):

10 MCP Trans STi (Synovial Thickness Index)
10 MCP Long STi (Synovial Thickness Index)
10 MCP Trans STA (Synovial Thickness Area)
10 MCP Long STA (Synovial Thickness Area)
10 MCP Trans VASCi (Vascularity Index)
10 MCP Long VASCi (Vascularity Index)
10 MCP Trans PDA (Power Doppler Area)
10 MCP Long PDA (Power Doppler Area)

In order to assess the within-scan intra-reader reproducibility in image scoring, 10 anonymised patient scans were randomly selected and the images and power Doppler clips were reviewed 2 years after the initial reading in a blinded fashion. Joint by joint and also composite score comparisons were then made between the first and second readings for each of the ten ultrasound endpoints and intra-class correlation coefficient values were determined.
2.2.2.3 Ultrasound of entheses in ankylosing spondylitis patients

Grey scale and power Doppler ultrasound was used to scan the following entheses bilaterally in the longitudinal plane: quadriceps insertion, superior and inferior pole of the patellar ligament, Achilles tendon (Figure 2.7) and plantar aponeurosis (Figure 2.8) in the ankylosing spondylitis patient group at each study visit. The image acquisition was performed using a Logiq9 machine with the M12L matrix array transducer (GE Healthcare). Ultrasound settings were maintained the same at each scan and were the same as detailed in Section 2.1.4.1. The patients were scanned by the same sonographer (D. Hull) at each visit to minimise image acquisition variability. The time of day of the measurements at each visit were made within 1 hour of the time of the baseline visit. When scanning, care was taken to avoid undue pressure with the probe to minimise alteration in blood flow within the enthesis.

To determine enthesal thickening and structural abnormalities, the entheses were scanned in the longitudinal plane using grey-scale ultrasound. To determine abnormal vascularisation at the enthesis insertion, power Doppler ultrasound was used and the entheses were scanned in the longitudinal plane and a 5 second digital film clip was recorded to allow selection of the frame with the most marked power Doppler signal at future analysis. Examination of the superior pole of the patella (for the quadriceps tendon insertion), the inferior pole of the patella (for the patellar ligament origin) and the patellar ligament insertion at the tibial tuberosity was performed with the patient in the supine position with the knee flexed to 70 degrees. The Achilles tendon and plantar aponeurosis were examined with the patient lying prone and the feet hanging over the edge of the examination table at 90 degrees of flexion. If the patient was unable to lie prone due to restriction in spinal mobility, the heels were scanned with the patient lying supine and the knees and ankles flexed to 90 degrees.
Figure 2.7  Ultrasound anatomy of lower limb entheses

Ultrasound appearances of lower limb enthesal insertions in patients with ankylosing spondylitis showing entheses of normal and abnormal thickness. (A) Quadriceps tendon enthesis of normal (top image) and abnormal thickness (lower image), (B) Proximal patellar ligament enthesis of normal (top image) and abnormal thickness (lower image), (C) Distal patellar ligament enthesis of normal (top image) and abnormal thickness (lower image), (D) Achilles enthesis of normal (top image) and abnormal thickness (lower image). * denotes enthesis insertion; Q, quadriceps tendon; P, patella; PL, patellar ligament; T, tibia; AT, Achilles tendon; C, calcaneus.
Figure 2.8  Ultrasound anatomy of the plantar aponeurosis enthesis

Ultrasound appearances of plantar aponeurosis enthesis insertion in patients with ankylosing spondylitis showing entheses of normal (top image) and abnormal thickness (lower image). * denotes enthesis insertion; A, plantar aponeurosis; C, calcaneus.
2.2.2.4 Ultrasound analysis of 2D Grey Scale and Power Doppler imaging of Entheses in Ankylosing Spondylitis Patients

The images obtained were anonymised and the stored images at each enthesis site were subsequently reviewed and analysed using the Logiq9 ultrasound machine (GE Healthcare). The grey scale ultrasound images of each enthesis in the longitudinal plane were analysed to measure enthesis thickness and to determine the presence or absence of bony erosion, enthesophytes and bursitis. Bursitis was defined as a well circumscribed, localised anechoic or hypoechoic area at the site of the anatomical bursa which was compressible by the transducer. Bony erosion was defined as a cortical breakage with a step down contour effect. An enthesophyte was defined as a step up bony prominence at the end of the normal bone contour. The enthesis thickness was measured in millimetres at the point of maximal thickness proximal to the bony insertion (*Figure 2.7 and 2.8*). The criteria used to determine abnormal structure thickness have been published previously and were as follows: quadriceps tendon thickness ≥6.1mm, proximal and distal patellar ligament ≥4mm, Achilles tendon ≥5.29mm, plantar aponeurosis ≥4.4mm (Balint et al., 2002; de Miguel et al., 2009)

The Glasgow Ultrasound Enthesitis Scoring System (GUESS) was used to determine an overall score of grey scale ultrasound features of lower limb entheses and was calculated by assigning one point for each abnormality at each site examined, giving a possible maximum total score of 36 (*Table 2.5*) (Balint et al., 2002).

Vascularisation at the enthesis insertion was assessed by power Doppler ultrasound. The recorded 5 second digital film clip was reviewed and the frame with the greatest power Doppler signal was selected for further analysis. The Doppler signal was scored using a semi-quantitative score as described previously as follows: grade 0 if signal absent, grade 1 if minimal signal (only 1 colour spot detected), grade 2 if signal moderate (2 colour spots detected) and grade 3 if signal is severe (≥3 colour spots detected) (D'Agostino et al., 2009).
### Table 2.5 The Glasgow Ultrasound Enthesitis Scoring System (GUESS)

<table>
<thead>
<tr>
<th>Superior pole of patella - quadriceps tendon enthesis</th>
<th>Superior pole of patella erosion</th>
<th>Superior pole of patella enthesophyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quadriceps tendon thickness ≥6.1mm</td>
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<td></td>
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<td>Suprapatellar bursitis</td>
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<table>
<thead>
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<th>Inferior pole of patella erosion</th>
<th>Inferior pole of patella enthesophyte</th>
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</thead>
<tbody>
<tr>
<td>Patellar ligament thickness ≥4mm</td>
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<table>
<thead>
<tr>
<th>Tibial tiberosity - distal patellar ligament enthesis</th>
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<td>Patellar ligament thickness ≥4mm</td>
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<td>Infrapatellar bursitis</td>
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<table>
<thead>
<tr>
<th>Superior pole of calcaneus - Achilles tendon enthesis</th>
<th>Posterior pole of calcaneus erosion</th>
<th>Posterior pole of calcaneus enthesophyte</th>
</tr>
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<tbody>
<tr>
<td>Achilles tendon thickness ≥5.29mm</td>
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</tr>
<tr>
<td>Retrocalcaneal bursitis</td>
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</table>

<table>
<thead>
<tr>
<th>Inferior pole of the calcaneus - plantar aponeurosis enthesis</th>
<th>Inferior pole of calcaneus erosion</th>
<th>Inferior pole of calcaneus enthesophyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plantar aponeurosis thickness ≥4.4mm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each item listed scores one point. Total possible score on both lower limbs is 36 (Balint et al., 2002).
2.3 Laboratory Methods

2.3.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation

30 ml peripheral venous blood was collected at each study visit in glass vacutainers containing sodium heparin (BD Vacutainer, BD Biosciences). Peripheral blood mononuclear cells (PBMC) were then isolated by density gradient centrifugation. Firstly, the blood was diluted in a ratio of 1:1 with 0.9% sterile normal saline. 20 ml of the blood/saline mixture was carefully layered over 10 ml of ficoll/hypaque (Lympholyte, Cedarlane, Ontario, Canada) in a universal container ensuring that a distinct interface between the two layers was maintained. The blood was then centrifuged at 750g for 30 minutes at 20°C, with no brake applied to ensure separation of the PBMCs from the denser ficoll/erythrocyte layer below, and the less dense plasma layer above. After centrifugation, the top plasma layer was siphoned off and discarded. The PBMC layer was gently removed with a sterile pipette and placed in a fresh universal container. The PBMCs were then washed twice with sRPMI and centrifuged at 1000g for 10 minutes with the brake applied. The cell pellet was finally resuspended in 2.25 ml FCS (750 μl of FCS per 10 ml of whole blood) in preparation for cryopreservation.

2.3.2 PBMC Counting

PBMCs were counted by adding 25 μl of the cell suspension to 475 μl counting solution for PBMCs. 10μl of this mixture was loaded onto a haemocytometer (Neubauer). Cells were counted in all of the 4 large quadrants using a manual tally counter and the concentration of cells (x10⁶/ml) was calculated by dividing the number of cells counted by 20 (dilution factor). To obtain the total number of cells isolated this figure was then multiplied by 2.25 ml (volume in which the cells were resuspended).

2.3.3 PBMC cryopreservation

Isolated PBMCs were cryopreserved in aliquots at a density of 5-10x10⁶ cells/ml in heat-inactivated foetal calf serum (Gibco) containing 10% dimethyl sulfoxide (Sigma), according to established procedure (Rehermann and Naoumov, 2007). A freezing mixture was firstly prepared by adding 3 volumes sRPMI to 2 volumes of 10% dimethyl sulfoxide (Sigma) and was allowed to cool for at least 20 minutes at room temperature as the reaction is exothermic.
The cell suspension of isolated PBMCs (as per Section 2.3.1) was pelleted by centrifugation at 1000g for 10 minutes. The pellet was resuspended in 750 µl heat-inactivated foetal calf serum (Gibco) per 5-10x10^6 PBMCs and 250 µl of the pre-prepared freezing mix was added. This 1 ml mixture was then transferred into a cryovial and placed into a Nalgene cryocontainer (Mr Frosty, Merck BDH, Leicestershire, UK) with isopropanol to allow cells to freeze to -80°C at a cooling rate of 1°C per minute. The cryocontainer was transferred immediately into a -80°C freezer. After 24 hours at -80°C, the cryovials were transferred to liquid nitrogen for storage.

PBMCs were cryopreserved to ensure that all the time points from the same patient were analysed concurrently in the downstream assays in order to minimise inter-assay variability. The key steps that have been identified in ensuring high cell viability is maintained after cryopreservation include: i) collection of blood using sodium heparin, ii) using 90% FCS/10% DMSO as the cryopreservation medium, iii) using an isopropanol container overnight and iv) on thawing - stepwise dilution of the thawed cell suspension to decrease osmotic stress, as well as resting cells in culture medium after thawing (Rehermann and Naoumov, 2007).

### 2.3.4 Thawing of cryopreserved PBMCs

The cryovials to be thawed were transferred from liquid nitrogen on dry ice for sequential thawing. To count one sample, 10 ml of sRPMI/20% FCS solution was transferred into a 30 ml universal container and warmed in a water bath at 37°C for 10 minutes. Each cryovial was defrosted in a water bath one sample at a time until the cell pellet became mobile. Once mobile, the pellet was transferred into a 30 ml universal container and the PBMCs were then defrosted using a _step-by-step_ method with the addition of 3 drops of the pre-warmed sRPMI/20% FCS followed by 20 seconds of gentle swirling of the universal container until a total volume of 5 ml was reached. 1ml of sRPMI/20% FCS was subsequently added followed by 20 seconds of gentle swirling until a total volume of 10 mls was reached. The cells were then rested for a minimum of 20 minutes in a water bath at 37°C prior to use in downstream assays. The thawed cells were then centrifuged at 750g for 10 minutes with the brake applied. The supernatant was discarded and the cells pellet was resuspended in 1 ml sRPMI/10%FCS
or sRPMI/10%AB serum according to the assay to be performed. Cells were then counted as per Section 2.3.2. 190 μl of counting solution was added into a 200 μl eppendorf tube and 10 μl of the PBMC suspension was added to the same eppendorf and mixed well. 10 μl of the PBMC/counting solution mixture was then dispensed into a haemocytometer (Neubauer). To assess the concentration of PBMCs, the live cells (white cells) were counted in two diagonal large squares of the haemocytometer and this number was multiplied by $10^5$ to give the number of cells per millilitre of cell suspension. The number of blue cells (dead cells) was also counted in the same two diagonal large squares. The percentage viability was calculated using the following equation:

$$\text{Viability (\%)} = 100 \times \frac{\text{number of white cells}}{\text{number of total cells both white and blue}}.$$  
A viability of >95% was achieved for all the samples used in subsequent assays.

### 2.3.5 Enzyme-Linked Immunospot (ELISpot) Assay

The IL17 and IFNγ ELISpot assays were run concurrently so that one patient's samples from each of the 4 time points on the study were analysed in the same experiment to minimise inter-assay variability. The assay was performed over 3 days. In addition, a test patient PBMCs were used as an inter-assay control and tested alongside the study patient samples with each run, in order to validate performance of the assay and ensure reproducibility of each experiment.

The ELISpot assay, originally based on the sandwich enzyme-linked immunosorbent assay (ELISA) has been developed for the quantitation of individual cells secreting specific cytokines in response to a stimulus. It is reproducible and sensitive, with detection levels as low as one cytokine-producing cell per 200,000 so it is especially useful in studying low frequencies of active cells. It also provides an objective analysis of cytokine production, allowing comparison between patients and different time points in a longitudinal study (Karlsson et al., 2003).

The principles of the assay are illustrated in Figure 2.9. Briefly, cytokines produced by stimulated cells are captured by anti-cytokine antibodies coating the membrane of the
Cytokines produced by stimulated cells are captured by anti-cytokine antibodies coating the membrane of the ELISpot wells. A secondary biotinylated antibody that binds to the capture antibody is added to detect the cytokine of choice. An enzyme conjugate is then added to allow visualisation of the cytokine. A precipitating enzyme substrate is then applied to allow development of coloured spots, with each spot representing a single cytokine-producing cell. The spots are then counted on an automated reader and the results are expressed as number of spot-forming cells/10^6. Reproduced with kind permission from Dr Sandra Phillips.
ELISpot wells. A secondary biotinylated antibody that binds to the capture antibody is added to detect the cytokine of choice. An enzyme conjugate is then added to allow visualisation of the cytokine. A precipitating enzyme substrate is then applied to allow development of coloured spots, with each spot representing a single cytokine-producing cell. The spots are then counted on an automated reader and the results are expressed as number of spot-forming cells/10^6.

All time points from a single patient were run on the same ELISpot plate to minimise variability. Each stimulation condition was prepared in triplicate. The assays were always run with 3 replicates per stimulation condition to reduce variability. In addition, a test patient PBMCs were used as an inter-assay control and tested alongside the study patient samples with each run, in order to validate performance of the assay and ensure reproducibility of each experiment. For both assays, the PBMCs were thawed as described in Section 2.3.4 and resuspended in sRPMI/10% human AB serum (Sigma, Poole, UK) and the final concentration adjusted to 2x10^6/ml. The stimulation agents were prepared in sRPMI/10% human AB serum.

2.3.5.1 Optimisation of the IL17 and IFNγ Enzyme-Linked Immunospot (ELISpot) Assays

To assess the frequency of IL17-producing peripheral blood mononuclear cells during anti-TNF treatment, I set up the IL17 ELISpot assay. As a first step, I carried out a series of preliminary experiments and adapted the protocols for the IL17 and IFNγ ELISpot assays using controls cells in order to optimise these assays (Cox et al., 2006; Janetzki et al., 2005; Lehmann, 2005). The assays were carried out at The Institute of Hepatology, University of London where the laboratory is accredited to GCLP standards for performing ELISpot assays.

Dose titration experiments were performed initially to determine the optimum concentration of each of the stimulation agents for the IL17 and IFNγ ELISpot assays. This was performed by running test IL17 and IFNγ ELISpot assays with serial dilutions of each of the stimulation agents in order to determine the optimum concentration of each one, which would provide a strong response, while still giving a good resolution of individual spots and minimal background staining. In this way, the optimum concentrations for the IL17 ELISpot assay
were determined as: anti-CD3 1 µg/ml, Phorbol-Myristate-Acetate (PMA) 50 ng/ml with Ionomycin 500 ng/ml and Phytohaemagglutinin (PHA) 1 µg/ml. For the IFNγ ELISpot assay the optimum concentrations were: anti-CD3 0.25µg/ml, PMA 25 ng/ml with Ionomycin 250 ng/ml and PHA 1µg/ml.

Figure of examples of IL17 and IFNγ ELISpot plates are illustrated in Figure 2.10.

The direct ELISpot incubation method, where cells and stimulation agents are incubated directly on the ELISpot wells was compared to the indirect ELISpot method, where cells and stimulation agents were pre-incubated in tissue culture plates before transfer to the ELISpot plates. The indirect method gave the lowest background staining and better contrast with the spots and was thus used instead of the direct method.

To determine the repeatability (intra-assay variability) of the IL17 and IFNγ ELISpot, the assays were performed using PBMCs from a test patient with three replicates per stimulation condition tested 10 times in the same experiment and the coefficient of variation (%CV) was measured for each stimulation condition. To test the inter-assay variability of the IL17 and IFNγ ELISpot assays, the same control cells were tested alongside the study patient samples with each run, in order to validate performance of the assay and ensure reproducibility of each experiment. This allowed the coefficient of variation (%CV) to be determined to evaluate the consistency between assays.

For both the IL17 assay and the IFNγ assay, the PBMCs were thawed as described in Section 2.3.4 and resuspended in sRPMI/10% human AB serum (Sigma, Poole, UK) with the final concentration adjusted to 2x10⁶/ml. The protocol for each assay was split over 3 days with the main differences in the IL17 and IFNγ ELISpot assays being the wash steps and buffers used as well as differing incubation times.
Figure 2.10  Representative images of IL17 and IFN\(\gamma\) ELISpot wells

Representative images of IFN\(\gamma\) (top images) and IL-17 (lower images) ELISpot wells showing the lack of spots in the unstimulated control wells (left hand side) and numerous spots in the stimulated wells (right hand side). PBMCs were either unstimulated (control wells) or stimulated with anti-CD3, PMA/Ionomycin or PHA. 200,000 PBMCs were added per well. Each spot represents one cytokine-secreting cell. Cell viability (by trypan blue exclusion) was >95% in all experiments.
2.3.5.2 IL17 ELISpot Assay

2.3.5.2.1 Day 1: Cell stimulation and preparation of ELISpot plates

Cell stimulation and the preparation of the ELISpot plates were performed under aseptic conditions in a Category 2 tissue culture hood. The stimulation agents were prepared in sRPMI/10% human AB serum to give final concentrations in the tissue culture plates as per Table 2.6. 100 µl of each stimulation agent (except the PMA/Ionomycin which were added immediately prior to transfer of the cells to the ELISpot plates on day 2) were plated in triplicate into 96-well round bottom tissue culture plates according to the plate layout (Table 2.7). 100 µl (200,000 PBMCs) of each patient PBMC cell suspension was then added to each well and the plates were incubated for 20 hours at 37^0C in a 5% CO₂ incubator.

IL17 (R&D Systems, Abingdon, UK) capture antibody was diluted according to the manufacturer’s instructions at a working concentration of 1:60 using sterile PBS. 100µl of the diluted capture antibody was added to each experimental well of a 96-well PVDF backed ELISpot plate (Millipore, MA, USA) and then incubated for 16 hours at 4^0C.

2.3.5.2.2 Day 2: Transfer of stimulated cells onto ELISpot plates

Transfer of stimulated cells onto the prepared ELISpot plates was performed under aseptic conditions in a Category 2 tissue culture hood. The capture antibody was flicked off the ELISpot plates and the plates blotted thoroughly onto absorbent paper. 200 µl of sterile PBS (Gibco) was added to each well to wash the plates and then flicked off and the plate blotted onto absorbent paper. This washing step was repeated three times. In order to block the plates, 200 µl of sterile PBS was added to each well and the plates were incubated for 2 hours at room temperature. The blocking buffer was then flicked off and the plates were washed once by adding 200 µl of sRPMI/10%AB serum to each well which was then flicked off and the plates were dried by blotting onto absorbent paper. The PBMCs from the 96 well round bottom tissue culture plates from Day 1 were then resuspended by gently pipetting up and down. Just prior to transfer of the PBMCs onto the Elispot plates, the PMA and Ionomycin were added to the corresponding wells of the round bottom tissue culture plates and mixed gently by pipetting up and down. The PBMCs prepared on Day 1 were then transferred to the corresponding wells on the pre-coated and blocked membrane of the Elispot plate and the plates were incubated for 24 hours at 37^0C in a 5% CO₂ incubator.
Table 2.6  Stimulation conditions and their concentrations used in the IL17 ELISPOT assay

<table>
<thead>
<tr>
<th>Well</th>
<th>Stimulation Agent</th>
<th>Final concentration in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No stimulation agent (negative control)</td>
<td>Medium only</td>
</tr>
<tr>
<td>2</td>
<td>Anti-CD3 (OKT3 clone)</td>
<td>1mg/ml</td>
</tr>
<tr>
<td>3</td>
<td>PMA and Ionomycin</td>
<td>50ng/ml PMA and 500ng/ml Ionomycin</td>
</tr>
<tr>
<td>4</td>
<td>PHA (positive control)</td>
<td>1mg/ml</td>
</tr>
</tbody>
</table>
Table 2.7  Plate layout used in the IL17 Elispot assay

<table>
<thead>
<tr>
<th>Visit</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium Medium Medium Medium</td>
<td>Anti-CD3 Anti-CD3 Anti-CD3 Anti-CD3</td>
<td>PMA1o PMA1o PMA1o PMA1o</td>
<td>PMA PMA PMA PMA</td>
</tr>
<tr>
<td>2</td>
<td>Medium Medium Medium Medium</td>
<td>Anti-CD3 Anti-CD3 Anti-CD3 Anti-CD3</td>
<td>PMA1o PMA1o PMA1o PMA1o</td>
<td>PMA PMA PMA PMA</td>
</tr>
<tr>
<td>3</td>
<td>Medium Medium Medium Medium</td>
<td>Anti-CD3 Anti-CD3 Anti-CD3 Anti-CD3</td>
<td>PMA1o PMA1o PMA1o PMA1o</td>
<td>PMA PMA PMA PMA</td>
</tr>
<tr>
<td>4</td>
<td>Medium Medium Medium Medium</td>
<td>Anti-CD3 Anti-CD3 Anti-CD3 Anti-CD3</td>
<td>PMA1o PMA1o PMA1o PMA1o</td>
<td>PMA PMA PMA PMA</td>
</tr>
</tbody>
</table>

Note: The table represents the plate layout used in the IL17 Elispot assay, with columns indicating different conditions and rows indicating different visits.
One patient’s PBMCs from each of the four time points on the study were analysed on the same Elispot plate in replicates of three for each stimulation condition. Medium only wells are unstimulated PBMCs in medium only to act as a negative control and PBMCs in the PHA wells acted as a positive control.
Day 3: Development of ELISpot plates

The PBMCs were discarded from the Elispot plate and the plate was washed four times by adding 200 μl per well of PBS/0.05% Tween-20. The detection biotinylated anti-IL17 antibody (R&D Systems) was prepared at a working concentration of 1:60 according to the manufacturer’s instructions using PBS/1%BSA and 100 μl of the prepared antibody was added to each well of the Elispot plates. The plates were then incubated for 2 hours in the dark at room temperature. Subsequently, the biotinylated antibody was discarded and the plates were washed three times by adding 200 μl per well of PBS/0.05% Tween-20. 100 μl per well of Streptavidin-AP (R&D Systems), diluted at a working concentration of 1:60 as per the manufacturer’s instructions with PBS/1%BSA, was then added to each experimental well and the plates were incubated for 2 hours at room temperature in the dark. After the incubation, the plates were washed three times by adding 200 μl per well of PBS/0.05% Tween-20 and dried by blotting onto absorbent paper. 100 μl of BCIP/NBT solution (R&D Systems) was then added to each well and the plates were incubated for 30 minutes at room temperature in the dark. After this the plates were washed with deionised water three times to stop the colour reaction and were inverted to remove excess water and then left to dry at room temperature before the spots were enumerated.

IFNγ ELISpot Assay

Day 1: Cell stimulation and preparation of ELISpot plates

Cell stimulation and the preparation of the ELISpot plates were performed under aseptic conditions in a Category 2 tissue culture hood. The stimulation agents were prepared in sRPMI/10% human AB serum to give final concentrations in the tissue culture plates as per Table 2.8. 100 μl of each stimulation agent (except the PMA/Ionomycin which were added immediately prior to transfer of the cells to the ELISpot plates on day 2) were plated in triplicate into 96-well round bottom tissue culture plates according to the plate layout (Table 2.9). 100 μl (200,000 PBMCs) of each patient PBMC cell suspension was then added to each well and the plates were incubated for 20 hours at 37°C in a 5% CO2 incubator.

IFNγ (BD Biosciences) capture antibody was diluted according to the manufacturer’s instructions at a working dilution of 1:200 using sterile PBS. 100 μl of the diluted capture
## Table 2.8  Stimulation conditions and their concentrations used in the IFN\(\gamma\) Elispot assay

<table>
<thead>
<tr>
<th>Well</th>
<th>Stimulation Agent</th>
<th>Final concentration in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No stimulation agent (negative control)</td>
<td>Medium only</td>
</tr>
<tr>
<td>2</td>
<td>Anti-CD3 (OKT3 clone)</td>
<td>0.25mg/ml</td>
</tr>
<tr>
<td>3</td>
<td>PMA/Ionomycin</td>
<td>25ng/ml PMA and 250ng/ml Ionomycin</td>
</tr>
<tr>
<td>4</td>
<td>PHA (positive control)</td>
<td>1mg/ml</td>
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Table 2.9  Plate layout used in the IFNγ ELISpot assay

<table>
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One patient’s PBMCs from each of the four time points on the study were analysed on the same ELISpot plate in replicates of three for each stimulation condition. Medium only wells are unstimulated PBMCs in medium only to act as a negative control and PBMCs in the PHA wells acted as a positive control.
antibody was added to each experimental well of a 96-well PVDF backed ELISpot plate (Millipore) and then incubated for 16 hours at 4°C.

2.3.5.3.2 Day 2: Transfer of stimulated cells onto ELISpot plates
The capture antibody was flicked off the ELISpot plates and the plates blotted thoroughly onto absorbent paper to remove any excess. The plates were washed by adding 200 µl of sRPMI/10%AB solution to each well and the plates were incubated for 2 minutes at room temperature. This was then removed and the plates blotted onto absorbent paper. 200 µl of sRPMI/10%AB was then added to each experimental well and the plates were incubated for 2 hours at room temperature to block the plates. The blocking buffer was then flicked off and the plates were washed once by adding 200 µl of sRPMI/10%AB serum to each well which was then flicked off and the plates dried by blotting onto absorbent paper. The PBMCs from the 96 well round bottom tissue culture plates from Day 1 were then resuspended by gently pipetting up and down. Just prior to transfer of the PBMCs onto the Elispot plates, the PMA and Ionomycin were added to the corresponding wells of the round bottom tissue culture plates and mixed gently by pipetting up and down. The PBMCs prepared on Day 1 were then transferred to the corresponding wells on the pre-coated and blocked membrane of the Elispot plate and the plates were incubated for 24 hours at 37°C in a 5% CO₂ incubator.

2.3.5.3.3 Day 3: Development of ELISpot plates
The PBMCs were discarded from the Elispot plate and the plate was washed twice by adding 200 µl per well of deionised water and then three times by adding 200 µl per well of PBS/0.05% Tween-20. The detection biotinylated anti-IFNγ antibody (BD Biosciences) was prepared at a working concentration of 1:250 according to the manufacturer's instructions using PBS/10% FCS and 100 µl of the prepared antibody was added to each well of the Elispot plates. The plates were then incubated for 2 hours in the dark at room temperature. Subsequently, the biotinylated antibody was discarded and the plates were washed three times by adding 200 µl per well of PBS/0.05% Tween-20. 100 µl per well of the Avidin-HRP conjugate prepared to a working concentration of 1:100 using PBS/10% FCS as per the manufacturer's instructions was then added to each experimental well and the plates were incubated for 1 hour at room temperature in the dark. After the incubation, the plates were washed 4 times by adding 200 µl per well of PBS/0.05% Tween and then twice by adding
200 µl per well of PBS. The AEC substrate was prepared according to the manufacturer's instructions (BD Biosciences) just before use and 100 µl of the solution was added to each experimental well and the colour was allowed to develop for 15 minutes in the dark at room temperature. After this the plates were washed with deionised water three times to stop the colour reaction and were inverted to remove excess water and then left to dry at room temperature before the spots were enumerated.

2.3.5.3.4 **Enumeration of spots from IL17 and IFNγ ELISpot assays**

Once the plates were dry, the numbers of IL17 or IFNγ spot forming cells were enumerated using an automated ELISpot reader (AID Diagnostika, Cadama medical, Stourbridge, United Kingdom). The count settings used for maximum and minimum values for spot size, spot density and colour gradient were determined during the preliminary experiments by reading a series of IL17 and IFNγ ELISpot plates to exclude contributions to the total number of spots from plate debris and smaller size spots produced by other immune cells. These settings were then kept consistent throughout all the ELISpot experiments.

Before reading a set of ELISpot plates, a quality control was always performed to check the performance of the ELISpot reader with a master plate (Masterlot, AID Diagnostika) provided by the reader manufacturer.

After counting the number of spots in each well, the mean of the number of spots from the non-stimulated wells (medium only) was subtracted from the mean of the number of spots of the stimulated wells and multiplied by five. The final results for the numbers of IL17 and IFNγ cytokine producing cells are expressed as number of specific-spot-forming cells/10^6 (spSFC/10^6).

In the analysis of the results obtained from the IL17 and IFNγ ELISpot assays, anti-CD3 stimulation produced a wider linear range for assessing the number of spots compared to PMA and Ionomycin, thus smaller changes in the numbers of spots could be assessed more sensitively. In view of this, the results of the IL17 and IFNγ ELISpot assays presented in **Chapter 5** are those obtained under anti-CD3 stimulation.
2.3.6 Flow Cytometry

Flow cytometry is a technique used for counting and examining individual cells suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of individual cells flowing through an optical, electronic detection system.

A beam of light of a single wavelength is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at a point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors. Each cell passing through the beam scatters light in a particular way and fluorescent markers present within the cells, or attached to the surface of the cells can be excited and emit light at a different wavelength than the light source. By detecting the combination of light scatter and fluorescent light detected it is possible to derive information about the physical and chemical structure of each particle. FSC correlates with the cell volume, whilst SSC is influenced by the inner complexity of the cell (e.g. shape of nucleus etc). Flow cytometry can be used with fluorescent tagged antibodies that will bind to specific antigens on the target cells and fluoresce following excitation.

The data generated from flow cytometry is usually represented in 2 dimensional dot plots and the regions within these plots can be separated by creating a series of subset extractions, called gates.

2.3.6.1 Titration of Antibody Concentrations and Determination of Stain Index

The following commercially available fluorescent antibodies were used for PBMC staining: Anti-CD4 (BD Biosciences), anti-CD8-PerCP/Cy5.5 (BD Biosciences), anti-CCR6-PE/Cy7 (BD Biosciences), anti-CD45RO-APC (BD Biosciences), anti-IL17A-PE – (eBioscience, Hatfield, UK), anti-IFNγ-eFluor450 (eBioscience). Aqua-Live/dead fixable dead cell stain kit (Invitrogen, Paisley, UK) was used to allow gating of live cells.
The same production lot of each of the fluorescent antibodies was used for the entire study in order to minimise antibody lot to lot variability. I carried out all the intracellular cytokine staining of the patient samples, acquisition on the FACS machine and subsequent analysis in order to minimise variability.

Prior to analysing patient samples by flow cytometry, a series of preliminary experiments were conducted to optimise the concentration of each fluorescent antibody used and the stain index for each antibody test concentration was calculated. The stain index is a normalised functional measure of reagent brightness and allows normalisation of the signal to the spread of the background, where background can be autofluorescence, nonspecific staining, electronic noise or spillover from another fluorochrome (Figure 2.11). The resolution sensitivity allowing the differentiation of a dim positive signal from the background depends on the difference between the positive and background peak medians and the spread of the background peak.

PBMCs from the same donor were prepared as described in Section 2.3.4 and aliquots of 1.5x10^6 cells were stained with a single fluorescent antibody at a different final concentration according to the method described in Section 2.3.6.2 below. The chosen concentrations to be tested for each fluorescent antibody were prepared by serial dilution using PBS/1%BSA (Sigma, Poole, UK). Cells were acquired as described below on a FACSCanto II cytometer (BD, Oxford, UK). Analysis was performed using FlowJo (Treestar Inc, Ashland, USA) by gating on the positive and negative populations and determining the median of the positive and negative populations and the 5th and 95th percentiles of the negative population. The stain index was then calculated for each fluorescent antibody concentration according to the formula:

\[
\text{Stain Index} = \frac{1.645 \times (\text{positive-background})}{\text{Background}_{95\%}-\text{background}_{5\%}} = \frac{\text{positive-background}}{2 \times \text{SD}_{\text{background}}}
\]

The optimum fluorescent antibody concentration for each fluorochrome was chosen as the one which gave a high stain index (i.e. normalised signal to the spread of background signal), while also differentiating clearly between the positive and negative populations.
The stain index allows for normalisation of the signal to the spread of the background, where background can be autofluorescence, unstained cells or spill over from another fluorochrome. The resolution sensitivity allowing the differentiation of a dim positive signal from background depends on the difference between the positive and background peak medians (D) and the spread of the background peak (W). W1 and W2 are background peaks with different spreads. The stain index is a measure that captures both of these factors.
2.3.6.2 Staining Protocol for PBMCs for flow cytometric analysis

PBMCs from each of the patient’s time points on the study (baseline, week 4 and week 12) were thawed as described in Section 2.3.4 and the final concentration was adjusted to 15x10^6 with sRPMI/10%FCS. For each time point, 1.5x10^6 PBMCs were cultured in the presence of 50ng/ml PMA (Calbiochem, Nottingham, UK) and 500ng/ml ionomycin (Calbiochem) for 5 hours and 10μg/ml Brefeldin A (Sigma) was added to encourage accumulation of cytokines intracellularly. 1.5x10^6 PBMCs from each time point were also cultured for 5 hours in the presence of Brefeldin A only to act as unstimulated controls. After 5 hours, cells were washed with PBS and incubated with Aqua live/dead fixable dead cell kit (Invitrogen) for 30 minutes at 4°C in the dark as per the manufacturer’s instructions. Cells were then washed with PBS and stained with CD4-FITC (at a working concentration of 1:50), CD8-PerCP/Cy5.5 (at a working concentration of 1:100), CD45RO-APC (at a working concentration of 1:50) and CCR6-PE/Cy7 (at a working concentration of 1:50) for 30 minutes at 4°C in the dark. Cells were then washed with PBS/1%BSA and fixed with Cytofix (BD Biosciences) for 20 minutes at 4°C in the dark. For the intracellular staining, cells were permeabilised with 0.05% saponin (Sigma) and stained with anti-IL17-PE (at a working concentration of 1:20) and anti-IFNγ-eFluor 450 (at a working concentration of 1:50) for 30 minutes at room temperature in the dark. Cells were then washed with 0.05% saponin and resuspended in PBS/1%BSA and acquired on a FACS Canto II cytometer (BD Biosciences) and 100,000 events were acquired each time.

Prior to acquiring the study samples, unstained cells were used to set PMT voltages and compensation beads stained individually with each of the fluorescent antibodies were used to set compensation (BD Biosciences). These settings were maintained for the duration of the study. Fluorescence minus one (FMO) and unstimulated controls were used to differentiate positive and negative populations and set gates appropriately (Section 2.2.6.3). Analysis was performed using FACS DIVA software (BD Biosciences). Live cells were gated by excluding the dead cells stained by the live/dead stain, doublets were excluded and lymphocytes were identified by forward and side scatter profiles as well as backgating using CD8 as a marker. The gating strategy for the selection of CD4+ and CD8+ cells from PBMCs is illustrated in Figure 2.12.
Figure 2.12  Gating strategy for the selection of CD4+ and CD8+ cells from PBMCs.

Representative flow cytometry dot plots illustrating cell gating strategy for the selection of CD4+ and CD8+ cells from PBMCs. Forward scatter area versus forward scatter width (A) and side scatter area versus side scatter width (B) allow the exclusion of doublets; dead cells are then excluded based on staining with live/dead cell stain kit (C) and once gated on live cells, the lymphocyte population is gated using forward and side scatter profiles (D) and backgating using CD8 as a marker. Gating on the lymphocyte population then allows CD4+ and CD8+ cells to be gated (E).
2.3.6.3 Flow Cytometry Controls and Determination of Positivity

Cytometer set-up and tracking (CST) beads (BD Biosciences) allow the software to automatically characterise, track and report measurements of BD digital flow cytometers. Each vial of CST beads contains an equal concentration of beads of 3 fluorescence emission intensities. The beads are used to define a cytometer baseline and detect any deviations from that baseline. Median fluorescence intensity (MFI) and robust CV (rCV) were measured for each bead in all fluorescence detectors. The software then calculates the fluorescence detection efficiency (Qr), relative background (Br), the standard deviation of electronic noise and the cytometer settings can subsequently be adjusted in order to maximise the population resolution in each detector.

Fluorescence minus one (FMO) and unstimulated controls were used to help set the gate boundaries for the markers without a clearly bimodal expression: IL17 (Figure 2.13), IFN\(\gamma\) (Figure 2.14), CD45RO (Figure 2.15) and CCR6 (Figure 2.16), in order to accurately determine the positive and negative cell populations.

FMO controls are samples that include all of the antibody conjugates present in the test samples except one and the channel in which the antibody conjugate is missing is the one for which the FMO provides the gating control. FMO controls are particularly pertinent to multicolour flow cytometry experiments, especially ones using >4 colours as the major source of background staining in these experiments tends to be fluorescence spill over (Maecker and Trotter, 2006). FMO controls will allow the assessment of the effect of spill over-induced background staining from other populations on the channel of interest. Preliminary experiments were performed using FMO controls for IL17, IFN\(\gamma\), CD45RO and CCR6 on test PBMCs prepared as described in Section 2.2.6.2 except the antibody for which the FMO control was for was not added. These experiments showed that there was no significant donor variability in the controls for the markers tested between PBMCs from different donors which allowed the gating boundaries for the markers IL17, IFN\(\gamma\), CD45RO and CCR6 to be set and these were utilised for all study samples.

Further controls in the form of unstimulated PBMCs were used to act as a biological comparison control to further check the setting of the positive/negative boundaries for the
Figure 2.13  Fluorescence minus one control (FMO) for IL17-PE

Representative flow cytometry dot plots illustrating the determination of positivity and setting of gates using fluorescence minus one (FMO) control for IL17-PE.
Figure 2.14  Fluorescence minus one controls (FMO) for IFNγ-Pacific Blue

Representative flow cytometry dot plots illustrating the determination of positivity and setting of gates using fluorescence minus one (FMO) controls for IFNγ-Pacific Blue.
Figure 2.15  Fluorescence minus one controls (FMO) for CD45RO-APC

Representative flow cytometry dot plots illustrating the determination of positivity and setting of gates using fluorescence minus one (FMO) controls for CD45RO-APC.
Figure 2.16 Fluorescence minus one controls (FMO) for CCR6-PE/Cy7

Representative flow cytometry dot plots illustrating the determination of positivity and setting of gates using fluorescence minus one (FMO) controls for CCR6-PE/Cy7.
intracellular cytokines IL17 and IFNγ (Figure 2.17). Unstimulated controls are PBMCs prepared as described in Section 2.3.6.2 and stained with the full panel of antibody conjugates except the cells have not been stimulated with PMA/Ionomycin. Unstimulated controls, like FMO controls also account for spill over effects on the channel of interest by including all the antibody conjugates present as in the study samples. In addition they also account for nonspecific staining in the channel of interest (Maecker and Trotter, 2006).

2.3.7 Serum samples

Blood samples were collected by venepuncture at each patient study visit in Vacutainer tubes with no additives (BD Biosciences). Tubes were allowed to clot for at least 30 minutes before centrifugation at 1000g for 15 minutes and the serum was aliquoted in 500 µl aliquots and these were stored in a -80°C freezer.

2.3.8 Determination of Serum Cytokines using Cytometric Bead Array (CBA)

The CBA was performed by Erin Paterson from the Translational Research Laboratory in the Kennedy Institute of Rheumatology.

Cytometric bead array (CBA) uses the principles of flow cytometry and employs particles with discrete fluorescence intensities to detect soluble analytes. The principles of the assay are illustrated in Figure 2.18. This assay uses single bead populations (called a flex set) with a distinct fluorescence intensity coated with a capture antibody specific for a soluble protein, e.g. IL17 and allows the simultaneous detection of multiple cytokine proteins in research samples when individual flex sets are mixed together (this forms the bead array). During the assay procedure the capture beads are mixed with recombinant standards and the test samples and then incubated with PE-conjugated detection antibodies to form sandwich complexes. Each bead population is given an alphanumerical position in the near infra-red and red channels of supporting BD cytometers designating its position relative to other bead populations. Beads with different alphanumerical positions can be combined in the same assay to create a multiplexed assay to detect multiple proteins in a single sample. The intensity of PE fluorescence of each of the sandwich complexes reveals the concentration of that cytokine or protein of interest in the test samples. The advantages of this technique over
Figure 2.17 Unstimulated controls for IL17 and IFNγ

Representative flow cytometry dot plots illustrating the setting of gates for IL17 (A) and IFNγ (B and C) using unstimulated controls as biological comparison controls in which PBMCs were not stimulated with PMA and Ionomycin but were stained with the full antibody panel (images on left side) compared to PBMCs which were stimulated with PMA and Ionomycin for 5 hours as per protocol (images on right side).
Figure 2.18  Schematic illustration of the cytometric bead array (CBA) assay methodology

Capture beads, each with a distinct fluorescence intensity and coated with a capture antibody specific for a soluble protein are mixed with recombinant standards and test samples (A). They are then incubated with PE-conjugated detection antibodies and allowed to form sandwich complexes (B). After a wash step (C), the samples are analysed on a flow cytometer. Each bead population has a unique alphanumerical position in the near infra-red and red channels of the flow cytometer. The intensity of PE fluorescence of each sandwich complex (D) allows the concentration of the protein of interest in the test sample to be determined (E). Figure adapted from: BD Cytometric Bead Array Technical Notes, BD Biosciences
conventional ELISA methodology are that CBA allows the measurement of a number of proteins of interest simultaneously in a test sample, while using fewer sample dilutions and is also more time efficient.

2.3.8.1 Quality Control
A quality control assay using 30 Plex Bead Mixture (BD Biosciences) was prepared as per the manufacturer’s instructions and run to check that the individual bead positions are visible and clearly separated each time prior to analysing the CBA assay samples on the cytometer.

2.3.8.2 CBA Assay
The following cytokines were tested for in the patient serum samples from each study time point by CBA: IL17, IFNγ, IL10, IL6, TNFα, TNFRI, TNFRII. All flex sets were obtained from BD Biosciences, Oxford as an individual flex set kit and the assay buffers were obtained as part of a Master Buffer Kit (BD Biosciences).

2.3.8.2.1 Preparation of CBA Human Soluble Protein Flex Set Standards
For each multiplex assay a standard curve was first prepared. One lyophilised standard vial from each flex set to be tested was opened and pooled together into a tube, labelled with “Top standard”. These standards were then reconstituted with 4 ml of Assay Diluent (BD Biosciences) and allowed to stand for 15 minutes. Eight tubes for 2 fold standard dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) were then prepared and 500 μl of Assay Diluent was added to each of the tubes. A 2 fold serial dilution was then performed using the top standard by transferring 500μl from the top standard to the 1:2 dilution and so forth. Two additional tubes, one containing Assay Diluent only served as a zero standard and the one containing the top standard allowed the formation of a 10 point standard curve.

2.3.8.2.2 Preparation of CBA Human Soluble Protein Flex Set Capture Beads and PE Detection Reagents
A 15ml falcon tube was labelled with “Mixed Capture Beads“. The number of samples/standards to be tested was calculated. The capture beads for each flex set were
vortexed for at least 15 seconds to resuspend the beads thoroughly. For each individual flex set to be included in the assay, 1 μl of capture beads per standard/sample to be tested was added to the _Mixed Capture Beads‘ tube. 500 μl of Wash Buffer was added and tapped to mix. This mixture was then centrifuged at 2000 rpm for 5 minutes. The supernatant was carefully discarded without dislodging the bead pellet and the capture beads were resuspended in Capture Bead Diluent allowing 50 μl per standard/sample to be tested. This was stored away from direct light.

The PE detection reagents provided with each flex set were then prepared. A 15 ml falcon tube was labelled with _Mixed PE Detection Reagent‘ and the number of standards/samples to be tested was calculated. For each flex set, 1μl of PE Detection Reagent per standard/sample was added to the _Mixed PE Detection Reagent‘ tube. Allowing a final volume of 50 μl per standard/sample to be tested, the PE Detection Reagent was diluted using Detection Reagent Diluent. The mixture was stored at 4°C away from direct light.

2.3.8.2.3 Sample Preparation for the CBA Assay

Tubes with the individual sample numbers were labelled. Serum samples were diluted 1:4 using Assay Diluent as per the manufacturer‘s recommendations.

To run samples using the CBA assay, Millipore multiscreen filter plates were used (Millipore, MA, USA). 100 μl of Wash Buffer was dispensed to each well of the filter plate to be assayed. The Wash Buffer was then removed using a vacuum manifold with pressure not exceeding 10 mmHg and aspiration was performed until the wells were drained. To ensure the filter was no longer porous following the aspiration, excess moisture was removed by blotting the bottom of the filter plate onto absorbent paper.

The _Mixed Capture Beads‘ tube was vortexed and 50 μl of Mixed Capture Beads were dispensed into each plate well. 50 μl of standard or sample was then added to the designated wells on the filter plate. The _zero‘ standard was dispensed into an additional well at the end of the plate to allow for a cluster test prior to the full analysis of the plate. The plate was covered and placed onto a digital shaker at 500 rpm for 5 minutes and then incubated for 1 hour away from direct light. 50 μl of _Mixed PE Detection Reagent‘ was added to each well of the plate. The plate was covered and placed onto a digital shaker at 500 rpm for 5 minutes
and then incubated for 2 hours away from direct light. The reagents were then aspirated using a vacuum manifold as described above and the plate blotted dry. The beads were resuspended in 200 µl Wash Buffer and the plate was covered and placed on a digital shaker at 500 rpm for 5 minutes. The samples were analysed on the day of the experiment using a BD FACS Array cytometer with FCAP Array software (BD Biosciences) to calculate the cytokine concentrations.

2.3.9 Determination of Serum Cytokines in the Rheumatoid Arthritis Cohort Using the MesoScale Discovery (MSD) Multi-Spot Assay System

The MSD (MesoScale Diagnostics, Gaithersberg, MD, USA) assay is a multiplex platform which allows the simultaneous measurement of a number of proteins of interest in a single small-volume sample. An antibody for a specific protein target is coated on one electrode (‗spot‘) per well in a single plex assay, or in a multiplex assay an array of capture antibodies against different targets is patterned on spatially distinct spots in the same well. The samples and solutions which contain labelled detection antibodies with the electrochemiluminescent compound MSD SULFO-TAG™ are then added. The analytes in the sample bind to the capture antibodies immobilised on the working electrode surface and the detection antibodies are recruited forming a sandwich complex. Addition of a read buffer allows the appropriate environment to develop for electrochemiluminescence to occur and the plate is loaded onto an MSD machine for analysis. In the machine, a voltage applied to the plate electrodes causes labels bound to the electrode surface to emit light. The intensity of the emitted light is measured which corresponds to a quantitative measurement of the protein of interest.

2.3.9.1 MSD Assay Procedure

The MSD assays were employed to analyse the following proteins of interest in the serum samples of the RA cohort at baseline, 1 week and 12 weeks after anti-TNF was started: IL6, IL8, IL12p70, IFNγ, IL17, IL10, TNFα, thymus-and activation-regulated cytokine (TARC), macrophage inflammatory protein 1β (MIP1β), monocyte chemotactic protein-1 (MCP1), monocyte chemotactic protein-4 (MCP4), interferon-inducible protein-10 (IP10). These assays were carried out by Mr Andrew Palfreeman and Dr Fiona McCann at the Kennedy Institute of Rheumatology as part of a collaborative study.
The Proinflammatory 9 plex Ultra-Sensitive Kit included all reagents and plates necessary to test for IFNγ, IL10, IL12p70, IL6, IL8 and TNFα and the Chemokine 7 plex Ultra-Sensitive Kit included all reagents and plates to test for TARC, MIP1β, MCP4, MCP1 and IP10. Testing for IL17 used a single plex kit. All kits were from MesoScale Diagnostics, Gaithersberg, MD, USA.

The lower limit of detection for each of the proteins of interest was as follows: IL8, 0.09 pg/ml; IL12p70, 1.4 pg/ml; IFNγ, 0.53 pg/ml; IL6, 0.27 pg/ml; IL10 0.21 pg/ml; TNFα, 0.50 pg/ml; IL17, 0.2 pg/ml; MIP1β, 8.3 pg/ml; TARC, 2.9 pg/ml; IP-10, 2.9 pg/ml; MCP1, 2.5 pg/ml; MCP4 6.3 pg/ml.

2.3.9.1.1 Reagent Preparation

All reagents were brought to room temperature and were made up according to the manufacturer's instructions. The stock Calibrator solution (MesoScale Diagnostics) was prepared by dilution as directed in each kit using Diluent 2 (MesoScale Diagnostics). An 8 point standard curve consisting of three replicates per point was prepared by a 4 fold serial dilution using Calibrator stock solution and Diluent 2, where the stock calibrator acted as the top standard and the diluent alone was the zero standard.

2.3.9.1.2 Assay procedure

Serum samples were thawed and used undiluted in the assays. 25 μl of Diluent 2 was added to each well and the plate sealed with adhesive tape and incubated for 30 minutes on a plate shaker at 500 rpm at room temperature. 25 μl of sample or Calibrator was dispensed into the relevant wells of the MSD plate and the plates were sealed with adhesive tape and incubated for 2 hours on a plate shaker at 500 rpm for 2 hours at room temperature. The plate was washed three times with PBS/0.05% Tween-20. 25μl of 1x Detection Antibody Solution (MesoScale Diagnostics) was added to each well of the MSD plate and the plate was sealed and incubated for 2 hours on a plate shaker at 500 rpm at room temperature. The plate was washed three times with PBS/0.05% Tween-20 and 150 μl of 2xRead Buffer T (MesoScale Diagnostics) was added to each well of the MSD plate. The plate was then analysed on a MSD SECTOR Imager 2400 (MesoScale Diagnostics).
2.3.10 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs are plate-based assays designed for detecting and quantifying a protein of interest in a test sample. A monoclonal antibody specific for the cytokine to be measured is pre-coated onto a microplate. Standards and samples are pipetted into wells and the cytokine of interest is bound by the immobilised antibody. Unbound substances are washed away and an enzyme-linked polyclonal antibody specific for the cytokine of interest is added to the wells. Unbound antibody-enzyme conjugate is washed away and a substrate solution is added to the wells. Colour develops in proportion to the amount of cytokine of interest bound in the initial step and colour development is stopped and colour measured.

2.3.10.1 ELISA Procedure

The ELISAs were performed by Ms Erin Paterson from the Translational Research Laboratory in the Kennedy Institute of Rheumatology. ELISA kits for testing patient serum samples for IL12p70, IL12/23p40 and IL23 were obtained and used exactly according to the manufacturer’s instructions (R&D Systems). The lowest limit of detection of the ELISAs for IL12 was <7.8 pg/ml, for IL12/IL23p40 it was <31.2 pg/ml and for IL23 it was <39 pg/ml.

2.3.10.1.1 IL12 ELISA Reagent preparation

20 ml of Wash Buffer concentrate was diluted into deionised water to prepare 500 ml of Wash Buffer. 20 ml of Calibrator Diluent RD5C concentrate (R&D Systems) was added to 80 ml deionised water to yield 100 ml of Calibrator Diluent RD5C. The IL12 standard was reconstituted with 5 ml of Calibrator Diluent RD5C to produce a stock solution of 500 pg/ml and this was allowed to sit for 15 minutes prior to making dilutions. 500 μl of Calibrator Diluent RD5C was pipetted into each tube and this stock solution was used to produce a 1:2 dilution series, where the undiluted standard served as the highest standard (500 pg/ml) and
the Calibrator Diluent RD5C served as the zero standard (0 pg/ml). In this way an eight point standard curve was generated.

2.3.10.1.2 IL12 ELISA Assay Procedure

50 μl of Assay Diluent RD1F (R&D Systems) was added to each well of the microplates supplied with the kit and 200 μl of standard, control or sample was added per well and the plate covered and incubated for 2 hours at room temperature. The wells were aspirated and washed three times by filling each well with 400 μl of Wash buffer and excess moisture was removed by blotting the plate against absorbent paper. 200 μl of IL12 conjugate was added to each well and the plates covered and incubated for 2 hours at room temperature. The plates were washed three times by filling each well with 400 μl of Wash buffer and excess moisture was removed by blotting the paper against absorbent paper. The Substrate Solution (R&D Systems) was made by mixing colour reagents A and B in equal volumes within 15 minutes of use and stored protected from light.

200 μl of substrate solution was added to each well and the plates were incubated for 20 minutes at room temperature in the dark. 50 μl of Stop Solution was added to each well and the colour was allowed to develop. The optical density was determined within 30 minutes using a microplate reader.

2.3.10.1.3 IL12/23p40 ELISA Reagent Preparation

20 ml of Wash Buffer concentrate was diluted into deionised water to prepare 500 ml of Wash Buffer. 20 ml of Calibrator Diluent RD5P concentrate was added to 80 ml deionised water to yield 100 ml of Calibrator Diluent RD5P. The IL12/23p40 standard was reconstituted with 1 ml of deionised water to produce a stock solution of 20,000 pg/ml and this was allowed to sit for 15 minutes prior to making dilutions. 900 μl of Calibrator Diluent RD6-13 was pipetted into each tube and this stock solution was used to produce a 1:2 dilution series, where the 2000 pg/ml standard served as the highest standard (2000 pg/ml) and the Calibrator Diluent RD6-13 served as the zero standard (0 pg/ml). In this way an eight point standard curve was generated.
2.3.10.1.4 IL12/23p40 ELISA Assay Procedure

100 μl of Assay Diluent RD1W was added to each well of the microplates supplied with the kit and 100 μl of standard, control or sample was added per well and the plate covered and incubated for 2 hours at room temperature. The wells were aspirated and washed four times by filling each well with 400 μl of Wash buffer and excess moisture was removed by blotting the paper against absorbent paper. 200 μl of IL12/23p40 conjugate was added to each well and the plates were covered and incubated for 2 hours at room temperature. The plates were washed four times by filling each well with 400 μl of Wash Buffer and excess moisture was removed by blotting the plate against absorbent paper. The Substrate Solution was made by mixing colour reagents A and B in equal volumes within 15 minutes of use and stored protected from light. 200 μl of Substrate Solution was added to each well and the plates were incubated for 30 minutes at room temperature in the dark. 50 μl of Stop Solution was added to each well and the colour was allowed to develop. The optical density was determined within 30 minutes using a microplate reader.

2.3.10.1.5 IL23 ELISA Reagent Preparation

20 ml of Wash Buffer concentrate was diluted into deionised water to prepare 500 ml of Wash Buffer. The IL23 standard was reconstituted with deionised water to produce a stock solution of 10,000 pg/ml and this was allowed to sit for 15 minutes prior to making dilutions. 750 μl of Calibrator Diluent RD5-16 was pipetted into each tube and this stock solution was used to produce a 1:2 dilution series, where the 2500 pg/ml standard served as the highest standard and the Calibrator Diluent served as the zero standard (0 pg/ml). In this way an eight point standard curve was generated.

2.3.10.1.6 IL23 ELISA Assay Procedure

100 μl of Assay Diluent RD1-22 was added to each well of the microplates supplied with the kit and 100 μl of standard, control or sample was added per well and the plate covered and incubated for 2 hours at room temperature on a plate shaker set at 500 rpm. The wells were aspirated and washed four times by filling each well with 400 μl of Wash Buffer and excess moisture was removed by blotting the plate against absorbent paper. 200 μl of IL23 conjugate was added to each well and the plates covered and incubated for 2 hours at room temperature.
on the plate shaker. The plates were washed four times by filling each well with 400 μl of Wash Buffer and excess moisture was removed by blotting the paper against absorbent paper. The Substrate Solution was made by mixing colour reagents A and B in equal volumes within 15 minutes of use and stored protected from light. 200 μl of Substrate Solution was added to each well and the plates were incubated for 30 minutes at room temperature in the dark. 50 μl of Stop Solution was added to each well and the colour was allowed to develop. The optical density was determined within 30 minutes using a microplate reader.

2.4 Statistical Analysis

Due to the exploratory design of the study, the sample size was based on feasibility. Data were analysed using Prism Version 5 (GraphPad Software Inc, La Jolla, USA). Values are expressed as mean ± standard deviations. Comparisons between patients and healthy controls and between disease groups were made using unpaired t test or Mann Whitney U test for parametric and non-parametric data, respectively. To determine the effects of anti-TNF treatment on various parameters, the time points on treatment were compared to baseline using Wilcoxon signed rank matched pairs test for paired non-parametric data. Due to some patients missing a study visit, or some missing results from certain visits it was not possible to analyse the effects of anti-TNF treatment on the various parameters using ANOVA. Correlation coefficients were obtained using Spearman’s rank. For all tests, p values of less than 0.05 were considered significant.

To determine the parallel scan intra-reader variability in grey scale and power Doppler ultrasound image acquisition in scanning 5 RA patients twice at the same sitting, the intra-class correlation coefficient for each ultrasound endpoint was determined using the free online calculation provided by the Department of Obstetrics and Gynaecology of the Chinese University of Hong Kong (Seymour, 2012). To determine the within scan intra-reader reproducibility in grey scale and power Doppler ultrasound image scoring in the RA patient group, the joint by joint and composite score comparisons between the first and second readings of 10 patient scans were compared and the intra-class correlation coefficient was determined using the free online calculation provided by the Department of Obstetrics and Gynaecology of the Chinese University of Hong Kong (Seymour, 2012).
Chapter 3. Clinical characteristics of study patients, the effect of anti-TNF treatment on clinical outcome measures and definitions of anti-TNF treatment response

3.1 Introduction

The objective of the clinical study was to recruit and characterise a cohort of anti-TNF naïve patients with active rheumatoid arthritis (RA), psoriatic arthritis (PsA) or ankylosing spondylitis (AS) and to follow them prospectively at predetermined protocol visits for the first 12 weeks after starting anti-TNF treatment. According to the study protocol, the aim was to recruit 20-25 patients with RA, 10-15 patients with PsA and 10-15 patients with AS. The effect of anti-TNF treatment on both validated clinical disease activity measures and patient-reported outcomes was analysed to allow robust phenotyping of the three patient cohorts. The overall objective was to identify treatment responders and non-responders and to evaluate the changes in the frequency of circulating Th1 and Th17 cells during anti-TNF treatment in order to determine immune correlates of treatment response.

The study protocol with predetermined study visits and a panel of investigations was developed to allow a standardised and systematic assessment of the patients. Ethical approvals were obtained as detailed in Chapter 2. I had a leading role in setting up the study protocol, obtaining ethics approvals, recruiting and monitoring of all the patients in the clinical study.

3.1.1 Anti-TNF therapy for rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis

In recent years, the emphasis in the treatment strategy of inflammatory arthritis has changed from pain management and rehabilitation to early reduction of inflammation and prevention of joint damage through timely use of disease modifying anti-rheumatic drugs (DMARDs) with the aim to ‘treat to target’ and achieve low disease activity or induce remission (Smolen
et al., 2010). DMARDs including agents such as methotrexate, sulfasalazine, leflunomide or hydroxychloroquine are utilised early in the disease course in RA and PsA in order to achieve prompt control of disease. Until recently, treatment of AS relied solely on the use of non-steroidal anti-inflammatory agents (NSAIDs) and physiotherapy, with sulfasalazine or, rarely, methotrexate only used in AS patients who had marked peripheral arthritis, as these agents have no effect on axial symptoms (Braun and Baraliakos, 2009).

However, the lack of efficacy of these agents in a substantial proportion of patients, their side effect profiles, as well as the improved understanding of molecular mechanisms of inflammation and cytokine involvement in disease pathogenesis led to the development of biologic agents including anti-TNF therapies (infliximab, etanercept, adalimumab, certolizumab and golimumab), which have revolutionised the treatment of these three diseases (Elliott et al., 1994; Feldmann and Maini, 2003; Maini et al., 1998). In the majority of patients with RA, these agents have a profound effect on symptoms and also slow radiographic progression (Singh JA, 2009). Similarly, in PsA anti-TNF treatment leads to improvement in signs and symptoms of arthritis, dactylitis, enthesitis, skin and nail disease and has a significant impact on radiographic progression (Mease, 2011b; Schett et al., 2007; van der Heijde et al., 2007). AS patients experience benefits at any stage of the disease both in axial and extra-spinal manifestations, although effects seem to be greatest in early disease. Spinal inflammation as assessed by MRI improves significantly, but anti-TNF has so far failed to show inhibition of radiological progression and ankylosis (Braun and Kalden, 2009).

According to the National Institute for Health and Care Excellence (NICE) in the UK, the use of biologics is restricted to patients with severe uncontrolled disease who have failed therapy with at least two DMARDs in RA and PsA, or NSAIDS in patients with AS. Anti-TNF agents are the first line biologics that can be prescribed. Patients with RA are considered eligible for anti-TNF treatment if they have persistent active disease (DAS28 score ≥5.1) on two separate occasions 1 month apart and have failed to respond to two DMARDs, including methotrexate (TA130, NICE 2007). In PsA, anti-TNF is prescribed if there is persistent active disease (≥3 tender and ≥3 swollen joints) and a lack of response to two DMARDs (TA199, NICE 2010). In AS, anti-TNF agents are prescribed for active disease (BASDAI ≥4 on two occasions 3 months apart) and lack of response to 2 different NSAIDs (TA143, NICE 2008). These recommendations in the UK differ from the guidelines from key international
rheumatological societies including the British Society of Rheumatology (BSR), the American College of Rheumatology (ACR) and the European League Against Rheumatic Diseases (EULAR). Their recommendations are that biologic agents are started early in the course of disease in order to take advantage of the ‘window of opportunity’ for early disease control (Ash et al., 2012; Gossec et al., 2012; Smolen et al., 2010). For example, in RA a common consensus is that a biologic should be started in patients who fail to achieve a DAS28 score <3.2 after treatment with DMARDs (Deighton et al., 2010; Saag et al., 2008; Smolen et al., 2010).

Despite the success of anti-TNF therapy in the majority of patients, 20-30% of patients still do not respond to this treatment. In addition, some patients experience side effects, such as injection site reactions, reactivation of tuberculosis or increased susceptibility to infections, some of which may be severe. Long term use of anti-TNF agents has been reported to increase the risk of non-melanoma skin cancers (Ding et al., 2010; Singh JA, 2009).

3.1.2 Clinical assessment of disease activity

Assessment of disease activity in inflammatory arthritis in the clinical setting includes history taking, physical examination, measurement of the acute phase response using inflammatory markers such as the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) and the use of composite measures of disease activity.

3.1.2.1 Rheumatoid Arthritis

Continuous composite scores are widely used in RA disease activity assessment in clinical trials and routine clinical practice as they produce a single score that is more responsive to change than individual components and provide more clinically meaningful estimates of disease activity.

The American College of Rheumatology (ACR) criteria and the Disease Activity Score (DAS) developed by American and European investigators respectively are the commonest composite scores used. The ACR criteria define a treatment response by a minimum of 20% improvement from baseline (ACR20) in tender and swollen joint counts and three of five...
additional assessments (physician global assessment, ESR, functional disability score, pain score, patient global assessment). Similarly 50% and 70% improvement scores (ACR50 and ACR70) have also been defined (Ranganath et al., 2006). However, a limitation of the ACR criteria is that they measure changes in signs and symptoms over time, without reflecting current disease activity, which limits its use in routine clinical practice.

The DAS score is a composite score developed using a calculated weighting system of key clinical variables including number of tender and swollen joints (commonly out of 28 joints), patient global assessment of disease activity on a 0-100mm visual analogue scale (VAS) and measurement of acute phase response (typically ESR) (Anderson et al., 2011). A score <3.2 indicates low disease activity, scores 3.2-5.1 indicate moderate disease activity, and scores >5.1 show high disease activity. A change in DAS28 >1.2 is considered a significant change. The DAS28 score has been extensively validated and is endorsed by ACR and EULAR for use in RA clinical trials and is often considered 'the gold standard' by which to measure RA disease activity and to which newer scores are compared (Anderson et al., 2011).

However, both the ACR criteria and DAS scores are based on individual components that can be subjective as well as insensitive to change. The Simplified Disease Activity Index (SDAI) was developed to overcome some of the shortcomings of the DAS score and avoid calculations. It involves a numerical addition of individual measures (28 tender joint count, 28 swollen joint count, patient global assessment on a 0-10 VAS, physician global on a 0-10 VAS and CRP measurement in mg/l), thus overcoming the problems due to mathematical transformations and weighting of individual components (Anderson et al., 2011).

Based on the SDAI, The Clinical Disease Activity Index (CDAI) was developed for use in routine clinical practice at the point of assessment as it does not include a laboratory value. It is composed of a 28 swollen joint count, a 28 tender joint count, patient global assessment on a 0-10 VAS and physician global on a 0-10 VAS. Both the SDAI and CDAI are endorsed by the ACR and EULAR for disease activity measurements in clinical trials and by EULAR for patient monitoring in clinical practice. However, the exclusion of a laboratory marker may decrease the construct validity of the CDAI (Anderson et al., 2011).
3.1.2.2 Psoriatic Arthritis

Many of the outcome measures used to evaluate aspects of PsA disease burden have been borrowed from the study of RA for the assessment of the peripheral joint disease; or AS for the assessment of spinal and entheseal involvement and from psoriasis for the assessment of skin involvement.

In PsA, the ACR response criteria and DAS scores have been widely used in clinical studies, although they only assess a limited number of the joints that could potentially be involved in PsA (Gladman et al., 2004). The Psoriatic Arthritis Response Criteria (PsARC) have been specifically developed for PsA and comprise an assessment of swollen joint count (out of 74 joints), a tender joint count (out of 76 joints), a physician and a patient global assessment of disease activity on a 0-5 scale (Mease, 2011a). PsARC responses in clinical trials of leflunomide, etanercept and infliximab have shown a good correlation with the modified ACR20 responses (Gladman et al., 2004; Mease, 2011a). The PsARC criteria also shares some of the limitations of the ACR and DAS criteria in that the joint assessments may be subjective and insensitive to change. In addition, the assessment of PsARC may be compounded by and would also not take into account dactylitis, enthesitis or axial disease, which are other characteristic manifestations of PsA (Mease, 2009). PsARC has also been shown to display a high placebo response rate (Mease, 2011a). Another limitation is that it allows response to be determined in a dichotomous way, i.e. whether a patient is a responder or non-responder, but does not allow for a magnitude of the response to be determined.

As patients with PsA may display other features of the disease other than peripheral arthritis, such as skin, nail, enthesal and axial disease, newer composite disease activity measures that incorporate all aspects of the disease have been recently developed but require further validation (Mease, 2011a). Currently, disease activity scores for the assessment of psoriasis skin lesional burden in PsA rely on the use of tools used in patients with psoriasis, such as the Psoriasis Area and Severity Index (PASI) score. It is the most commonly used measure and combines the assessment of body surface area involved, the percentage coverage by skin disease and severity of lesions based on degree of erythema, induration and desquamation. The total score ranges from 0-72 and limitations include overestimation of area of involvement due to the heavy weighting of body surface area in the total score, poor
sensitivity to change and poor responsiveness at the extremes of the scale (Coates and Helliwell, 2010).

3.1.2.3 Ankylosing Spondylitis

The Assessment of Ankylosing Spondylitis International Society (ASAS) recommends routine measurement of patient global health, spinal pain, morning stiffness, physical function and fatigue in monitoring patients with AS (Sieper et al., 2009). The Bath Indices comprising disease activity index (BASDAI), functional index (BASFI) and metrology index (BASMI) are commonly used tools for assessing different aspects of AS, which encompass the components recommended by ASAS. The BASDAI is a questionnaire completed by patients which assesses fatigue, spinal and peripheral joint pain and morning stiffness on a 0-100mm visual analogue scale and has become the ‘gold standard’ measure in clinical trials and daily practice. A BASDAI score greater than 4 is considered to represent high disease activity. The BASFI is a composite score of 10 questions concerning activities of daily living measured on a 0-100mm VAS scale. The BASMI score assesses spinal mobility and is calculated from tragus-to-wall distance, degree of cervical and lateral spinal rotation and flexion, modified Schoeber’s index and intermalleolar distance (Sieper et al., 2009; van Tubergen and Landewe, 2009). All of these indices have been shown to be reliable and sensitive to change in patients with AS, but they are either purely patient or physician-orientated, and each assesses separate parts of the disease. In addition, the measures of symptoms and impairment in AS are not specific for inflammatory processes, as they also capture mechanical symptoms and limitations due to spinal fusion. Other components of the disease process such as enthesitis and peripheral joint involvement are not directly assessed by these composite measures and each have their own separate assessment tools.

ASAS have recently developed the AS Disease Activity Score (ASDAS) in order to counteract the problems presented by the Bath Indices by including both patient-centred items and objective measures of inflammation. It comprises 3 items from BASDAI (question 2 on back pain, question 3 on peripheral swelling and question 6 on duration of morning stiffness), as well as the patient global assessment on a 0-100mm VAS and CRP measurement. ASDAS <1.9 has been proposed as low disease activity, ASDAS >4.5 as high disease activity and a change in ASDAS >1.85 as considerable improvement (Sieper et al.,
The ASDAS has demonstrated construct validity and high responsiveness during treatment with anti-TNF and although still being validated it is now being used in clinical trials (Pedersen et al., 2010b; Zochling, 2011).

3.1.3 Biologic measures of inflammation to assess disease activity

The most frequently used laboratory markers of disease activity in the clinical setting are the inflammatory markers, ESR and CRP. In patients with RA, ESR and CRP have been shown to correlate with disease activity, radiographic progression and to be responsive to treatment (Combe et al., 2001; Lindqvist et al., 2005). However, up to 40% of patients with RA can have normal levels of these acute phase markers (Pincus and Sokka, 2009).

The utility of ESR and CRP in PsA is more limited. In PsA, only 40-60% of patients demonstrate an elevation in ESR or CRP and this tends to occur more commonly in patients with polyarticular joint involvement. High ESR at presentation has been shown to be associated with early mortality and progression of PsA joint disease (Gladman et al., 2004). Studies have suggested that if PsA patients have elevated ESR and CRP at study entry, then these parameters do decrease significantly with anti-TNF treatment and that CRP is sensitive to change with treatment. However, the relevance of ESR and CRP in patients with minimal elevation at baseline is unknown (Antoni et al., 2005; Mease et al., 2000).

In patients with AS, an elevated ESR or CRP is present in only 30-40% of patients and normal values do not rule out the presence of active inflammation. ESR and CRP are more likely to be raised in patients with peripheral disease or concomitant inflammatory bowel disease (van Tubergen and Landewe, 2009). However, while poorly associated with disease activity per se, high baseline levels of CRP have been shown to be associated with a poorer response to anti-TNF agents (de Vries et al., 2009; Rudwaleit et al., 2004).

3.1.4 Effect of disease burden on quality of life, disability and fatigue

As RA, AS and PsA are long term chronic conditions which can lead to persistent pain, functional disability, fatigue and depression, it is important to assess the effects of treatment interventions on these aspects.
The Health Assessment Questionnaire (HAQ) has been specifically developed for the assessment of disability in patients with RA and focuses on physical disability and pain. It comprises 20 questions divided across eight different categories of function incorporating dressing, getting up from a chair, eating, walking, personal hygiene, grip and usual daily activities. The scores for each category are added together and transformed to a HAQ score on a 0-3 scale where 0 indicates no disability, 3 corresponds to complete disability and changes of >0.22 are considered clinically relevant. HAQ scores are low in early disease and increase over time. Patients with active disease and many swollen and tender joints have higher HAQ scores and scores fall when the inflammatory synovitis improves. There are strong correlations between HAQ scores and erosive damage (Kingsley et al., 2011).

The Medical Outcomes Study Short Form 36 (SF-36) is a self-administered patient questionnaire designed to measure functional status and wellbeing across diverse disease states. It comprises 36 questions across eight domains comprising physical function, physical role, bodily pain, general health, vitality, social role, emotional role and mental health. Total scores range from 0-100, where the higher the score, the better the overall health state. As SF-36 scores incorporate measures designed to reflect health status across different medical conditions, it facilitates direct comparisons of quality of life between different conditions.

Fatigue, defined as low energy and constant tiredness is often a feature of chronic disease (Klareskog et al., 2009). It is a physiological state that can be caused by the direct action of inflammatory cytokines, in particular interleukins 6 and 1 (IL6 and IL1) on cytokine receptors on brain endothelial cells (Ek et al., 2001). In addition, medications, as well as psychological and emotional factors can also contribute to fatigue in chronic disease (Klareskog et al., 2009). Fatigue, as a state can be measured as part of a patient’s outcome and a positive effect on fatigue is one of the earliest and most prominent effects of biological agents (Moreland et al., 2006). The Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-F) questionnaire is used in different chronic diseases and higher scores reflect lower fatigue levels. Fatigue severity has been found to correlate with disease activity, disability and joint pain (Campbell et al., 2012).
3.1.5 Definition of treatment response in RA, PsA and AS

In RA clinical trials, the ACR response criteria (ACR 20, 50 and 70) and improvement in DAS28 scores (as defined in Section 3.2.1.1) are the most commonly used measurements of treatment response. The ACR response criteria reflect improvement relative to baseline and do not give information on current level of disease activity, thus have limited utility in daily practice. ACR20 has been adopted as a primary outcome measure in anti-TNF trials in RA and PsA patients and it has confirmed discriminant validity, whereas ACR50 and ACR70 do not have the same discriminant validity (Felson et al., 1995). A limitation of the use of ACR20 as an endpoint is that despite achieving ACR20, many patients may still continue to exhibit a substantial number of actively inflamed joints which increases their risk of erosive damage.

The EULAR response criteria for RA are based on DAS score and reflect both the change in DAS score from baseline, as well as the current level of disease activity. On the basis of changes in DAS, patients can be divided into non-responders (with a reduction of DAS of <0.6 or between 0.6-1.2, with a persistent DAS >3.7); moderate responders (reduction in DAS between 0.6 to 1.2 and a persistent DAS <3.7), and good responders (improvement in DAS score >1.2 from baseline, and DAS at follow up <2.4) (van Gestel et al., 1999). EULAR and ACR response criteria have been shown to be comparable in different clinical trials and to behave similarly with <5% discrepancy in responder status (van Gestel et al., 1999).

The PsARC has been specifically designed for PsA and response incorporates a reduction in tender or swollen joint count by 30%, a reduction in patient global assessment by 1 point and a reduction in physician global assessment of 1 point. To be considered a responder, a patient must show improvement in at least 2 of the 4 domains, one of which must be joint count, and none of the criteria must show a worsening. PsARC responses in clinical trials of leflunomide, etanercept and infliximab have shown a good correlation with the modified ACR20 responses (Gladman et al., 2004; Mease, 2011a).

Based on the BASDAI questionnaire, ASAS defines response in AS with respect to treatment with anti-TNF agents as an improvement of at least 50% in the BASDAI score or an absolute change of 20mm on a 100mm VAS scale (Rudwaleit et al., 2004; Sieper et al., 2009).
The ASAS response criteria have also been developed which cover 4 domains: patient global assessment of disease activity evaluated by a VAS; pain as assessed by a spinal pain VAS; function as assessed by BASFI and inflammation as assessed by the mean of BASDAI questions 5 and 6. ASAS20 and 40 refer to a ≥20% and ≥1 unit improvement in at least 3 domains or ≥40% and ≥2 units improvement in at least 3 domains respectively, without worsening in the other domains. ASAS5/6 criteria which take into account two additional domains-CRP and spinal mobility and refer to ≥20% improvement in at least 5 of the domains.

Changes in ASDAS from baseline have also been proposed to reflect disease activity, such that ASDAS <1.9 corresponds to low disease activity, ASDAS >1.9 but <4.5 as moderate disease activity and ASDAS >4.5 as high disease activity. A change in ASDAS >1.85 from baseline has been proposed to reflect considerable improvement in disease activity, a change <0.40 as no improvement and change in ASDAS ≥0.40 but ≤1.85 as moderate improvement (van der Heijde et al., 2009). However, these proposed thresholds for disease activity and treatment response need further validation (Pedersen et al., 2010b).

3.2 Results

3.2.1 Overview of patients enrolled in the study

Study protocol approvals were obtained from the Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (09/H0707/80), the Imperial College AHSC Joint Research Office (ABRS2001) and clinical trial authorisation was granted by the Medicines Healthcare Products Regulatory Agency (19174/0283/001-0001). The study was registered with EudraCT (2009-012424-87) and clinicaltrials.gov (NCT01060098). Written, informed consent was obtained from all study participants prior to study entry and the study was conducted in accordance with Good Clinical Practice.

In order to ensure consistency in the patient assessments at each visit, I reviewed all the patients at each of their visits and performed all the ultrasound examinations. The clinical measures of disease activity were made by the same trained research nurse at each study visit. The first patient was recruited on 20th April 2010 and the last patient last study visit took place on 21st October 2011. In total, 52 patients were enrolled into the study, comprising 28
patients with rheumatoid arthritis, 16 patients with ankylosing spondylitis and 8 patients with psoriatic arthritis. Subsequently, there were 3 patients with rheumatoid arthritis and 1 patient with ankylosing spondylitis withdrawn from the study due to deciding not to commence anti-TNF treatment. Therefore in total, 25 patients with RA, 15 patients with AS and 8 patients with PsA form the study patient cohort.

3.2.2 Baseline characteristics of RA cohort

The RA patient cohort comprised 25 patients in total (18 female and 7 male) (Table 3.1). All of these patients were followed up prospectively according to the study protocol for the first 12 weeks after starting anti-TNF, except 3 patients who only completed the first 3 study visits. Prior to study visit 4, 1 patient was lost to follow up, 1 patient was diagnosed with breast cancer and discontinued anti-TNF and 1 patient discontinued anti-TNF due to development of severe lower respiratory tract infection. These 3 patients are included in all the analyses but their anti-TNF responder status was assessed at week 4 and not at week 12.

The demographics of the RA patients are shown in Table 3.1. The mean±SD age of the RA patients was 57.4±11.7 years (range 27-76 years) and the mean disease duration was 10.6±9.2 years (range 2-40 years). Over half of the patients were seropositive for rheumatoid factor (15 patients, 60% of the RA group) and anti-CCP antibodies (14 patients, 56% of the RA group). The majority of patients (21 patients, 84%) were taking a disease modifying agent and 7 patients (28%) were taking prednisolone. 8 patients (32%) were on methotrexate alone, 2 patients (8%) were on sulfasalazine alone, 10 (40%) patients were on combination DMARD treatment that included methotrexate and 1 patient was on combination DMARD treatment not including methotrexate. Five patients (20%) had completed prophylaxis for latent TB infection prior to starting their anti-TNF treatment.

Within the cohort, 18 patients were treated with 50mg etanercept weekly and 7 patients were treated with 40mg adalimumab fortnightly as prescribed by their treating physician. (Table 3.1)

The course of the clinical disease activity scores, joint counts and laboratory parameters assessed throughout the study is shown in Table 3.2. The DAS28-ESR score of the RA group at baseline reflected high disease activity with mean DAS28-ESR score 5.7±0.8. The mean numbers of swollen joints at baseline was 7.7±5.4 and the mean number of tender joints
Table 3.1  Baseline patient characteristics of the rheumatoid arthritis group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57.44±11.70</td>
</tr>
<tr>
<td>Sex</td>
<td>18F, 7M</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>10.60±9.15</td>
</tr>
<tr>
<td>Rheumatoid factor positive, n (%)</td>
<td>15 (60)</td>
</tr>
<tr>
<td>Anti-CCP positive, n (%)</td>
<td>14 (56)</td>
</tr>
<tr>
<td>DMARDs, n (%)</td>
<td>21 (84)</td>
</tr>
<tr>
<td>Methotrexate dosage, milligrams per week</td>
<td>16.63±5.29</td>
</tr>
<tr>
<td>Prednisolone, n (%)</td>
<td>7 (28)</td>
</tr>
<tr>
<td>Tuberculosis prophylaxis, n (%)</td>
<td>5 (20)</td>
</tr>
<tr>
<td>Anti-TNF treatment type</td>
<td></td>
</tr>
<tr>
<td>Etanercept</td>
<td>18</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>7</td>
</tr>
</tbody>
</table>

DMARD, disease modifying anti-rheumatic drug
Table 3.2  Changes in clinical measures of disease activity over 12 weeks on anti-TNF treatment in the rheumatoid arthritis group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>DAS28-ESR</td>
<td>5.72±0.84</td>
<td>4.47±1.14</td>
<td>****</td>
<td>4.26±1.13</td>
</tr>
<tr>
<td>DAS28-CRP</td>
<td>5.28±0.98</td>
<td>3.98±1.09</td>
<td>****</td>
<td>3.85±1.13</td>
</tr>
<tr>
<td>SJC28</td>
<td>7.68±5.36</td>
<td>4.42±3.89</td>
<td>**</td>
<td>4.54±4.28</td>
</tr>
<tr>
<td>TJC28</td>
<td>14.88±8.51</td>
<td>8.95±6.66</td>
<td>***</td>
<td>8.76±6.69</td>
</tr>
<tr>
<td>CRP</td>
<td>15.74±18.26</td>
<td>5.29±6.39</td>
<td>***</td>
<td>10.75±24.15</td>
</tr>
<tr>
<td>ESR</td>
<td>27.21±22.67</td>
<td>20.50±19.36</td>
<td>**</td>
<td>19.96±14.88</td>
</tr>
</tbody>
</table>

DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SJC28, swollen joint count out of 28 joints; TJC28, tender joint count out of 28 joints.

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
was 14.9±8.5. There was a significant decrease in DAS28-ESR and DAS28-CRP scores from as early as one week after initiation of anti-TNF treatment, with further significant decrease in DAS28 scores at 4 and 12 weeks after anti-TNF initiation compared to baseline scores (Figure 3.1A-D). This was paralleled by significant decreases in the numbers of swollen and tender joint counts at 1 week, 4 weeks and 12 weeks after anti-TNF was initiated compared to baseline (Figure 3.1E and F). RA patients exhibited raised inflammatory markers at baseline, with mean CRP 15.8 ±18.3 mg/l and mean ESR 27.1±22.7 mm/hr. ESR levels decreased significantly at 1 and 4 weeks after the start of therapy, but the change at 12 weeks was no longer significant compared to baseline (Figure 3.2A). CRP levels showed a significant decrease with anti-TNF treatment at 1, 4 and 12 weeks after treatment was initiated compared to baseline levels (Figure 3.2B).

There was a significant improvement in disability as assessed by HAQ as early as 1 week after commencing anti-TNF treatment compared to baseline, with further significant improvement at 4 and 12 weeks on treatment (Table 3.3). FACIT-F and SF-36 scores increased significantly at 4 and 12 weeks on treatment compared to baseline, reflecting improvement in fatigue and overall quality of life respectively (Table 3.3).

12 patients in total (48%) experienced an adverse event during the study. These included injection site reactions (7 cases), infections (4 cases) and headaches (1 case). Injection site reactions ranged from mild, which resolved without intervention, to more significant in 1 case necessitating discontinuation of anti-TNF after 12 weeks. All of the patients who experienced injection site reactions were treated with etanercept. The adverse events of infection included 3 cases of upper respiratory tract infection and one case of lower respiratory tract infection. Three out of the five cases of infection required treatment with antibiotics and one case required hospital admission for treatment. In the latter case, the anti-TNF treatment was discontinued after 4 weeks. If patients had active intercurrent infection or were taking antibiotics at the time of a study visit, that study visit was excluded from the data analysis.
Figure 3.1   Changes in clinical measures of disease activity over 12 weeks on anti-TNF treatment in rheumatoid arthritis group

The clinical measures of disease activity DAS28ESR (A) and DAS28CRP (B) are demonstrated for the 12 week course of anti-TNF treatment with each line representing an individual patient. The changes for the whole rheumatoid arthritis group (n=25) at baseline and at each time point on anti-TNF treatment are shown for mean±SEM DAS28ESR score (C) and mean±SEM DAS28CRP score (D). Joint assessments for the rheumatoid arthritis
group at baseline and over time on anti-TNF treatment are demonstrated for the mean±SEM numbers of swollen joints (out of a 28 joint count) (E) and tender joints (out of a 28 joint count) (F). Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, **p<0.001, ***p<0.0005, ****p<0.0001. DAS28, Disease Activity Score of 28 joints; SJC, swollen joint count, TJC, tender joint count; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate
Figure 3.2  Changes in inflammatory markers over 12 weeks on anti-TNF treatment in rheumatoid arthritis group.

Mean±SEM ESR (mm/hr) (A) and mean ±SEM CRP levels (mg/l) (B) are shown for baseline and at three timepoints on anti-TNF treatment in the RA group (n=25). Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, **p<0.001, ***p<0.0005. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.
Table 3.3  Changes in total scores from patient-reported outcome measures over 12 weeks on anti-TNF therapy in the rheumatoid arthritis group

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Baseline</th>
<th>1 week</th>
<th>4 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>HAQ</td>
<td>1.52±0.76</td>
<td>1.41±0.71</td>
<td>*</td>
<td>1.02±0.79</td>
</tr>
<tr>
<td>FACIT-F</td>
<td>85.78±25.58</td>
<td>27.25±13.35</td>
<td>***</td>
<td>32.86±21.57</td>
</tr>
<tr>
<td>SF-36</td>
<td>33.92±21.36</td>
<td>52.61±25.09</td>
<td>***</td>
<td>60.58±24.92</td>
</tr>
</tbody>
</table>

HAQ, Health Assessment Questionnaire; FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; SF-36, Short Form-36.

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
3.2.3 Baseline characteristics of AS group

The AS patient group comprised 15 patients in total (3 female and 12 male). The patient demographics of this group are shown in Table 3.4. The mean age±SD for this group was 36.4±11.8 years (range 25-59 years). The mean disease duration was 10.9±10.7 years (range 1-34 years). 4 patients were on concomitant DMARD therapy: 3 were on sulfasalazine and 1 on methotrexate. 11 patients were treated with adalimumab and 4 patients were treated with etanercept. None of the patients required prophylaxis for latent tuberculosis prior to starting anti-TNF therapy.

BASDAI, BASFI and BASMI scores and inflammatory markers for the AS group as a whole at baseline and with time on anti-TNF treatment are summarised in Table 3.5. Mean BASDAI score at baseline was 5.3±2.0, reflecting high disease activity and this significantly decreased at 4 and 12 weeks after anti-TNF therapy was started (Figure 3.3A). Mean BASFI score at baseline was 4.2±1.9 and mean BASMI score at baseline was 3.3±1.8, with both scores decreasing significantly at 12 weeks after anti-TNF initiation (Figure 3.3B and 3C). Inflammatory markers were raised at baseline, with mean ESR 22.3±18.3 mm/hr and mean CRP 7.0±7.7 mg/l. Both inflammatory markers showed a significant decrease at 1, 4 and 12 weeks on anti-TNF treatment when compared to baseline levels (Figure 3.4).

The patient reported outcome measures of fatigue and quality life, FACIT-F and SF-36 respectively, increased significantly with time on anti-TNF treatment, reflecting an improvement in fatigue and quality of life (Table 3.6).

Two patients experienced side effects; one patient developed injection site reactions with etanercept but these were mild and self-limiting and one patient developed an upper respiratory tract infection which did not require antibiotic treatment.

3.2.4 Baseline characteristics of PsA group

The PsA group comprised 8 patients (5 female and 3 male) and the demographics of this group are summarised in Table 3.7. Mean age±SD was 50.9±8.43 years (range 36-63 years). Mean disease duration was 7.8±7.3 years (range 2-20 years). 5 patients were taking
Table 3.4  Baseline patient characteristics of the ankylosing spondylitis group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36.40±11.83</td>
</tr>
<tr>
<td>Sex</td>
<td>3F, 12M</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>10.87±10.65</td>
</tr>
<tr>
<td>DMARDs, n (%)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Tuberculosis prophylaxis, n (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anti-TNF treatment type</td>
<td></td>
</tr>
<tr>
<td>Etanercept</td>
<td>4</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>11</td>
</tr>
</tbody>
</table>

DMARD, disease modifying anti-rheumatic drug
Table 3.5  Changes in clinical measures of disease activity and inflammatory markers over 12 weeks on anti-TNF treatment in the ankylosing spondylitis group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>1 week</th>
<th>4 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>BASDAI</td>
<td>5.33±2.03</td>
<td>4.99±2.29</td>
<td>ns</td>
<td>4.02±2.34</td>
</tr>
<tr>
<td>BASFI</td>
<td>4.22±1.87</td>
<td>4.14±1.99</td>
<td>ns</td>
<td>3.29±1.85</td>
</tr>
<tr>
<td>BASMI</td>
<td>3.29±1.80</td>
<td>3.35±1.75</td>
<td>ns</td>
<td>3.04±1.43</td>
</tr>
<tr>
<td>ESR</td>
<td>22.36±18.33</td>
<td>12.67±12.05</td>
<td>*</td>
<td>9.00±8.52</td>
</tr>
<tr>
<td>CRP</td>
<td>7.04±7.74</td>
<td>1.71±1.62</td>
<td>***</td>
<td>1.47±1.87</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.
Figure 3.3   Changes in Bath Indices (BASDAI, BASFI, BASMI) over 12 weeks on anti-TNF treatment in the ankylosing spondylitis group

The mean±SEM scores at baseline and changes over 12 weeks of anti-TNF treatment are demonstrated for the BASDAI (A), BASFI (B) and BASMI (C) scores in the AS group (n=15). Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ****p<0.0001. BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index.
Figure 3.4 Changes in inflammatory markers over 12 weeks on anti-TNF treatment in ankylosing spondylitis group.

Mean±SEM ESR (mm/hr) (A) and mean ±SEM CRP levels (mg/l) (B) are shown for baseline and at three timepoints on anti-TNF treatment in the AS group (n=15). Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate
Table 3.6  Changes in total scores from patient-reported outcome measures over 12 weeks on anti-TNF therapy in the ankylosing spondylitis group

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Baseline</th>
<th>1 week</th>
<th>4 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>FACIT-F</td>
<td>91.33±20.05</td>
<td>96.38±18.98</td>
<td>*</td>
<td>104.8±23.86</td>
</tr>
<tr>
<td>SF-36</td>
<td>45.50±13.67</td>
<td></td>
<td></td>
<td>58.07±17.06</td>
</tr>
</tbody>
</table>

FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; SF-36, Short Form-36.

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>50.90±8.43</td>
</tr>
<tr>
<td>Sex</td>
<td>5F, 3M</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>7.75±7.32</td>
</tr>
<tr>
<td>DMARDs, n (%)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>Methotrexate dosage (mg/week)</td>
<td>14.38±7.18</td>
</tr>
<tr>
<td>Prednisolone, n (%)</td>
<td>3 (38)</td>
</tr>
<tr>
<td>Tuberculosis prophylaxis, n (%)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>Anti-TNF treatment type</td>
<td></td>
</tr>
<tr>
<td>Etanercept</td>
<td>3</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>5</td>
</tr>
</tbody>
</table>

DMARD, disease modifying anti-rheumatic drug
concomitant DMARDs; 4 patients were on methotrexate and 1 patient was on sulfasalazine. 3 patients were taking prednisolone. 5 patients were treated with adalimumab and 3 patients were treated with etanercept. 2 patients completed a course of chemoprophylaxis for latent tuberculosis prior to starting their anti-TNF therapy.

Swollen joint counts, tender joint counts, patient and physician global assessments and inflammatory markers were assessed at baseline in the PsA group and their change during anti-TNF are summarised in Table 3.8. Mean swollen joint count (out of maximum 76 joints) was 8.1±4.8, which decreased significantly at 1, 4 and 12 weeks on anti-TNF treatment compared to baseline (Figure 3.5A). Mean tender joint count (out of a maximum of 78 joints) was 33.5±19.3 and this showed a slower improvement to anti-TNF, reaching a statistically significant decrease at 12 weeks after anti-TNF was initiated (Figure 3.5B). Mean patient global score (on a 0 to 5 scale) was 3.4 at baseline corresponding to ‘fair to poor’ patient perception of their disease and this improved significantly to a mean of 1.4 at 12 weeks after anti-TNF was started, corresponding to ‘very good’. Mean physician global assessment (on a 0 to 5 scale) of the patient’s disease at baseline was 3.4 corresponding to ‘fair to poor’ on the 6 point scale and this showed significant and rapid decline with anti-TNF at 1 week and then at 12 weeks on treatment.

In this group, patients had relatively mild or no psoriasis skin lesions as reflected by a low mean baseline PASI score of 7.3±0.2. Mean total PASI score decreased significantly at 12 weeks on treatment compared to baseline (Figure 3.5C).

Inflammatory markers, ESR and CRP were raised at baseline with mean ESR 22.3±17.1 mm/hr and mean CRP 16.1±16.3 mg/dl. ESR showed a trend to a gradual decline with time on anti-TNF, whereas there was a significant decrease in CRP at 1, 4 and 12 weeks compared to baseline as shown in Figure 3.6.

There was a trend to an increase in FACIT-F score with anti-TNF treatment and this was significant at 12 weeks compared to baseline reflecting improving fatigue levels (Table 3.9). Although there was a trend towards improvement in quality of life with anti-TNF treatment as reflected by an increase in total SF-36 questionnaire scores, this did not reach statistical significance at any of the time points (Table 3.9).
Table 3.8 Changes in clinical measures of disease activity and inflammatory markers over 12 weeks on anti-TNF treatment in the psoriatic arthritis group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>Mean score</td>
<td>Mean score</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
</tr>
<tr>
<td>PASI score</td>
<td>7.31±10.20</td>
<td>5.47±6.40</td>
<td>ns</td>
<td>3.78±5.85</td>
</tr>
<tr>
<td>SJC</td>
<td>8.13±4.82</td>
<td>4.88±2.64</td>
<td>*</td>
<td>3.43±3.16</td>
</tr>
<tr>
<td>TJC</td>
<td>33.50±19.35</td>
<td>20.29±10.78</td>
<td>**</td>
<td>26.71±14.67</td>
</tr>
<tr>
<td>Patient global assessment</td>
<td>3.38±1.19</td>
<td>2.63±1.30</td>
<td>ns</td>
<td>2.71±1.11</td>
</tr>
<tr>
<td>Physician global assessment</td>
<td>3.38±0.92</td>
<td>2.38±0.92</td>
<td>*</td>
<td>2.43±1.13</td>
</tr>
<tr>
<td>ESR</td>
<td>22.25±17.14</td>
<td>14.40±15.44</td>
<td>ns</td>
<td>11.25±8.28</td>
</tr>
<tr>
<td>CRP</td>
<td>16.09±16.32</td>
<td>4.98±6.39</td>
<td>*</td>
<td>4.69±7.12</td>
</tr>
</tbody>
</table>

PASI, Psoriasis Area and Severity Index; SJC, swollen joint count (out of 76 joints); TJC, tender joint count (out of 78 joints); ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
Figure 3.5 Changes in clinical measures of disease activity over 12 weeks on anti-TNF treatment in the psoriatic arthritis group

Mean±SEM numbers of swollen joints (A) (out of a 76 joint count) and tender joints (B) (out of a 78 joint count) along with the mean±SEM total PASI score (C) are shown for the psoriatic arthritis group at baseline and with time on anti-TNF treatment in the PsA group (n=8). Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001. SJC, swollen joint count; TJC, tender joint count; PASI, Psoriasis Area and Severity Index.
Figure 3.6  Changes in inflammatory markers over 12 weeks on anti-TNF treatment in the psoriatic arthritis group.

Mean±SEM ESR (mm/hr) (A) and mean ±SEM CRP levels (mg/l) (B) are shown for baseline and at three timepoints on anti-TNF treatment in the PsA group (n=8). Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, *p<0.05. CRP, C-reactive protein; ESR, erythrocyte sedimentation rate
Table 3.9  Changes in total scores from patient-reported outcome measures over 12 weeks on anti-TNF therapy in the psoriatic arthritis group

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Baseline Mean score</th>
<th>1 week Mean score</th>
<th>4 weeks Mean score</th>
<th>12 weeks Mean score</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACIT-F</td>
<td>92.58±18.29</td>
<td>106.7±26.49 ns</td>
<td>104.3±28.17 ns</td>
<td>120.8±24.37 *</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF-36</td>
<td>41.63±15.00</td>
<td>47.29±26.59 ns</td>
<td>63.00±23.79 ns</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; SF-36, Short Form-36.

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
3 patients in the PsA group experienced side effects; 2 patients developed injection site reactions, but these were self-limiting and 1 patient developed severe cellulitis of the hand necessitating hospital admission with debridement and intravenous antibiotic treatment.

3.2.5 Healthy controls

Nine healthy controls with no history of rheumatological conditions were recruited from clinical and laboratory staff and informed consent was obtained. The group comprised 3 men and 7 women and the mean age±SD was 50.2±13.9 years.

3.2.6 Definition of anti-TNF treatment response

Patients in the RA group were classified as responders or non-responders to anti-TNF therapy based on whether they achieved an improvement in DAS28-ESR score >1.2 units from baseline to 12 weeks after starting anti-TNF, based on the EULAR response criteria (van Gestel et al., 1996). According to this, 16 patients were responders and 9 patients were non-responders to anti-TNF treatment over 12 weeks.

Patients in the AS group were classified as responders or non-responders to anti-TNF therapy depending on whether they achieved an improvement of at least 50% of the BASDAI score at 12 weeks compared to baseline according to ASAS recommendations (Rudwaleit et al., 2004; Sieper et al., 2009). Based on this, 11 patients were responders and 4 patients were non-responders to anti-TNF at 12 weeks.

The PsARC response criteria were used to determine responders and non-responders to anti-TNF in the PsA group. Patients who achieved an improvement in at least 2 of the 4 domains of the PsARC, one of which had to be joint count, without worsening of the other criteria at 12 weeks were classified as responders (Gladman et al., 2004; Mease, 2011a). Based on this, 7 patients were responders and 1 patient was a non-responder to anti-TNF at 12 weeks.
3.2.7 Comparison of baseline patient characteristics between anti-TNF responders and non-responders

3.2.7.1 RA patients
A comparison of the patient baseline characteristics between RA responders (n=16) and non-responders (n=9) is presented in Table 3.10. RA responders to anti-TNF showed a tendency to have longer disease duration (mean disease duration 13.2±10.5 years) than the non-responders group (mean disease duration 6.0±2.7 years), however this difference was not statistically significant (p=0.14). The majority of patients in the responder and non-responder groups were taking concomitant DMARDs and a similar mean weekly methotrexate dose. Baseline disease activity as reflected by DAS28-ESR score was not significantly different between the two groups, with responders mean baseline DAS28 score of 5.7±0.8 and non-responders mean baseline DAS28 score 5.7±0.9. Responders showed a tendency to having a higher number of swollen joints at baseline (mean 8.8±5.5) compared to non-responders (mean 5.7±4.9) but this difference was not statistically significant. Both responders and non-responders had elevated inflammatory markers at baseline, with non-responders showing a tendency to having higher CRP levels at baseline (mean 23.5±25.7) than responders (mean 11.4±11.2); however this difference was not statistically significant (p=0.46).

3.2.7.2 AS patients
A comparison of the baseline characteristics of AS responders (n=11) and non-responders (n=4) is presented in Table 3.11. There was a significant difference (p=0.03) in the mean age between responders and non-responders, with the non-responders being significantly older. There was also a tendency for non-responders to have longer mean disease duration (19.3±13.9 years) compared to responders (7.8±7.9). 2 patients in each group were on concomitant DMARD therapy. The mean baseline BASDAI score in non-responders group showed a non-significant trend to be higher (6.8±2.5) than in the responders group (5.1±1.8) and in both groups the baseline BASDAI scores indicated very active disease. Despite the non-responder group showing a tendency to longer disease duration, there was no significant difference between mean BASMI scores at baseline between responders and non-responders. Both responders and non-responders had elevated inflammatory markers at baseline with mean ESR at baseline of 33.0±27.3 in the non-responders group compared to 18.1±12.9 in the responders group.
Table 3.10  Comparison of baseline patient characteristics, clinical measures of disease activity and inflammatory markers of anti-TNF responder versus non-responder patients within the rheumatoid arthritis group

<table>
<thead>
<tr>
<th>Parameter, treatment group</th>
<th>Responders Mean ±SD</th>
<th>Non-responders Mean ±SD</th>
<th>Mann Whitney U test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.5±9.97</td>
<td>55.55±14.75</td>
<td>0.71</td>
</tr>
<tr>
<td>Sex</td>
<td>12F, 4M</td>
<td>6F, 3M</td>
<td></td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>13.18±10.46</td>
<td>6±2.96</td>
<td>0.14</td>
</tr>
<tr>
<td>Rheumatoid factor positive, n (%)</td>
<td>62</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Anti-CCP positive, n (%)</td>
<td>56</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>DMARDs, n (%)</td>
<td>14 (88)</td>
<td>7 (78)</td>
<td></td>
</tr>
<tr>
<td>Methotrexate dosage (mg/week)</td>
<td>16</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Prednisolone, n (%)</td>
<td>3 (19)</td>
<td>4 (44)</td>
<td></td>
</tr>
<tr>
<td>Tuberculosis prophylaxis, n (%)</td>
<td>4 (25)</td>
<td>1 (11)</td>
<td></td>
</tr>
<tr>
<td>Anti-TNF treatment type</td>
<td>11 Etanercept</td>
<td>7 Etanercept</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 Adalimumab</td>
<td>2 Adalimumab</td>
<td></td>
</tr>
<tr>
<td>DAS28-ESR</td>
<td>5.70±0.84</td>
<td>5.73±0.88</td>
<td>0.97</td>
</tr>
<tr>
<td>SJC28</td>
<td>8.81±5.45</td>
<td>5.67±4.85</td>
<td>0.12</td>
</tr>
<tr>
<td>TJC28</td>
<td>14.56±8.67</td>
<td>15.44±8.71</td>
<td>0.82</td>
</tr>
<tr>
<td>CRP, mg/dl</td>
<td>11.35±11.18</td>
<td>23.53±25.68</td>
<td>0.46</td>
</tr>
<tr>
<td>ESR, mm/hr</td>
<td>25.63±22.32</td>
<td>30.38±24.58</td>
<td>0.39</td>
</tr>
</tbody>
</table>

DMARD, disease modifying anti-rheumatic drug; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SJC28, swollen joint count out of 28 joints; TJC28, tender joint count out of 28 joints.

Data are presented as mean±SD; Mann Whitney U test responders vs non-responders, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001
Table 3.11  Comparison of baseline patient characteristics, clinical measures of disease activity and inflammatory markers of anti-TNF responder versus non-responder patients within the ankylosing spondylitis group

<table>
<thead>
<tr>
<th>Parameter, treatment group</th>
<th>Responders Mean ±SD</th>
<th>Non-responders Mean ±SD</th>
<th>Mann Whitney U test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34.27±7.61</td>
<td>49.25±11.87</td>
<td>0.03</td>
</tr>
<tr>
<td>Sex</td>
<td>2F, 9M</td>
<td>1F, 3M</td>
<td>-</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>7.82±7.93</td>
<td>19.25±13.84</td>
<td>0.17</td>
</tr>
<tr>
<td>DMARDs, n (%)</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Anti-TNF treatment type</td>
<td>4 Etanercept</td>
<td>0 Etanercept</td>
<td>-</td>
</tr>
<tr>
<td>BASDAI score</td>
<td>5.08±1.74</td>
<td>6.79±2.49</td>
<td>0.17</td>
</tr>
<tr>
<td>BASFI score</td>
<td>4.69±1.79</td>
<td>3.06±1.76</td>
<td>0.19</td>
</tr>
<tr>
<td>BASMI score</td>
<td>3.35±2.06</td>
<td>3.15±0.98</td>
<td>0.79</td>
</tr>
<tr>
<td>CRP, g/dl</td>
<td>6.48±8.73</td>
<td>4.88±3.21</td>
<td>0.79</td>
</tr>
<tr>
<td>ESR ,mm/hr</td>
<td>18.10±12.89</td>
<td>33.00±27.31</td>
<td>0.48</td>
</tr>
</tbody>
</table>

DMARD, disease modifying anti-rheumatic drug; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Data are presented as mean±SD; Mann Whitney U test responders vs non-responders, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001
3.2.7.3 PsA patients

7 patients were responders and 1 patient was a non-responder. Due to the small numbers in this group, baseline characteristics in the responders and non-responder have not been compared.

3.2.8 Changes in clinical outcome measures in anti-TNF responders and non-responders

Differences in the changes of clinical outcome measures with time on anti-TNF treatment between treatment responders and non-responders have been compared within the RA and AS groups only due to the small numbers in the PsA group.

3.2.8.1 RA patients

The changes in disease activity scores, swollen and tender joint counts and inflammatory markers during anti-TNF treatment were compared between responder and non-responder groups (Table 3.12). Both responder and non-responder patients showed a significant decrease in DAS28 scores as early as 1 week after starting anti-TNF, with further decrease at 4 and 12 weeks of anti-TNF treatment relative to their baseline DAS28 scores (Figure 3.7A-C). However, there were significant differences in the mean DAS28ESR score attained between responder and non-responder groups at 1, 4 and 12 weeks of anti-TNF treatment, with responders having lower DAS28ESR score at each time point (Figure 3.7C). In addition, there were significant differences between the responder and non-responder groups in the mean absolute change in DAS28ESR score from baseline to 1, 4 and 12 weeks on treatment, such that the mean change in DAS28ESR score at 12 weeks in responders was -2.4±0.9 and in non-responders it was -0.8±0.4 (p=0.0002) (Figure 3.7D).

There was a significant decrease in both the numbers of swollen joints and the numbers of tender joints in responders at 1, 4 and 12 weeks compared to baseline, whereas in non-responder patients a significant decrease did not occur until 12 weeks after starting anti-TNF treatment (Table 3.12). However, although there was a trend towards non-responder patients having a greater number of swollen and tender joints than responders at 1, 4 and 12 weeks after starting anti-TNF treatment, these differences were not statistically significant (Figure 3.8 A and B).
### Table 3.12  Comparison of the changes in clinical measures of disease activity over 12 weeks on anti-TNF treatment between rheumatoid arthritis responder and non-responders patients to anti-TNF.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>DAS28-ESR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>5.70±0.84</td>
<td>4.01±1.04</td>
<td>***</td>
<td>3.86±1.04</td>
</tr>
<tr>
<td>Non-responders</td>
<td>5.73±0.88</td>
<td>5.33±0.81</td>
<td>*</td>
<td>5.03±0.93</td>
</tr>
<tr>
<td>DAS28-CRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>5.19±1.08</td>
<td>3.51±0.97</td>
<td>***</td>
<td>3.46±1.05</td>
</tr>
<tr>
<td>Non-responders</td>
<td>5.43±0.85</td>
<td>4.92±0.64</td>
<td>ns</td>
<td>4.64±0.88</td>
</tr>
<tr>
<td>SJC28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>8.81±5.45</td>
<td>3.64±3.15</td>
<td>**</td>
<td>4.00±3.25</td>
</tr>
<tr>
<td>Non-responders</td>
<td>5.667±4.85</td>
<td>6.00±4.97</td>
<td>ns</td>
<td>5.63±5.98</td>
</tr>
<tr>
<td>TJC28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>14.56±8.67</td>
<td>7.00±5.16</td>
<td>**</td>
<td>6.94±6.47</td>
</tr>
<tr>
<td>Non-responders</td>
<td>15.44±8.71</td>
<td>13.50±8.02</td>
<td>ns</td>
<td>12.00±6.98</td>
</tr>
<tr>
<td>CRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>11.35±11.18</td>
<td>3.61±4.03</td>
<td>***</td>
<td>3.97±4.14</td>
</tr>
<tr>
<td>Non-responders</td>
<td>23.53±25.68</td>
<td>8.67±9.02</td>
<td>ns</td>
<td>22.79±38.25</td>
</tr>
<tr>
<td>ESR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>25.63±22.32</td>
<td>18.00±20.88</td>
<td>**</td>
<td>16.07±14.57</td>
</tr>
<tr>
<td>Non-responders</td>
<td>30.38±24.58</td>
<td>25.40±16.63</td>
<td>ns</td>
<td>27.25±13.35</td>
</tr>
</tbody>
</table>

DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; SJC28, swollen joint count out of 28 joints; TJC28, tender joint count out of 28 joints. Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
Figure 3.7  Comparison of the changes in DAS28ESR scores over 12 weeks on anti-TNF treatment in responders versus non-responders rheumatoid arthritis patients

The changes in DAS28ESR scores over 12 weeks on anti-TNF treatment are shown for individual patients in the responders (A) (n=16) and non-responders (B) (n=9) groups with each line representing an individual patient. Comparisons of the changes in mean±SEM DAS28ESR score (C) and the mean±SEM absolute change from baseline (D) between responders and non-responders over the 12 weeks of anti-TNF therapy are illustrated. Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001. Comparison between responder and non-responders groups was made using Mann Whitney U test, *p<0.05, **p<0.001. DAS28, Disease Activity Score of 28 joints, ESR, Erythrocyte Sedimentation Rate.
Figure 3.8  Comparison of the changes in swollen and tender joint counts and inflammatory markers over 12 weeks on anti-TNF treatment in responders versus non-responders rheumatoid arthritis patients

Comparisons between responders (n=16) and non-responders (n=9) to anti-TNF therapy at baseline and over the 12 week course of anti-TNF treatment were made for mean±SEM numbers of swollen joints (out of a 28 joint count) (A), mean±SEM numbers of tender joints (out of a 28 joint count) (B), mean±SEM ESR level (mm/hr) (C) and mean±SEM CRP level (mg/l) (D). Comparison between responder and non-responders groups was made using Mann Whitney U test *p<0.05. SJC, swollen joint count (28 joints); TJC, tender joint count (28 joints); ESR, Erythrocyte Sedimentation Rate; CRP, C-reactive protein.
Responders showed a significant decrease in ESR and CRP levels at 1, 4 and 12 weeks compared to baseline, whereas non-responders did not show a significant change in ESR or CRP with time on anti-TNF treatment (Table 3.12). Non-responders showed a trend to higher ESR and CRP levels compared to responders at 1, 4 and 12 weeks on treatment, but the differences were statistically significant only with ESR levels at 4 and 12 weeks (Figure 3.8C and D).

3.2.8.2 AS patients

The changes in Bath indices and inflammatory markers were compared between responder and non-responder groups during anti-TNF treatment (Table 3.13). Anti-TNF responders showed a significant decrease in BASDAI score at 4 and 12 weeks of anti-TNF treatment compared to baseline score, whereas non-responders showed a trend towards a decrease in BASDAI score with anti-TNF but this did not reach statistical significance at any of the time points (Figure 3.9A). Significant differences did exist between the responder and non-responder groups in the BASDAI scores attained at 4 and 12 weeks on anti-TNF (Figure 9A). There was also a significant difference in the percentage change of BASDAI score at 12 weeks between responder and non-responder groups, with responders having a mean percentage change of -76.6±12.3% from baseline and non-responders achieving a mean percentage change of -30.8±8.3% from baseline (Figure 3.9B).

In the responders group, there was a significant decrease in BASFI scores at 4 and 12 weeks of anti-TNF treatment compared to baseline, reflecting improving overall function. In contrast, non-responders showed a trend towards an increase in BASFI score with time on anti-TNF treatment, reflecting worsening function (Table 3.13). There was a significant difference between the responder and non-responder groups in the BASFI score at 12 weeks of anti-TNF treatment, with non-responders having a higher score (p=0.03) (Figure 3.9C). The percentage change in BASFI score achieved at 4 and 12 weeks was also significantly different between responders and non-responders (Figure 3.9D).

There were no significant changes in BASMI score with anti-TNF treatment in the responder or non-responder groups compared to baseline (Table 3.13). There were also no significant
Table 3.13  Comparison of the changes in clinical measures of disease activity over 12 weeks on anti-TNF treatment between ankylosing spondylitis responder and non-responders patients to anti-TNF.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>Mean score</td>
<td>Mean score</td>
</tr>
<tr>
<td><strong>BASDAI score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Responders</strong></td>
<td>5.08±1.74</td>
<td>4.78±2.25</td>
<td>3.23±1.85</td>
<td>1.32±0.69</td>
</tr>
<tr>
<td><strong>Non-responders</strong></td>
<td>6.79±2.49</td>
<td>5.73±2.79</td>
<td>6.19±2.38</td>
<td>4.69±2.13</td>
</tr>
<tr>
<td><strong>BASFI score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Responders</strong></td>
<td>4.69±1.79</td>
<td>4.31±2.05</td>
<td>3.06±1.98</td>
<td>1.83±1.36</td>
</tr>
<tr>
<td><strong>Non-responders</strong></td>
<td>3.06±1.76</td>
<td>3.58±1.76</td>
<td>3.95±1.44</td>
<td>4.13±1.94</td>
</tr>
<tr>
<td><strong>BASMI score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Responders</strong></td>
<td>3.35±2.06</td>
<td>3.51±2.01</td>
<td>2.93±1.59</td>
<td>2.82±1.29</td>
</tr>
<tr>
<td><strong>Non-responders</strong></td>
<td>3.15±0.98</td>
<td>2.87±0.50</td>
<td>3.35±0.91</td>
<td>2.65±1.17</td>
</tr>
<tr>
<td><strong>CRP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Responders</strong></td>
<td>6.48±8.73</td>
<td>2.53±1.94</td>
<td>0.88±0.61</td>
<td>1.48±2.25</td>
</tr>
<tr>
<td><strong>Non-responders</strong></td>
<td>4.88±3.21</td>
<td>0.73±0.61</td>
<td>2.03±3.06</td>
<td>1.40±1.58</td>
</tr>
<tr>
<td><strong>ESR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Responders</strong></td>
<td>18.10±12.89</td>
<td>11.44±10.61</td>
<td>6.46±2.54</td>
<td>6.46±3.05</td>
</tr>
<tr>
<td><strong>Non-responders</strong></td>
<td>33.00±27.31</td>
<td>16.33±17.93</td>
<td>16.00±15.10</td>
<td>21.67±16.04</td>
</tr>
</tbody>
</table>

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BASMI, Bath Ankylosing Spondylitis Metrology Index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Data are presented as mean±SD; Mann Whitney U test responders vs non-responders, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001
Figure 3.9  Comparison of the changes in Bath Indices over 12 weeks on anti-TNF treatment between responders versus non-responders ankylosing spondylitis patients

Comparisons between responders (n=11) and non-responders (n=4) to anti-TNF therapy at baseline and over the 12 week course of anti-TNF treatment were made for mean±SEM total
BASDAI score (A), mean±SEM percentage change from baseline in total BASDAI score (B), mean±SEM total BASFI score (C), mean±SEM percentage change from baseline in total BASFI score (D), mean±SEM total BASMI score (E) and mean ±SEM percentage change from baseline in total BASMI score (F). Comparison between responder and non-responders groups was made using Mann Whitney U test, *p<0.05, **p<0.001.
differences between the responder and non-responder groups in absolute BASMI score attained, nor the percentage change in BASMI score (Figures 3.9E and F).

3.2.9 Changes in quality of life, fatigue and disability between anti-TNF responders and non-responders

3.2.9.1 RA patients

In anti-TNF responders, there was a significant decrease in the HAQ scores at 1, 4 and 12 weeks compared to baseline, whereas non-responders did not show a significant change in HAQ score with time on anti-TNF treatment (Table 3.14). Thus disability improved significantly during 12 weeks on anti-TNF treatment in responders, but not in non-responders. Compared to non-responders, responders had significantly lower HAQ scores at 4 weeks (responders mean HAQ=0.7±0.7; non-responders mean HAQ=1.5±0.8) and at 12 weeks (responders mean HAQ=0.7±0.9; non-responders mean HAQ=1.7±0.7).

There was a significant increase in FACIT-F questionnaire scores in responders at 4 and 12 weeks on anti-TNF treatment, reflecting improving fatigue levels, whereas there was no significant change in FACIT-F scores in the non-responder group (Table 3.14).

There was a significant increase in total SF-36 scores in the responders group at 1, 4 and 12 weeks of anti-TNF treatment suggesting improved quality of life. In the non-responders group, there was a significant increase in SF-36 score at 12 weeks after anti-TNF treatment suggesting some improvement in quality of life of these patients, but that this occurred slower than in responders (Table 3.14). However, there was a significant difference (p=0.009) in the SF-36 scores attained at 12 weeks of anti-TNF treatment between responders and non-responders, with responders having a mean total SF-36 score of 74.8±19.9 and non-responders having a mean total SF-36 score of 41.8±18.3.

3.2.9.2 AS patients

Responders showed a significant increase in FACIT-F scores at 4 and 12 weeks after starting anti-TNF therapy reflecting improving fatigue levels, whereas there was no significant change in the non-responder group (Table 3.15).
Table 3.14  Comparison of the changes in total scores from patient-reported outcome measures over 12 weeks on anti-TNF treatment between rheumatoid arthritis responders and non-responders to anti-TNF treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>HAQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>1.41±0.86</td>
<td>1.02±0.74</td>
<td>*</td>
<td>0.74±0.66</td>
</tr>
<tr>
<td>Non-responders</td>
<td>1.71±0.53</td>
<td>1.39±0.61</td>
<td>ns</td>
<td>1.53±0.79</td>
</tr>
<tr>
<td>FACIT-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>86.32±26.67</td>
<td>116.70±24.60</td>
<td>**</td>
<td>125.50±28.89</td>
</tr>
<tr>
<td>Non-responders</td>
<td>84.70±24.99</td>
<td>89.28±29.33</td>
<td>ns</td>
<td>90.76±34.99</td>
</tr>
<tr>
<td>SF-36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>36.44±23.69</td>
<td>59.53±23.52</td>
<td>***</td>
<td>74.27±19.86</td>
</tr>
<tr>
<td>Non-responders</td>
<td>28.88±15.90</td>
<td>39.63±24.02</td>
<td>ns</td>
<td>41.55±18.27</td>
</tr>
</tbody>
</table>

HAQ, Health Assessment Questionnaire; FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; SF-36, Short Form-36.

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
Table 3.15  Comparison of the changes in total scores from patient-reported outcome measures over 12 weeks on anti-TNF treatment between ankylosing spondylitis responders and non-responders to anti-TNF treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>FACIT-F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>91.86±23.21</td>
<td>96.06±20.69</td>
<td>109.60±23.20 **</td>
<td>127.30±17.54 ***</td>
</tr>
<tr>
<td>Non-responders</td>
<td>89.88±8.78</td>
<td>97.44±15.22</td>
<td>91.71±23.47 Ns</td>
<td>105.5±24.84 ns</td>
</tr>
<tr>
<td>SF-36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>46.45±13.12</td>
<td>62.18±16.27 ***</td>
<td>73.27±14.44 **</td>
<td></td>
</tr>
<tr>
<td>Non-responders</td>
<td>38.67±21.55</td>
<td>50.00±11.83 ns</td>
<td>50.50±19.02 ns</td>
<td></td>
</tr>
</tbody>
</table>

FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; SF-36, Short Form-36.

Data are presented as mean±SD; each time point on treatment compared to baseline using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
Responders showed a significant increase in total SF-36 scores at 4 and 12 weeks after starting anti-TNF reflecting improving quality of life. There was a trend towards an increase in total SF-36 scores in non-responders with time on anti-TNF therapy, but this did not reach statistical significance. There was a significant difference in the total SF-36 score between responder and non-responder groups at 12 weeks, with responders achieving higher scores to reflect better quality of life than non-responders.

3.3 Discussion

The aim of the clinical study was to recruit a cohort of anti-TNF naïve patients with active rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis and to follow them prospectively in order to characterise changes in their disease activity longitudinally and determine response to treatment for the first 12 weeks after starting anti-TNF. The strengths of this clinical study are that it comprised protocol visits with predetermined investigations during which patients were monitored allowing the effect of anti-TNF treatment to be determined on validated clinical disease activity measures and thus identify treatment responders and non-responders. This well characterised and prospectively followed patient cohort also enabled immunological investigations to be carried out at each time point to assess the effect of anti-TNF on the kinetics of change of Th1 and Th17 cells in peripheral blood and on serum cytokine levels as detailed in the subsequent Chapters 5, 6 and 7. The relationships between clinical, ultrasonographic and immunological parameters with time on anti-TNF treatment could then be studied as discussed in Chapter 8.

Patients with three different types of inflammatory arthritis treated with anti-TNF were followed up in order to investigate if the effects of anti-TNF treatment on the immunological parameters would be specific to some types of inflammatory arthritis only or would occur in all three types. To our knowledge, a prospective study of this kind comprising patients with 3 different types of inflammatory arthritis treated with anti-TNF and involving clinical, morphological and immunological investigations has not been carried out to date. Other similar studies have focused on only one disease group (commonly RA) and have assessed disease activity using only composite disease activity scores. The majority of these studies have also only investigated the effects of anti-TNF treatment either on changes in cytokine production by stimulated PBMCs or on changes in the frequency of circulating IL17 or IFNγ producing cells by flow cytometry (Aerts et al., 2010; Alzabin et al., 2012; Bosè et al., 2011;
Chen et al., 2011; Maurice et al., 1999; Nissinen et al., 2004; Xueyi et al., 2012; Zou et al., 2003). In addition, very few other studies have been truly longitudinal and only a few have followed up the patient cohorts for more than one time point on treatment (Alzabin et al., 2012; Maurice et al., 1999; Nissinen et al., 2004). By comparing changes from baseline to 3 time points on treatment in this study, we have been able to study the kinetics of change of the clinical, ultrasonographic and immunological parameters with anti-TNF treatment. We selected to study the effects of anti-TNF at weeks 1, 4 and 12 on treatment to allow us to study changes both in the shorter and medium term after initiation of anti-TNF, while also enabling us to make an assessment of treatment response at 12 weeks. Early studies investigating the effects of anti-TNF on changes in the phenotype of CD4+ cells in RA patients have shown changes occurring within 3 days after just one infusion of infliximab, whereas earlier work from our group has shown changes in Th17 cells 4 weeks after anti-TNF initiation (Alzabin et al., 2012; Maurice et al., 1999). Early studies investigating the effects of infliximab on changes in serum cytokines have also shown changes within 1 week of the first infusion of infliximab (Elliott M.J., 1993; Elliott M.J., 1997).

Due to the exploratory nature of this study, the target numbers of patients to be recruited to each group were based on feasibility. However, early clinical studies investigating the effect of infliximab on RA patients disease activity have shown a significant effect on composite disease activity scores and inflammatory markers in a cohort of 20 patients with RA (Elliott M.J., 1993). Other studies investigating the effects of anti-TNF on Th1 cells in the peripheral blood of RA patients showed significant changes in a group of 17 patients and cytokine production from PBMCs during treatment with infliximab was investigated in a cohort of 25 patients with RA (Maurice et al., 1999; Nissinen et al., 2004). More recently, a study of 8 patients with RA treated with adalimumab reported a significant increase in IL17 production by cultured PBMCs 12 weeks after treatment initiation (Aerts et al., 2010).

Similarly, the early studies of the use of infliximab in AS showed significant improvements in Bath Indices within 2 weeks of the start of treatment in 2 small cohorts, one comprising 11 patients with AS and the other one comprising 35 patients with AS (Brandt et al., 2000; Braun et al., 2002). Additionally, significant up regulation IFNγ production by T cells has been reported in 10 AS patients after 12 weeks of etanercept treatment (Zou et al., 2003).
3.3.1 Effect of anti-TNF treatment on clinical measures of disease activity

3.3.1.1 RA patients

In the RA cohort as a whole, initiation of anti-TNF resulted in a significant decrease in DAS28 score as early as 1 week after starting treatment, with further significant decreases at 4 and 12 weeks. Similarly, CRP and ESR levels decreased significantly as early as 1 week after anti-TNF initiation. This rapid effect of anti-TNF was seen in the early clinical studies with infliximab (Elliott M.J., 1993; Elliott et al., 1994; Maini et al., 1998) and corresponds to the rapid changes seen in serum cytokines and acute phase markers, the early changes in CD4+ T cells and reduced chemokine levels and leukocyte trafficking to inflamed joints following a single infusion with infliximab. (Charles et al., 1999; Elliott M.J., 1993; Elliott M.J., 1997; Maurice et al., 1999; Taylor et al., 2000). A recent longitudinal model-based meta-analysis study compared the speed of onset of response to various biologic agents and determined the time to achieve 50% of the maximal ACR20 response for infliximab and adalimumab was 1.6 weeks and for etanercept it was between 2 to 3 weeks, confirming rapid responses to anti-TNF agents (Demin et al., 2012).

The EULAR response criteria were used in order to classify the RA patients into responders and non-responders at 12 weeks after initiation of anti-TNF (van Gestel et al., 1996). The DAS28 score has been extensively validated and is endorsed by EULAR and the ACR for RA disease activity measurement in RA clinical trials, and together with ACR criteria is often considered the ‘gold standard’ by which to measure RA disease activity. EULAR and ACR response criteria have been shown to be comparable in different clinical trials and to behave similarly with <5% discrepancy in responder status (van Gestel et al., 1999). The DAS response was used instead of ACR response criteria as this provided a numerical score at each time point on treatment, rather than just reflecting change in disease activity over time. Using the EULAR response criteria, 16 (64%) out of the 25 RA patients were classified as ‘responders’ to anti-TNF at 12 weeks after starting treatment. Thus, in our RA cohort the non-response rate was approximately 30%, which corresponds to the known non-response rates for anti-TNF therapy, showing that our cohort is representative (Villeneuve and Haraaoui, 2010). Although the DAS score and EULAR response criteria have allowed the classification of patients into clear responders and non-responders, there are limitations to using DAS score and EULAR response criteria. The individual components of the DAS score
can be subjective and insensitive to change. The different weighting of the DAS score components can affect the total DAS score result as patients with higher numbers of tender joints can score higher than patients with higher numbers of swollen joints for example. High DAS scores can reflect active arthritis as well as high pain levels, whereas baseline scores and changes in values may vary with the severity and stage of disease, so patients with late stage disease may have greater functional disability and show lower ACR/EULAR responses. The assessments can also be subjective; both from the patient’s and the assessor’s perspective, especially with respect to tender and swollen joint count assessments. Swelling has been shown to perform better than tenderness in terms of reliability and sensitivity to change. However, swelling may not necessarily reflect presence of active synovitis, but may be due to chronic structural changes or changes in the peri-articular structures. Although clinicians tend to give swollen joint counts a greater clinical importance, the DAS28 score weighs the tender joint count more heavily. The use of ESR may mask accurate reflection of disease activity as it contributes 15% to the overall score and patients with a low ESR result may be deemed to have achieved DAS remission despite having relatively high numbers of swollen joints. Older patients who tend to have higher ESR levels may achieve DAS remission less easily (Anderson et al., 2011). It is also well recognised that the magnitude of treatment response between patients is heterogeneous and that the composite disease activity scores commonly used to detect this have limited sensitivity to change. In particular, while the DAS28 score is a continuous variable it also comprises some binary components, therefore it may not be easy to judge whether a patient who has a small change in total DAS28 score with treatment is indicative of a true response or simply a variation or 'noise' in the total score. In view of these limitations, grey scale ultrasonography was used to evaluate changes in synovial hypertrophy and power Doppler ultrasound (PDUS) to determine changes in synovial vascularity in responders and non-responders with anti-TNF treatment alongside composite disease activity scores. As detailed in Chapter 4, PDUS provided a more detailed and objective morphological assessment of the changes in the joints of patients with RA and PsA with anti-TNF.

Comparison of the baseline characteristics of RA responders and non-responders to anti-TNF therapy showed that RA responders in this cohort have a tendency to longer disease duration than non-responders. This may imply that the non-responders may have had a shorter but more aggressive disease course thus requiring treatment with anti-TNF agents earlier in their
disease course and this being a reason why this group was less responsive to anti-TNF. On the other hand, one has to consider that patients with longer RA disease duration are more likely to have accumulated greater joint damage and disability than RA patients with shorter disease duration, which could potentially result in reduced anti-TNF responses. In addition, the RA responders and non-responders at baseline were well matched with respect to disease activity, with no significant differences in DAS28 scores, swollen or tender joint counts, ESR or CRP levels.

In the responders group, patients showed a significant decrease in swollen and tender joint counts at weeks 1, 4 and 12 after anti-TNF was initiated, whereas non-responders did not show a significant decrease until 12 weeks on treatment. The differences between responders and non-responders in the changes of swollen and tender joint counts, ESR and CRP measurements and DAS28 scores show that despite the small numbers of patients these parameters can effectively differentiate between responders and non-responders and that this is a representative cohort in which to study effects of anti-TNF treatment. Although these clinical parameters for assessment of disease activity and response have their limitations, this cohort shows that they remain useful in routine clinical practice.

### 3.3.1.2 AS patients

Response to anti-TNF in the AS cohort was assessed based on whether patients achieved a 50% reduction in BASDAI score (BASDAI 50) at 12 weeks compared to baseline. This is based on ASAS recommendations for the assessment of AS patients on anti-TNF treatment and on the clinical studies of anti-TNF agents in AS which utilised BASDAI 50 to define major clinical response (Rudwaleit et al., 2004; Sieper et al., 2009). Using these criteria, 11 out of the 15 AS patients (69%) were classified as responders to anti-TNF at 12 weeks and 4 patients as non-responders (31%). Despite the small number of patients, this corresponds to the published 30% non-response rate of anti-TNF therapy, showing that this AS cohort is representative of others (Villeneuve and Haraoui, 2010).

Although the BASDAI score has been shown to be both reliable and reproducible it has some limitations in that it is a purely patient-generated index, without including any objective measures of disease activity, nor a clinician’s assessment (Madsen et al., 2010). The score is
dependent on what the patient perceives is related to their AS disease and studies have shown that apart from inflammation, BASDAI score can also reflect mechanical symptoms and stiffness due to spinal fusion (Ward, 2006; Zochling, 2011). The ASDAS score was recently developed to overcome these limitations and provide a more objective measure of disease activity, but at the time of the protocol development, the ASDAS score was not yet fully validated hence this was not calculated in our cohort. As a patient global score (BAS-G) was not obtained in this patient cohort, an ASAS response could not be calculated either but addition of these two endpoints would strengthen assessment of treatment response in future studies.

In the AS cohort as a whole, mean baseline BASDAI score was 5.33 corresponding to high levels of AS disease activity. Changes in BASDAI, BASFI and BASMI scores with anti-TNF treatment in the whole cohort showed that BASDAI scores showed a more rapid improvement than BASFI and BASMI scores but all three indices improved significantly with anti-TNF treatment within 12 weeks.

There was a significant difference in the percentage change in BASDAI score at 12 weeks between responders and non-responders, showing that BASDAI remains a reliable measure of disease activity in routine clinical practice. BASFI improved significantly at 4 and then 12 weeks showing that it is also a reliable measure of response to anti-TNF. Although there was a significant improvement in BASMI score in the whole AS cohort, when the cohort was divided into responders and non-responders, there were no significant changes in BASMI scores in either group with treatment. This was likely to be due to the small numbers of patients in each group which were insufficient to detect small changes in BASMI with treatment within 12 weeks. These findings are supported by the early clinical studies of infliximab in AS patients, which also noted a rapid improvement in BASDAI, BASFI and BASMI scores by week 2 after infliximab infusion, with further improvement at weeks 6 and 12 on treatment (Brandt et al., 2000; Braun et al., 2002).

When the AS cohort was divided into responders and non-responders to anti-TNF treatment, non-responders were significantly older than responders. This may suggest that this group of patients had more longstanding disease and may thus imply that they would be less responsive to anti-TNF treatment, especially if they have more spinal ankylosis than inflammation. However, the more likely explanation is that due to the small number of
patients in the non-responder group, one of the patients with markedly longer disease duration compared to the others skewed the overall disease duration in this group. In support of this, there were no significant differences in the BASMI scores between the responders and non-responders at baseline suggesting no significant differences in spinal mobility.

3.3.1.3 PsA patients

The PsARC criteria were used to classify patients as responders and non-responders to anti-TNF therapy. With these criteria, 7 patients were classified as responders and 1 as non-responder to anti-TNF at 12 weeks. However, in the cohort as a whole and despite the small overall number of patients, anti-TNF treatment did lead to a significant decline in number of swollen joints at 1, 4 and 12 weeks of treatment and also led to a significant decrease in tender joint counts at 12 weeks of treatment. In addition, both patient and physician global scores declined significantly with anti-TNF therapy, with the physician global decreasing significantly at 1, 4 and 12 weeks and the patient global decreasing significantly at 12 weeks after treatment initiation. Studies evaluating the extent and differences in the evaluation of disease activity between patients and physicians have found that different disease factors are attributed differing importance and weighting by patients and physicians. Thus, the main determinants of patient global scores have been found to be pain and function, whereas the main determinants of physician global assessments include numbers of swollen joints, pain, function and CRP levels. Patients with increased pain have a worse perception of their disease activity whereas higher numbers of swollen joints lead to worse physician global scores of disease activity (Studenic et al., 2012).

Although PsARC has been able to demonstrate differences between drug and placebo treated patients in clinical studies, its limitations are that it can have a high placebo response rate and that it reflects a change in disease activity over time but cannot determine current level of disease activity. It also sets a relatively ‘low bar’ of response which in routine practice may fail short of the ultimate desired level of clinical outcome. The inherent problems with subjectivity with the assessment of numbers of tender and swollen joints are also further limitations.

The alternative disease activity scores to PsARC which could have been used in this study include the DAS score (and EULAR response criteria) or ACR criteria as used in RA
patients. Although DAS score has been validated for use in PsA, its limitation is that it excludes joints which are commonly affected in PsA. ACR response criteria have also been validated for use in PsA and employed in clinical trials with PsA patients, but they provide a dichotomous measure and only report a change in disease activity over time, rather than reflecting current levels of disease activity.

Difficulties with patient recruitment to this group led to the small overall number of PsA patients in this study and due to this, comparisons between responders and non-responders in changes of clinical parameters with anti-TNF were not made. Future work to recruit and follow up prospectively additional PsA patients would allow identification and characterisation of PsA anti-TNF responders and non-responders and allow comparisons with the RA and AS responder and non-responder groups to be made.

3.4 Conclusion

This clinical study has prospectively followed patients with RA, AS and PsA at predetermined protocol visits during the first 12 weeks of anti-TNF treatment to evaluate changes in composite disease activity scores, inflammatory markers and patient-reported outcome measures during therapy. This has also enabled the classification of anti-TNF responders and non-responders within the RA and AS groups. Although this study has a small number of patients, the effect of anti-TNF on disease activity has identified that in this study approximately 65% of patients with RA and AS are responders to anti-TNF which is in agreement with the known anti-TNF response rates, showing that these patient cohorts are representative. The effect of anti-TNF treatment on the clinical parameters of disease activity in this study has also been in line with the changes seen in these parameters in the early clinical studies of anti-TNF therapy. To our knowledge, a prospective study of this kind comprising patients with 3 different types of inflammatory arthritis treated with anti-TNF and involving clinical, morphological and immunological investigations has not been carried out to date. The three patient cohorts characterised in this chapter have provided the basis to evaluate the effects of anti-TNF treatment on changes in the frequency of circulating Th1 and Th17 cells, as well as to identify immune correlates of treatment response, as described subsequently in Chapters 5, 6 and 8.
Chapter 4. Use of power Doppler ultrasonography to characterise changes in synovial pathology of hand joints in RA and PsA patients and entheseal pathology in AS patients during anti-TNF treatment

4.1 Introduction

The work presented in this chapter describes the use of grey scale and power Doppler ultrasonography (PDUS) to evaluate changes in synovial thickening and vascularity in the metacarpophalangeal joints of RA and PsA patients and changes in entheseal pathology in patients with AS during anti-TNF treatment. Ultrasound examination allows direct visualisation and quantitative assessment of the morphological changes in the target tissue in RA and PsA patients and one of the common sites of disease involvement in AS patients. Due to the limitations of the use of composite disease activity scores in assessing treatment response in each of these three disease phenotypes as discussed in Chapter 3, we also utilised PDUS in order to obtain a more objective and sensitive measure of inflammation in the joints and entheses in RA, PsA and AS patients.

The objective of the work in this chapter was to characterise longitudinally the morphological changes occurring in the target tissue during anti-TNF therapy in patients with different types of inflammatory arthritis using PDUS, which would complement the information obtained from changes in clinical outcome measures during anti-TNF treatment and allow more detailed characterisation of treatment responders and non-responders.

This is the first longitudinal ultrasound study which incorporates comprehensive semi-quantitative and quantitative measures of synovial thickening and vascularity to characterise changes in the MCP joints of patients with RA and PsA over time on anti-TNF treatment including shorter term (1 week and 4 weeks) and medium term (12 months) endpoints. This is also the first study to use grey scale and power Doppler ultrasound to characterise changes in lower limb entheseal pathology of AS patients longitudinally during anti-TNF treatment.
4.1.1 The use of power Doppler ultrasound in monitoring treatment response in RA and PsA

Joint synovitis is generally evaluated indirectly by assessing subjective clinical data and laboratory parameters of inflammation. There is good evidence that ultrasound is more sensitive than clinical examination for detecting synovitis (Backhaus et al., 1999; Szkudlarek et al., 2001). Ultrasound assessment of synovitis includes grey scale ultrasonography, which uses reflected pulses of high frequency sound waves to assess structures and the superficial hand joints involved in inflammatory arthritis are very amenable to ultrasound evaluation. Although grey scale ultrasonography visualises synovial thickening, it cannot distinguish between active synovitis and fibrous tissue, thus when used alone it does not demonstrate a close relationship with clinical assessments of disease activity (Qvistgaard et al., 2001). By demonstrating the presence of blood flow in small vessels, power Doppler ultrasonography can make this distinction and delineate the presence of highly vascularised synovium. Power Doppler ultrasonography uses the Doppler effect to detect movement in red blood cells within a vessel by analysing the change in frequency of returning echoes and this change in signal is related to the velocity of the moving objects. Power Doppler detects changes in the amplitude of the Doppler signal, which is related to the volume of blood flowing through the region of interest. Whilst it does not provide information on direction or velocity of flow, the power of the many frequency shifts inside a cell are added to form a power signal. Power Doppler is thus sensitive at detecting low velocity flow in small vessels, such as in the synovium (Martinoli, 1998). A number of studies have proven PDUS to be more sensitive and reproducible than clinical evaluation in assessing joint inflammation (Backhaus et al., 1999; Szkudlarek et al., 2001; Szkudlarek et al., 2006). PDUS has been shown to identify ongoing synovial inflammation in a cohort of patients defined as being in clinical remission by their treating physician by clinical examination and 60.4% of these patients were identified to have active inflammation as confirmed by increased PDUS signal. This further demonstrates the increased sensitivity of PDUS in the detection of synovial inflammation compared with clinical examination alone and confirms that PDUS provides objective and complementary information to clinical disease activity markers (Brown et al., 2006). Comparison studies of PDUS with other imaging modalities such as MRI, arthroscopy and scintigraphy have also confirmed the validity of PDUS in detecting synovial inflammation (Brown et al., 2006; Szkudlarek et al., 2001; Szkudlarek et al., 2006). In addition, PDUS has confirmed validity in detecting synovial vascularity and therefore inflammation when compared with histologically
confirmed synovial inflammation in biopsies from hip and knee joints (Walther et al., 2001; Walther et al., 2002).

Several studies of grey scale and PDUS performed in RA patients during anti-TNF treatment have shown that PDUS findings can be used as an outcome measure of the response to therapy (Filippucci et al., 2006; Fiocco et al., 2005; Iagnocco et al., 2008; Naredo et al., 2008a; Ribbens et al., 2003; Taylor et al., 2004). These studies also showed that while PDUS and clinical measures of disease activity such as DAS score, swollen joint count, CRP and ESR are related, they provide complementary information to each other, as PDUS was shown to detect synovitis in some 30% of joints which were not clinically swollen. This confirms PDUS to be more sensitive than clinical outcome measures in evaluating treatment response (de Miguel et al., 2009; Naredo et al., 2008a; Ribbens et al., 2003; Salaffi et al., 2008; Taylor et al., 2004).

Longitudinal studies evaluating the changes in synovial thickening and vascularity by PDUS in response to anti-TNF treatment have been conducted studying both short term changes, over a few weeks and also changes over a longer time period, of up to a year on treatment. These studies have consistently shown that PDUS findings can be used as an outcome measure of response to therapy, with significant reductions in power Doppler signal being reported as early as 2 weeks after treatment initiation (Filippucci et al., 2006; Hammer et al., 2010; Ribbens et al., 2003).

In a double-blind placebo-controlled randomised study of anti-TNF therapy in 24 RA patients, Taylor et al demonstrated that ultrasound assessments of synovial thickening and vascularity improved significantly after 18 weeks of anti-TNF treatment and were able to discriminate between the 2 treatment groups with greater sensitivity than the conventionally used clinical outcome measures of disease activity (Taylor et al., 2004). Naredo et al further confirmed the validity and responsiveness of PDUS in monitoring response to anti-TNF in RA patients in a longitudinal multi-centre study with assessments at baseline and 1, 3, 6, and 12 months on therapy (Naredo et al., 2008a). Changes in PDUS parameters of joint inflammation were shown to be consistent, sensitive to change over time and closely correlated to changes in DAS28 scores, confirming the construct validity of PDUS in the longitudinal assessment of RA synovial inflammation. A number of other smaller studies have also documented the significant improvement in PDUS indices with anti-TNF treatment.
in RA patients (Ellegaard et al., 2009; Filippucci et al., 2006; Fiocco et al., 2005; Iagnocco et al., 2008).

In addition to its use in monitoring treatment response, PDUS may also be important in predicting treatment outcome. The presence of synovial vascularity detected by ultrasound is associated with radiographic joint damage but this positive relationship has been shown to be abrogated by anti-TNF treatment (Naredo et al., 2008a; Taylor et al., 2004). This suggests that patients with the highest baseline disease activity as assessed by the presence of PDUS may derive the greatest benefit from anti-TNF treatment, especially with regard to suppression of radiological progression (Taylor et al., 2004). Ellegaard et al further showed that baseline vascular signal in the wrist joints of patients prior to starting anti-TNF treatment was able to predict which patients will remain on anti-TNF therapy 1 year after initiation, in contrast to clinical measures which were not predictive (Ellegaard et al., 2009). As PDUS findings reflect pannus vascularisation and this is the target tissue of anti-TNF therapies, it has been proposed that the response of synovial power Doppler signal in rheumatoid joints may be considered to be a measurement of response to anti-TNF therapy which is independent of clinical and grey scale indices of disease activity (Naredo et al., 2008a). Thus, PDUS may have utility in prognostication and predicting treatment response, although confirmation in larger patient cohorts would be needed.

Similarly to RA, PDUS in PsA has been shown to be more sensitive than clinical examination in detecting synovitis and bony erosions and ultrasound has shown high concordance with MRI in detecting destructive and inflammatory joint changes (Milosavljevic et al., 2005; Wiell et al., 2007). Only a few studies have utilised PDUS for monitoring treatment response in patients with PsA. They have shown that in PsA as in RA, PDUS endpoints are objective, sensitive and responsive to change with treatment using ciclosporin, methotrexate or anti-TNF (De Agustin JJ, 2012; Fiocco et al., 2005; Fraser et al., 2005).

4.1.2 Use of PDUS to characterise changes in enthesitis with treatment in AS patients

Enthesitis is considered the primary site of disease in AS but it can often be asymptomatic in both the axial and peripheral skeleton. The most common sites of peripheral enthesial
involvement in AS are the lower limb entheses. Ultrasound has been proven to be very sensitive in the detection of enthesial abnormalities, even before these are evident on MRI or by clinical examination (Balint et al., 2002; D'Agostino et al., 2003; Falsetti et al., 2009; Kamel et al., 2004). Grey scale ultrasound allows the depiction of signs of acute and chronic enthesial inflammation, as well as structural damage as it allows visualisation of the abnormal fibrillar echogenicity pattern of the tendon, increased tendon thickness, the presence of calcification and enthesophytes within the enthesis. Power Doppler mode allows the visualisation of abnormal vascularisation of the entheses and altered vascularisation of the enthesis insertion into the cortical bone by PDUS has been shown to be a hallmark feature of spondyloarthritis (D'Agostino et al., 2011).

Characterisation of the therapeutic effects of anti-TNF treatment on PDUS changes of enthesitis is still mostly limited to a few case reports or small clinical studies. D’Agostino et al reported two HLA-B27 positive spondyloarthritis patients with refractory erosive calcaneal enthesitis who showed significant improvement of the enthesial lesions and bursitis as detected by PDUS after treatment with infliximab (D'Agostino et al., 2002). Aydin et al showed that anti-TNF treatment resulted in significant improvement of subclinical Achilles enthesitis detected by grey scale ultrasound after only 2 months of treatment in patients with AS (Aydin et al., 2010). More recently, Naredo et al conducted the only multi-centre study to date involving more than 300 patients with spondyloarthritis to investigate the response of enthesitis to anti-TNF therapy treatment and the reproducibility of PDUS abnormalities. They showed that after 6 months of anti-TNF treatment, there were significant improvements in entheses thickening, hypoechochogenicity and bursitis on PDUS, which were deemed to reflect acute inflammation. However, no significant improvements were seen in calcific deposits or cortical abnormalities at the entheses, which have been suggested to reflect more structural damage and thus they would be unresponsive to inflammation-targeted treatment (Naredo et al., 2010).

4.2 Results

4.2.1 Parallel-scan intra-observer variability in PDUS image acquisition

To minimise variability in image acquisition, I scanned all the study patients using a standardised approach at each visit, as described in Section 2.1.4.1 using the same ultrasound machine with constant settings for grey scale and power Doppler modes. To assess the
variability in grey scale and power Doppler ultrasound image acquisition, five patients with RA were scanned twice at the same sitting and the anonymised images were then scored as described in Section 2.1.4.2. Composite score comparisons were then made between the first and second scans for the 5 patients and the intra-class correlation coefficient for synovial thickening and vascularity was calculated (Table 4.1). These results show excellent agreement between the two scan acquisitions for all ultrasound endpoints, thus confirming that the ultrasound scanning technique was consistent and the images obtained were reproducible.

4.2.2 Within-scan intra-reader variability in PDUS image analysis

In order to minimise variability in scoring the grey scale and power Doppler images, I scored all the images obtained in the study and this was performed according to the protocol described in Section 2.1.4.2. Images were anonymised after acquisition and the image scoring was completed on images which were anonymised with respect to patient details and time point on the study. In order to assess the within-scan intra-reader reproducibility in image scoring, 10 anonymised RA patient scans were randomly selected and the images and power Doppler clips were reviewed 2 years after the initial reading in a blinded fashion. Joint by joint and also composite score comparisons were then made between the first and second readings for each of the ten ultrasound endpoints and intra-class correlation coefficient values were determined (Tables 4.2 and 4.3). For joint by joint analysis (Table 4.2), the ICC values for the 100 paired readings were excellent for all ultrasound endpoints. For the 10MCP composite scores, the ICC values for the 10 paired readings were also excellent for all ultrasound endpoints (Table 4.3). Thus, the method for scoring synovial thickness and vascularity both semi-quantitatively and quantitatively was highly reproducible with consistent results.

4.2.3 Agreement between semi-quantitative and quantitative ultrasound scores for synovial thickening and synovial vascularity

Synovial thickening and synovial vascularity were scored using both a 5 point semi-quantitative scale and also quantitatively by determining the number of pixels within a region of interest as described in Section 2.1.4.2. There were very strong correlations between the
composite semi-quantitative and quantitative scores for synovial thickness (Trans STA versus Trans STi $r=0.95$, $p<0.0001$; Long STA versus Long STi $r=0.95$, $p<0.0001$) (Figure 4.1 A and B) and synovial vascularity (Trans PDA versus Trans VASCi $r=0.98$, $p<0.0001$; Long

Table 4.1  Assessment of parallel scan intra-reader reliability in image acquisition of grey scale and power Doppler ultrasound images

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ICC value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synovial thickening</strong></td>
<td></td>
</tr>
<tr>
<td>Trans STi</td>
<td>0.99</td>
</tr>
<tr>
<td>Long STi</td>
<td>1</td>
</tr>
<tr>
<td>Trans STA</td>
<td>0.99</td>
</tr>
<tr>
<td>Long STA</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Synovial vascularity</strong></td>
<td></td>
</tr>
<tr>
<td>Trans VASCi</td>
<td>0.99</td>
</tr>
<tr>
<td>Long VASCi</td>
<td>0.98</td>
</tr>
<tr>
<td>Trans PDA</td>
<td>0.99</td>
</tr>
<tr>
<td>Long PDA</td>
<td>0.99</td>
</tr>
</tbody>
</table>

ICC scores shown for the composite semi-quantitative and quantitative ultrasound scores for synovial thickening and vascularity. Abbreviations: ICC, intra-class correlation coefficient; Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area; Trans VASCi, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCi, longitudinal synovial vascularity index; Long PDA, longitudinal synovial power Doppler area score.
Table 4.2  Assessment of within-scan intra-reader reproducibility in image scoring for 100 paired joint by joint comparisons for semi-quantitative and quantitative scores for synovial thickness and vascularity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ICC value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synovial thickening</strong></td>
<td></td>
</tr>
<tr>
<td>Trans STi</td>
<td>0.96</td>
</tr>
<tr>
<td>Long STi</td>
<td>0.98</td>
</tr>
<tr>
<td>Trans STA</td>
<td>0.91</td>
</tr>
<tr>
<td>Long STA</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Synovial vascularity</strong></td>
<td></td>
</tr>
<tr>
<td>Trans VASCi</td>
<td>0.97</td>
</tr>
<tr>
<td>Long VASCi</td>
<td>0.98</td>
</tr>
<tr>
<td>Trans PDA</td>
<td>0.99</td>
</tr>
<tr>
<td>Long PDA</td>
<td>0.99</td>
</tr>
</tbody>
</table>

ICC scores shown for the composite semi-quantitative and quantitative ultrasound scores for synovial thickening and vascularity. Abbreviations: ICC, intra-class correlation coefficient; Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area; Trans VASCi, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCi, longitudinal synovial vascularity index; Long PDA, longitudinal synovial power Doppler area score.
Table 4.3  Assessment of within-scan intra-reader reproducibility of 10 MCP composite semi-quantitative and quantitative scores for synovial thickness and vascularity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ICC value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synovial thickening</strong></td>
<td></td>
</tr>
<tr>
<td>Trans STi</td>
<td>0.96</td>
</tr>
<tr>
<td>Long STi</td>
<td>0.98</td>
</tr>
<tr>
<td>Trans STA</td>
<td>0.91</td>
</tr>
<tr>
<td>Long STA</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Synovial vascularity</strong></td>
<td></td>
</tr>
<tr>
<td>Trans VASCi</td>
<td>0.97</td>
</tr>
<tr>
<td>Long VASCi</td>
<td>0.98</td>
</tr>
<tr>
<td>Trans PDA</td>
<td>0.99</td>
</tr>
<tr>
<td>Long PDA</td>
<td>0.99</td>
</tr>
</tbody>
</table>

ICC scores shown for the composite semi-quantitative and quantitative ultrasound scores for synovial thickening and vascularity. Abbreviations: ICC, intra-class correlation coefficient; Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area; Trans VASCi, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCi, longitudinal synovial vascularity index; Long PDA, longitudinal synovial power Doppler area score.
Figure 4.1  Positive correlations between the semi-quantitative and quantitative ultrasound measures of synovial thickening and vascularity assessed by power Doppler ultrasound

Positive correlations obtained using Spearman’s rank method between the semi-quantitative and quantitative composite ultrasound scores for synovial thickening in the transverse view of metacarpophalangeal joints in rheumatoid arthritis patients (A), for synovial thickening in the longitudinal view of metacarpophalangeal joints in rheumatoid arthritis patients (B), for synovial vascularity in the transverse view of metacarpophalangeal joints in rheumatoid arthritis patients (C) and for synovial vascularity in the longitudinal view of metacarpophalangeal joints in rheumatoid arthritis patients (D). Abbreviations: Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area; Trans VASCI, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCI, longitudinal synovial vascularity index; Long PDA, long synovial power Doppler area score. Correlations obtained by Spearman’s rank method.
PDA versus Long VASCi $r=0.98$, $p<0.0001$ (Figure 4.1 C and D) both in the transverse and in the longitudinal views.

4.2.4 Relationships between ultrasound scores for synovial thickening and vascularity and clinical disease activity measures in RA patients

Correlations between the semi-quantitative and quantitative parameters of synovial thickness and synovial vascularity on ultrasound and the clinical measures of disease activity in the RA patients at baseline are shown in Table 4.4. All ultrasound endpoints for synovial thickening correlated positively with the number of swollen joints determined by clinical examination, but there was no correlation with the number of tender joints determined by clinical examination. There were no significant correlations between synovial thickness ultrasound parameters and DAS28ESR or DAS28CRP scores. In contrast, there were significant positive correlations between the semi-quantitative and quantitative parameters of synovial vascularity and DAS28ESR and DAS28CRP scores. Ultrasound endpoints for synovial vascularity also correlated positively with the number of swollen joints determined by clinical examination, but not with numbers of tender joints. There were also significant positive correlations between the change in DAS28ESR score from baseline to 4 weeks on treatment and the change in synovial vascularity endpoints from baseline to 4 weeks on treatment (Trans VASCi $r=0.79$, $p=<0.0001$; Trans PDA $r=0.61$, $p=0.0017$; Long VASCi $r=0.64$, $p=0.0008$; Long PDA $r=0.67$, $p=0.0003$). There were also significant positive correlations between the change in DAS28ESR score from baseline to 12 weeks on treatment and the change in synovial vascularity endpoints from baseline to 12 weeks on treatment (Trans VASCi $r=0.62$, $p=0.0009$; Trans PDA $r=0.45$, $p=0.02$; Long VASCi $r=0.40$, $p=0.04$; Long PDA $r=0.58$, $p=0.0027$). This shows that the changes in synovial vascularity parameters by ultrasound with anti-TNF treatment are closely paralleled by changes in DAS scores with anti-TNF treatment.

There were no significant correlations between the ultrasound endpoints for synovial thickness or vascularity and ESR or CRP levels.
Table 4.4  Correlations between power Doppler ultrasound parameters and clinical measures of disease activity confirming validity of PDUS as a biomarker of synovitis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DAS28ESR</th>
<th></th>
<th>DAS28CRP</th>
<th></th>
<th>SJC</th>
<th></th>
<th>TJC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans STi</td>
<td>0.32 0.12</td>
<td>0.32 0.11</td>
<td><strong>0.56 0.0039</strong></td>
<td>0.25 0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans STA</td>
<td>0.35 0.09</td>
<td>0.33 0.11</td>
<td><strong>0.62 0.0009</strong></td>
<td>0.24 0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long STi</td>
<td>0.36 0.09</td>
<td>0.39 0.06</td>
<td><strong>0.56 0.0038</strong></td>
<td>0.25 0.17</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Long STA</td>
<td>0.26 0.21</td>
<td>0.31 0.13</td>
<td><strong>0.55 0.0042</strong></td>
<td>0.24 0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans V ASCi</td>
<td><strong>0.58 0.02</strong></td>
<td><strong>0.61 0.01</strong></td>
<td><strong>0.61 0.001</strong></td>
<td>0.1 0.63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans PDA</td>
<td><strong>0.59 0.01</strong></td>
<td><strong>0.61 0.01</strong></td>
<td><strong>0.58 0.002</strong></td>
<td>0.11 0.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long V ASCi</td>
<td><strong>0.49 0.04</strong></td>
<td><strong>0.51 0.04</strong></td>
<td><strong>0.61 0.001</strong></td>
<td>0.03 0.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Long PDA</td>
<td><strong>0.48 0.04</strong></td>
<td><strong>0.51 0.04</strong></td>
<td><strong>0.68 0.0002</strong></td>
<td>0.09 0.68</td>
<td></td>
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</tbody>
</table>

Correlations obtained by Spearman’s rank method, significant correlations are shown in bold type. Abbreviations: DAS28, Disease Activity Score of 28 joints; SJC, swollen joint count, TJC, tender joint count.
4.2.5 RA patient cohort

4.2.5.1 Changes in ultrasound scores for synovial thickening and vascularity during anti-TNF treatment

The changes in the semi-quantitative and quantitative ultrasound scores for synovial thickening and vascularity during anti-TNF treatment in the whole RA cohort are presented in Table 4.5. The semi-quantitative ultrasound scores for synovial thickening showed a trend to decrease at 12 weeks after anti-TNF initiation, but this did not reach statistical significance (Trans STi p=0.10 and Long STi p =0.06). However, the quantitative scores for synovial thickening did show a significant change with time on anti-TNF treatment; transverse synovial thickness area (Trans STA) showed a significant decrease at 4 and 12 weeks on treatment and longitudinal synovial thickness area (Long STA) decreased significantly after 12 weeks on treatment.

All the semi-quantitative and quantitative scores for synovial vascularity showed an early and significant decrease after 1 week on anti-TNF treatment and these parameters further decreased at 4 and 12 weeks on treatment (Table 4.5). These significant changes in synovial vascularity with time on anti-TNF treatment were seen both in the transverse and longitudinal views of the MCP joints.

4.2.5.2 Comparison between anti-TNF responders and non-responders in changes of ultrasound scores during treatment

There were no significant differences between anti-TNF responders and non-responders at baseline in the composite semi-quantitative and quantitative scores for synovial thickness or vascularity (Table 4.6). There was a trend towards the non-responders group having lower scores for synovial thickening at baseline compared to responders, but this difference was not statistically significant. Similarly, there was a trend towards the non-responders group having lower semi-quantitative and quantitative scores for synovial vascularity at baseline compared to the responders group, but this difference was also not statistically significant.

Anti-TNF responders showed an early and significant decrease in the quantitative scores for synovial thickness (Trans STA and Long STA) during anti-TNF treatment compared to baseline (Table 4.7). The semi-quantitative scores for synovial thickening (Trans STi and
Table 4.5 Change in semi-quantitative and quantitative ultrasound measures of synovial thickening and vascularity over 12 weeks on anti-TNF treatment in rheumatoid arthritis group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>1 week</th>
<th>4 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD value</td>
<td>Mean ± SD value</td>
<td>Mean ± SD value</td>
<td>Mean ± SD value</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
</tr>
<tr>
<td>Trans STi</td>
<td>17.8±7.3</td>
<td>19.5±7.2</td>
<td>17.5±6.4</td>
<td>15.3±7.7</td>
</tr>
<tr>
<td>Trans STA</td>
<td>207,837±129,741</td>
<td>213,197±104,172</td>
<td>182,380±96,420</td>
<td>157,467±101,946</td>
</tr>
<tr>
<td>Long STi</td>
<td>19.9±8.3</td>
<td>19.8±8.1</td>
<td>18.8±7.3</td>
<td>16.8±7.1</td>
</tr>
<tr>
<td>Long STA</td>
<td>210,163±132,133</td>
<td>185,890±108,337</td>
<td>174,155±87,735</td>
<td>159,171±87,779</td>
</tr>
<tr>
<td>Trans VASCi</td>
<td>9.3±9.7</td>
<td>6.8±7.6</td>
<td>5.2±8.3</td>
<td>4.3±7.5</td>
</tr>
<tr>
<td>Trans PDA</td>
<td>4,425±6,552</td>
<td>2,964±5,244</td>
<td>2,166±5,308</td>
<td>1,546±4,186</td>
</tr>
<tr>
<td>Long VASCi</td>
<td>9.3±10.9</td>
<td>6.9±9.3</td>
<td>5.2±7.8</td>
<td>3.5±6.3</td>
</tr>
<tr>
<td>Long PDA</td>
<td>5,216±8,523</td>
<td>3,165±7,877</td>
<td>1,715±4,043</td>
<td>1,337±3,640</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, ns non-significant, *p<0.05 **p<0.001. Abbreviations: Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area; Trans VASCi, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCi, longitudinal synovial vascularity index; Long PDA, longitudinal synovial power Doppler area score.
Table 4.6  Comparison between rheumatoid arthritis responder and non-responder patients to anti-TNF in baseline semi-quantitative and quantitative measures of synovial thickness and vascularity

<table>
<thead>
<tr>
<th>Parameter, treatment group</th>
<th>Responders Mean ±SD</th>
<th>Non-responders Mean ±SD</th>
<th>Mann Whitney U test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans Sti</td>
<td>19.4±6.7</td>
<td>14.25±7.6</td>
<td>0.09</td>
</tr>
<tr>
<td>Trans STA</td>
<td>225,375.6±120,138.5</td>
<td>170,568±149,623.5</td>
<td>0.17</td>
</tr>
<tr>
<td>Long Sti</td>
<td>21.0±8.0</td>
<td>17.6±9.1</td>
<td>0.32</td>
</tr>
<tr>
<td>Long STA</td>
<td>232,540.3±139,605.5</td>
<td>162,610±107,291.2</td>
<td>0.23</td>
</tr>
<tr>
<td>Trans VASCi</td>
<td>11.5±9.7</td>
<td>5.6±8.6</td>
<td>0.08</td>
</tr>
<tr>
<td>Trans PDA</td>
<td>5,473.6±6,970.1</td>
<td>2,197.1±5269.1</td>
<td>0.08</td>
</tr>
<tr>
<td>Long VASCi</td>
<td>11.3±11.2</td>
<td>5.0±9.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Long PDA</td>
<td>6,756.4±9600.1</td>
<td>1,943.4±4515.4</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. Comparison of responder versus non-responder groups using Mann Whitney U test. Abbreviations: Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area; Trans VASCi, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCi, longitudinal synovial vascularity index; Long PDA, longitudinal synovial power Doppler area score.
Table 4.7  Comparison between RA responders and non-responders to anti-TNF therapy in the change in semi-quantitative and quantitative scores for synovial thickening with time on anti-TNF treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>Trans STi</td>
<td>Responders</td>
<td>19.4±6.7</td>
<td>20.4±6.6</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Non-responders</td>
<td>14.25±7.6</td>
<td>17.5±8.9</td>
<td>ns</td>
</tr>
<tr>
<td>Trans STA</td>
<td>Responders</td>
<td>225,375.6±120,138.5</td>
<td>217,029.6±97,643.7</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Non-responders</td>
<td>170,568±149,623.5</td>
<td>204,255.7±127,712.4</td>
<td>ns</td>
</tr>
<tr>
<td>Long STi</td>
<td>Responders</td>
<td>21.0±8.0</td>
<td>20.6±7.5</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Non-responders</td>
<td>17.6±9.1</td>
<td>17.8±9.7</td>
<td>ns</td>
</tr>
<tr>
<td>Long STA</td>
<td>Responders</td>
<td>232,540.3±139,605.5</td>
<td>201,460.5±116,901.3</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Non-responders</td>
<td>162,610±107,291.2</td>
<td>149,558.2±82,499.2</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, ns non-significant, *p<0.05, **p<0.005. Abbreviations: Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area
Long STi) in the responders group showed a trend to decrease during treatment, with the decrease at 12 weeks on treatment reaching statistical significance (Trans STi p=0.03; Long STi p=0.03). In contrast, there were no significant changes in the semi-quantitative or quantitative scores of synovial thickening with time on anti-TNF treatment in the non-responders group at any of the time points compared to baseline.

In the responders group, there were significant decreases in the semi-quantitative and quantitative scores of synovial vascularity at 1, 4 and 12 weeks on anti-TNF treatment compared to baseline (Table 4.8). However, in the non-responders group, there were no significant changes in the synovial vascularity endpoints with time on anti-TNF treatment.

A comparison of the change from baseline to each time point on treatment in the semi-quantitative and quantitative scores for synovial thickness and vascularity between the responders and non-responders groups are presented in Figures 4.2 to 4.9. These results show that the ultrasound endpoints were able to discriminate well between the responder and non-responder groups.

Transverse synovial thickness index (Trans STi) decreased significantly in the responders group at 12 weeks after treatment initiation compared to baseline, whereas there was no significant change in the non-responders at 12 weeks (p=0.9) (Figure 4.2). When the change in transverse synovial thickness index from baseline to each time point on treatment was compared between responders and non-responders, responders showed a trend towards a gradual decrease from baseline with time on treatment, whereas in the non-responders group a trend towards a decrease was only evident at week 12 (Figure 4.2C). There was a significant difference in the magnitude of change in Trans STi from baseline to week 1 on treatment between responders and non-responders, but not at 4 or 12 weeks.

Transverse synovial thickness area (Trans STA) decreased significantly at 4 and 12 weeks on anti-TNF treatment in responders compared to baseline, whereas there were no significant changes in the non-responders group (Figure 4.3). A comparison of the change in Trans STA from baseline to weeks 1, 4 and 12 on treatment between responders and non-responders showed that in responders, total scores decreased steadily during anti-TNF treatment, whereas in non-responders, there is little change from baseline to weeks 1 and 4 and a trend towards a decrease only occurred at 12 weeks.

In anti-TNF responders, longitudinal synovial thickness index (Long STi) decreased significantly at 12 weeks compared to baseline, whereas there was no significant change in
Table 4.8  Comparison between RA responders and non-responders to anti-TNF therapy in the change in semi-quantitative and quantitative scores for synovial vascularity by power Doppler ultrasound with time on anti-TNF treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 4</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean score</td>
<td>Mean score</td>
<td>p-value</td>
<td>Mean score</td>
</tr>
<tr>
<td>Trans VASCi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>11.5±9.7</td>
<td>7.9±7.5</td>
<td>*</td>
<td>4.3±6.5</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>5.6±8.6</td>
<td>4.2±7.9</td>
<td>ns</td>
<td>5.4±12.2</td>
</tr>
<tr>
<td>Trans PDA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>5,473.6±6,970.1</td>
<td>3,438.2±5,635.2</td>
<td>*</td>
<td>1,288.6±2,410.4</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>2,197.1±526.9</td>
<td>1,856.7±445.2</td>
<td>ns</td>
<td>3,635.1±9,430.5</td>
</tr>
<tr>
<td>Long VASCi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>11.3±11.2</td>
<td>7.9±10.2</td>
<td>**</td>
<td>4.5±6.4</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>5.0±9.4</td>
<td>4.8±7.3</td>
<td>ns</td>
<td>5.1±11.1</td>
</tr>
<tr>
<td>Long PDA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>6,756.4±9600.1</td>
<td>3,910.9±9205.4</td>
<td>*</td>
<td>1,056.2±2,075.6</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>1,943.4±451.5</td>
<td>1,424.7±3,199.1</td>
<td>ns</td>
<td>2,787.9±7,019.9</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, ns non-significant, *p<0.05, **p<0.005. Abbreviations: Trans VASCi, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCi, longitudinal synovial vascularity index; Long PDA, longitudinal synovial power Doppler area score.
Figure 4.2  Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Transverse Synovial Thickness Index (Trans STi) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Trans STi score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05. The mean±SEM 10MCP Trans STi scores were compared at each time point between the two groups (C) using Mann Whitney U test. ns; non-significant, *p<0.05. 10MCP Trans STi is the composite transverse synovial thickness index score of the 10 metacarpophalangeal joints scanned.
Figure 4.3 Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Transverse Synovial Thickness Area (Trans STA) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Trans STA score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001. The mean±SEM 10MCP Trans STA scores were compared at each time point between the two groups (C) using Mann Whitney U test. ns; non-significant. 10 MCP Trans STA is the composite transverse synovial thickness area score of the 10 metacarpophalangeal joints scanned.
the non-responders group (**Figure 4.4**). Both responders and non-responders showed a trend towards a decrease in the longitudinal synovial thickness index from their baseline level to weeks 1, 4 and 12 on anti-TNF treatment, with responders exhibiting a greater change in total score from baseline to each of the time points compared to the non-responders.

Longitudinal synovial thickness area (Long STA) decreased significantly at weeks 1, 4 and 12 compared to baseline in the responders group, but there were no significant changes in this parameter with treatment in the non-responders group. Comparison between responder and non-responder groups in the change in Long STA from baseline to weeks 1, 4 and 12 on treatment showed that the decrease in total score from baseline in responders was more marked than in non-responders, who showed minimal change in total scores from baseline (**Figure 4.5**).

Transverse synovial vascularity index (Trans VASCi) decreased significantly at weeks 1, 4 and 12 in the responders group compared to baseline, whereas there were no significant changes in the non-responders group at any time point (**Figure 4.6**). In addition, the magnitude of the change in Trans VASCi from baseline to week 4 and to week 12 was significantly greater in responders compared to non-responders, with responders showing a marked decrease from baseline, whereas non-responders showed minimal changes from baseline.

A significant decrease was observed in transverse power Doppler area (Trans PDA) at each time point during anti-TNF treatment in the responders group; whereas there were no significant changes in the non-responders group (**Figure 4.7**). A comparison of the magnitude of change of Trans PDA score from baseline to each time point on treatment between responders and non-responders showed significant differences at weeks 4 and 12, with responders showing reductions in total scores from baseline, whereas non-responders showed a trend towards an increase in total scores from baseline.

In the responders group, there was a significant decrease in longitudinal synovial vascularity index score (Long VASCi) at weeks 1, 4 and 12 on treatment compared to baseline score, but there were no significant changes in this score in the non-responders group (**Figure 4.8**). The change in Long VASCi from baseline to weeks 1, 4 and 12 on treatment also showed a marked reduction in total score from baseline in the responders group, whereas non-responders did not show marked changes from baseline. The magnitude of the change in Long VASCi from baseline to week 4 was significantly different between responders and non-responders, with responders achieving a greater decrease from baseline.
Figure 4.4  Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Transverse Synovial Thickness Area (Long STi) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Long STi score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001. The mean±SEM 10MCP Long STi scores were compared at each time point between the two groups (C) using Mann Whitney U test. ns; non-significant. 10 MCP Long STi is the composite longitudinal synovial thickness index score of the 10 metacarpophalangeal joints scanned.
Figure 4.5  Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Longitudinal Synovial Thickness Index (Long STA) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Long STA score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05. The mean±SEM 10MCP Long STA scores were compared at each time point between the two groups (C) using Mann Whitney U test. ns; non-significant. 10 MCP Long STA is the composite longitudinal synovial thickness area score of the 10 metacarpophalangeal joints scanned.
Figure 4.6  Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Transverse Synovial Vascularity Index (Trans VASCi) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Trans VASCi score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001. The mean±SEM 10MCP Trans VASCi scores were compared at each time point between the two groups (C) using Mann Whitney U test. ns; non-significant, *p<0.05. 10 MCP Trans VASCi is the composite transverse synovial vascularity index score of the 10 metacarpophalangeal joints scanned.
Figure 4.7  Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Transverse Synovial Power Doppler Area (Trans PDA) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Trans PDA score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001. The mean±SEM 10MCP Trans PDA scores were compared at each time point between the two groups (C) using Mann Whitney U test. *p<0.05. 10 MCP Trans PDA is the composite transverse synovial power Doppler area score of the 10 metacarpophalangeal joints scanned.
Figure 4.8  Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Longitudinal Synovial Vascularity Index (Long VASCI) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Long VASCI score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001. The mean±SEM 10MCP Long VASCI scores were compared at each time point between the two groups (C) using Mann Whitney U test. *p<0.05. 10 MCP Long VASC is the composite longitudinal synovial vascularity index score of the 10 metacarpophalangeal joints scanned
In anti-TNF responders, longitudinal power Doppler area score (Long PDA) decreased significantly at weeks 1, 4 and 12 compared to baseline, whereas there were no significant changes in the non-responders group during treatment (Figure 4.9). A comparison of the change in Long PDA from baseline to weeks 1, 4 and 12 on treatment between responders and non-responders confirmed a gradual decrease from baseline in the responders, whereas non-responders showed little change in Long PDA score from baseline. The magnitude of the change from baseline to weeks 4 and 12 on treatment in Long PDA showed significant differences between responders and non-responders, with responders achieving a significantly greater decrease from baseline than non-responders at weeks 4 and 12.

Grey scale and power Doppler ultrasound images from a representative RA responder patient at baseline and at each time point on treatment are presented in Figures 4.10 and 4.11, depicting the improvement in synovial thickening and vascularity with treatment. In contrast, Figures 4.12 and 4.13 show grey scale and power Doppler ultrasound images from a representative RA non-responder patient at baseline and at each time point on treatment depicting the lack of improvement in synovial thickening or vascularity with treatment.

4.2.6 PsA patient cohort

4.2.6.1 Relationships between clinical markers of PsA disease activity and ultrasound scores for synovial thickening and vascularity

In the PsA cohort, there were no significant correlations between the baseline semi-quantitative and quantitative scores for synovial thickness or vascularity and clinical markers of disease activity at baseline: swollen joint count, tender joint count, ESR or CRP levels, which may at least in part be due to the small number of patients in this group.

4.2.6.2 Changes in ultrasound scores for synovial thickening and vascularity during anti-TNF treatment in PsA cohort

The synovial thickening of the MCP joints of the PsA patients at baseline as assessed by grey scale ultrasound was not as marked as in the RA group (Table 4.9). The semi-quantitative (Trans STi and Long STi) and quantitative (Trans STA and Long STA) scores for synovial
Figure 4.9  Comparison between anti-TNF responder and non-responder rheumatoid arthritis patients in the change in total Longitudinal Synovial Power Doppler Area (Long PDA) score with time on anti-TNF treatment

The changes in mean±SEM 10MCP Long PDA score over 12 weeks on anti-TNF treatment are shown for responders (n=16) (A) and non-responders (n=9) (B). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05, **p<0.001. The mean±SEM 10MCP Long PDA scores were compared at each time point between the two groups (C) using Mann Whitney U test. *p<0.05. 10 MCP Long PDA is the composite longitudinal synovial power Doppler area score of the 10 metacarpophalangeal joints scanned.
Figure 4.10  Grey scale ultrasound images of a representative rheumatoid arthritis responder patient showing improvement in synovial thickening with anti-TNF treatment

Longitudinal (left column) and transverse (right column) grey scale ultrasound images of the 3rd MCP joint of a representative rheumatoid arthritis responder patient showing gradual improvement in synovial thickening from severe at baseline to mild after 12 weeks of anti-TNF treatment.
Figure 4.11  Power Doppler ultrasound images of a representative rheumatoid arthritis responder patient showing improvement in synovial vascularity with anti-TNF treatment

Longitudinal (left column) and transverse (right column) power Doppler ultrasound images of the 3rd MCP joint of a representative rheumatoid arthritis responder patient showing improvement in synovial vascularity from severe at baseline to mild after 12 weeks of anti-TNF treatment.
Figure 4.12  Grey scale ultrasound images of a representative rheumatoid arthritis non-responder patient showing no improvement in synovial thickening with anti-TNF treatment

Longitudinal (left column) and transverse (right column) grey scale ultrasound images of the 4th MCP joint of a representative rheumatoid arthritis non-responder patient showing no significant improvement in synovial thickening after 12 weeks of anti-TNF treatment.
Figure 4.13  Power Doppler ultrasound images of a representative rheumatoid arthritis non-responder patient showing no improvement in synovial vascularity with anti-TNF treatment

Longitudinal (left column) and transverse (right column) power Doppler ultrasound images of the 4th MCP joint of a representative rheumatoid arthritis non-responder patient showing no improvement in synovial vascularity after 12 weeks of anti-TNF treatment.
Table 4.9  Change in semi-quantitative and quantitative ultrasound measures of synovial thickening and vascularity over 12 weeks on anti-TNF treatment in psoriatic arthritis group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>1 week</th>
<th>4 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD value</td>
<td>Mean ± SD value</td>
<td>p-value</td>
<td>Mean ± SD value</td>
</tr>
<tr>
<td>Trans STi</td>
<td>10.1±3.3</td>
<td>10.6±2.4</td>
<td>ns</td>
<td>9.1±2.9</td>
</tr>
<tr>
<td>Trans STA</td>
<td>103,865.6±35,987.4</td>
<td>104,561.7±21,394.9</td>
<td>ns</td>
<td>87,622.3±30,647.7</td>
</tr>
<tr>
<td>Long STi</td>
<td>10.4±2.1</td>
<td>9.4±2.5</td>
<td>ns</td>
<td>9.6±3.3</td>
</tr>
<tr>
<td>Long STA</td>
<td>77,081.1±25,487.6</td>
<td>76,197±29,617.7</td>
<td>ns</td>
<td>70,607.3±24,524.5</td>
</tr>
<tr>
<td>Trans VASCI</td>
<td>0.3±0.5</td>
<td>0.1±0.4</td>
<td>ns</td>
<td>0.1±0.4</td>
</tr>
<tr>
<td>Trans PDA</td>
<td>12.9±30.5</td>
<td>13.9±36.7</td>
<td>ns</td>
<td>0.3±0.8</td>
</tr>
<tr>
<td>Long VASCI</td>
<td>0.38±0.74</td>
<td>0.4±1.1</td>
<td>ns</td>
<td>0</td>
</tr>
<tr>
<td>Long PDA</td>
<td>15.1±42.8</td>
<td>53.0±140.0</td>
<td>ns</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. Comparison of each time point on treatment versus baseline was made using Wilcoxon matched pairs test, ns; non-significant, *p<0.05. Abbreviations: Trans STi, transverse synovial thickness index; Trans STA, transverse synovial thickness area; Long STi, longitudinal synovial thickness index; Long STA, longitudinal synovial thickness area; Trans VASCI, transverse synovial vascularity index; Trans PDA, transverse synovial power Doppler area score; Long VASCI, longitudinal synovial vascularity index; Long PDA, longitudinal synovial power Doppler area score.
thickening decreased significantly at 12 weeks after treatment initiation compared to baseline (Figure 4.14).

Only a few patients in the PsA cohort had evidence of power Doppler signal in the MCP joints at baseline. There were no significant changes in the semi-quantitative or quantitative scores of synovial vascularity with anti-TNF treatment compared to baseline (Table 4.9).

4.2.7 AS patient cohort

4.2.7.1 Changes in Glasgow Ultrasonographic Enthesitis Scoring System (GUESS) and enthesal vascularity score during anti-TNF treatment in AS cohort

The lower limb entheses were scanned at each study visit using grey scale ultrasound to determine the presence of morphological changes of acute and chronic inflammation and also using power Doppler ultrasound to determine the presence of abnormal vascularisation of the enthesis insertion as a further sign of acute inflammation. There was a high frequency of abnormal findings detected by grey scale and power Doppler ultrasound in the lower limb entheses in this cohort of AS patients, despite the majority of patients being clinically asymptomatic (Figure 4.15 and Table 4.10). Although there was a trend towards a gradual decrease in total GUESS score with time on treatment, this change did not reach statistical significance at 12 weeks after treatment initiation (p=0.07) (Figure 4.15A). Seven out of the fifteen AS patients (47%) had evidence of power Doppler signal in at least one enthesis insertion at baseline and there was a significant decrease in total power Doppler score of the entheses 12 weeks after anti-TNF initiation (p=0.03) (Figure 4.15B). Anti-TNF responders showed a trend towards a decrease in total GUESS score 12 weeks after anti-TNF initiation (Figure 4.15C). In contrast, non-responders showed little change in GUESS score with anti-TNF treatment. Although in the majority of the patients the power Doppler signal at the entheses was mild to moderate at baseline, there was a significant decrease in total power Doppler score of entheses in both responder and non-responder groups with anti-TNF treatment (Figure 4.15D).

There were no significant correlations between total GUESS score or total power Doppler score at baseline and the Bath Indices or ESR or CRP levels in the AS group.
Figure 4.14  Changes in the semi-quantitative and quantitative ultrasound parameters of synovial thickening with time on anti-TNF treatment in the psoriatic arthritis group.

The changes over 12 weeks on anti-TNF treatment in the psoriatic arthritis group (n=8) are shown for mean±SEM 10MCP Trans STi (A), mean±SEM 10MCP Trans STA (B), mean±SEM 10MCP Long STi (C) and mean±SEM 10MCP Long STA (D). Comparison of each time point on treatment versus baseline within the responder and non-responder groups was made using Wilcoxon matched pairs test, *p<0.05. Abbreviations: Trans STi, transverse synovial thickness index score; Trans STA, transverse synovial thickness area score; Long STi, longitudinal synovial thickness index score; Long STA, longitudinal synovial thickness area score.
Figure 4.15  Changes in total GUESS and power Doppler scores of lower limb entheses in ankylosing spondylitis patients with anti-TNF treatment

Changes in mean±SEM GUESS score of entheses (A) and mean±SEM total power Doppler score of entheses (B) with anti-TNF treatment in ankylosing spondylitis group (n=15), mean±SEM GUESS score of entheses at baseline and after 12 weeks on anti-TNF treatment in responders and non-responders (C) and mean±SEM power Doppler score of entheses at baseline and after 12 weeks on anti-TNF treatment in responders and non-responders (D). GUESS, Glasgow Ultrasonographic Enthesitis Scoring System (maximum score 36)
Table 4.10  Sites of enthesal involvement and types of enthesal pathology detected by grey scale and ultrasonography in the ankylosing spondylitis group at baseline

<table>
<thead>
<tr>
<th></th>
<th>Quadriceps Tendon (n=30)</th>
<th>Proximal patellar ligament (n=30)</th>
<th>Distal patellar ligament (n=30)</th>
<th>Achilles Tendon (n=30)</th>
<th>Plantar Aponeuroses (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tendon thickened *</td>
<td>10</td>
<td>9</td>
<td>22</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Bursitis</td>
<td>0</td>
<td>n/a</td>
<td>1</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Bone erosion</td>
<td>3</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enthesophyte</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Power Doppler signal</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>12 weeks post anti-TNF treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tendon thickened *</td>
<td>9</td>
<td>9</td>
<td>22</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Bursitis</td>
<td>0</td>
<td>n/a</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Bone erosion</td>
<td>3</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enthesophyte</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Power Doppler signal</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Quadriceps tendon ≥6.1mm, proximal patellar ligament ≥4mm, distal patellar ligament ≥4mm, Achilles tendon ≥5.29mm, plantar aponeuroses ≥4.4mm
4.2.7.2 Sites of lower limb entheseal involvement and pathology detected by ultrasonography

The frequency of involvement of each entheseal site as determined using ultrasound in the AS cohort is shown in Figure 4.16A, with the distal patellar ligament being involved most frequently and the plantar aponeurosis least frequently in this cohort. The type of entheseal pathology most commonly detected by ultrasound is illustrated in Figure 4.16B. Tendon thickening and presence of power Doppler signal were most frequently observed. A comparison of the frequency of entheseal pathology detected by grey scale and power Doppler ultrasound at baseline and after 12 weeks on anti-TNF treatment showed that the most significant change after 12 weeks on anti-TNF treatment was a marked reduction in power Doppler signal of the entheses, with minimal change in the other types of pathology detected by PDUS (Figure 4.16C).

4.3 Discussion

4.3.1 PDUS methodology – image acquisition and scoring of synovitis in RA and PsA patients

One of the main limitations of two-dimensional grey scale and power Doppler ultrasonography is that this technique is operator dependent. In order to minimise variability in grey scale and power Doppler ultrasound image acquisition and scoring, all patients in the study were scanned by myself using a standardised protocol for image acquisition and all the images were also scored semi-quantitatively and quantitatively by myself. In addition, the intra-observer variability in grey scale and power Doppler image acquisition and image scoring for synovial thickening and vascularity using the semi-quantitative and quantitative methods was assessed. The intra-class correlation coefficient values for image acquisition and image scoring showed excellent reliability for all the ultrasound endpoints. This confirms that the methods used to acquire and score the ultrasound images in this study were reliable and the results obtained are reproducible and comparable between different patients and different time points.

Studies evaluating synovitis by ultrasound have employed a number of different scoring systems to monitor RA disease activity and therapeutic response, including qualitative scores
Figure 4.16 Frequency of involvement of lower limb entheseal sites and type of entheseal pathology seen using grey scale and power Doppler ultrasonography in ankylosing spondylitis patients

The frequency of lower limb entheseal site involvement (A) and frequency of entheseal pathology type (B) in the ankylosing spondylitis group (n=15) at baseline is demonstrated along with a comparison of the frequency of entheseal pathology type seen by ultrasound at baseline and after 12 weeks on anti-TNF treatment (C).
(0/1), semi-quantitative scoring systems (commonly 0-3) or quantitative measurements (number of pixels) to grade the severity of synovitis. Some scoring systems have graded grey scale synovial thickening only, some also include evaluation of bony erosions, joint effusion and/or power Doppler activity (Ohrndorf and Backhaus, 2012). Current aims of the EULAR/OMERACT Ultrasound Task Force includes the development of a Global OMERACT Sonography Scoring System (GLOSS) in RA in order to standardise the scoring of synovitis in all studies using ultrasound. However, its feasibility and sensitivity to change are still being tested thus this score is yet to be validated (D’agostino et al., 2009). Although commonly the semi-quantitative scores used to grade synovitis have used a four point scale, the scale used to grade synovial thickening in this study was a five point scale (0-4). This was used instead of a four point scale due to its increased sensitivity in grading synovial thickening. A five point scale has also been shown to be capable of detecting treatment effects of oral prednisolone after only one week in a randomised placebo-controlled study of RA patients (Seymour et al., 2012).

Additionally, a five point scale was also used to grade synovial vascularity in this study. Several published four point scales for assessing the severity of power Doppler signal in the joints have no signal as the lowest grade (normal), presence of a single vessel (mild) as the next grade and then less than 50% (moderate) and greater than 50% (severe) power Doppler signal within the region of interest as grades 3 and 4 respectively (Albrecht et al., 2008; Naredo et al., 2005; Szkudlarek et al., 2003). However, these four point scales potentially lack sensitivity at the lower levels of power Doppler signal, with the potential to underestimate change in response to an intervention. The use of the five point scale for grading power Doppler signal in our study allows greater sensitivity to change by including a grade in between absence of signal, mild and moderate synovitis, without which there is usually a greater leap in between mild and moderate synovitis grades in a four point score. This five point scale has also demonstrated reproducibility, sensitivity and the ability to detect treatment effects of oral prednisolone after just a week in a placebo-controlled study of RA patients (Seymour et al., 2012).

The sensitivity and reliability of the semi-quantitative and quantitative ultrasound scoring systems used in this study have been further investigated recently in a randomised double-blind placebo-controlled study testing the effect of repeat oral dosing of prednisolone over two weeks in RA patients. These endpoints were shown to all be reproducible and capable of detecting significant treatment effects at all the time points and to be able to detect a
treatment effect earlier than changes in DAS28 score could. The quantitative measures of synovitis were shown to demonstrate better overall reproducibility and sensitivity than the semi-quantitative measures (Seymour et al., 2012).

To improve the accuracy of image scoring using the semi-quantitative scores for synovial thickening and vascularity in this study, a library of reference images was used. Semi-quantitative scores were then assigned by comparing the images acquired to the library of reference images and deciding which representative image and thus score was the closest fit. The use of an ultrasonographic atlas as a reference in semi-quantitative scoring has been recently shown to increase intra and inter-rater reliability for grey scale and power Doppler scoring (Hammer et al., 2011).

The quantitative method for scoring synovial vascularity (power Doppler area) obtained by calculating the number of pixels with power Doppler signal within an area of interest has been used in a number of studies and has been shown to be more sensitive than laboratory measures of disease activity, such as CRP and ESR in determining between group differences and response to treatment with oral corticosteroids in a group of patients with active RA (Albrecht et al., 2008; Larché et al., 2010; Seymour et al., 2012; Seymour, 2012). Quantitative assessment of power Doppler signal has also been shown to be a useful measure to monitor patient response to anti-TNF therapy (Taylor et al., 2004).

In the RA cohort as a whole, the quantitative measures of synovial thickening (Long STA and Trans STA) decreased significantly after 4 weeks of anti-TNF treatment, whereas the semi-quantitative measures (Long STi and Trans STi) showed a trend to decrease at 12 weeks after anti-TNF was initiated, but this decrease was not statistically significant. Similarly, when the RA cohort was divided into anti-TNF responders and non-responders, the quantitative measures of synovial thickening demonstrated a significant decrease with anti-TNF treatment earlier than the semi-quantitative measures; Long STA score decreased significantly at 1, 4 and 12 weeks on treatment and Trans STA score decreased significantly at 4 and 12 weeks on treatment, whereas the semi-quantitative scores (Trans STi and Long STi) decreased significantly only at 12 weeks. Both the semi-quantitative and quantitative measures of synovial vascularity demonstrated a significant decrease with treatment as early as 1 week after anti-TNF initiation in the RA cohort as a whole and particularly in the responders group.
The quantitative scores of synovial vascularity (Long PDA and Trans PDA) were better able to discriminate between anti-TNF responder and non-responder patients. Thus, the quantitative scores of synovial vascularity and synovial thickening appear to be able to demonstrate a change with anti-TNF treatment earlier than the semi-quantitative scores. Quantitative ultrasonographic measures of synovitis have also been shown to demonstrate better overall reliability and greater effect sizes than semi-quantitative measures in two other studies (Larché et al., 2010; Seymour, 2012). This may be because semi-quantitative scores may constrain the detection of change in synovial thickening or vascularity when the severity of synovial thickening or vascularity exceeds the largest semi-quantitative score. Additionally, a change at the extremes of synovial thickening or vascularity can be evaluated more accurately quantitatively.

In the present study, the longitudinal views of the MCP joints also appeared to be able to demonstrate a change with anti-TNF treatment earlier than the transverse views. The transverse measures of synovial thickening have been demonstrated to have smaller effect sizes than longitudinal measures in a randomised double-blind placebo-controlled study of oral corticosteroids in patients with active RA and the Long STA endpoint was shown to demonstrate greatest change with treatment (Seymour et al., 2012). The greater ability of the longitudinal view to demonstrate change with treatment may be due to the greater area afforded by the longitudinal versus the transverse view of the joint.

A potential limitation of this investigation was the number of joints scanned in the RA and PsA patients. In this study, only the ten MCP joints were scanned in the RA and PsA patients at each time point. The MCP joints are invariably involved in rheumatoid arthritis and due to their shallow depth they are easily amenable to evaluation with ultrasound utilising higher frequencies in order to produce high resolution images. It was felt that the evaluation of these joints by ultrasound would provide a good overall reflection of RA disease activity and its change with anti-TNF treatment. The total scanning time to perform the grey scale and power Doppler scan of the 10 MCP joints was 30 minutes and scanning additional joints would have lengthened this time and made the overall study visits much longer, thus decreasing patient acceptability. The EULAR/OMERACT Ultrasound Task Force has not yet agreed on a number or sequence of joints to be studied by ultrasound in clinical studies and a number of studies have proposed different scoring systems using different combinations of joints.
(Ohrndorf and Backhaus, 2012). In the development of ultrasound sum scores, several studies have analysed different numbers of joints and all studies have reached the conclusion that reduced sum scores provide a good reflection of overall inflammatory activity in RA (Backhaus et al., 2012; Ellegaard et al., 2009; Naredo et al., 2008b). Naredo et al investigated the validity of reduced joint scans which included large and small joints on both sides. The resulting 12 joint score was shown to correlate highly with a corresponding 60 joint score and to reflect well the overall joint inflammation in patients with RA and to be useful in treatment monitoring. Ellegaard et al showed that scanning only one affected wrist can be sufficient and can be used as a measure of disease activity, anti-TNF therapy response and show correlations with DAS28 score and swollen joint counts (Ellegaard et al., 2009). Recently, Hammer et al published a study in which a comprehensive ultrasound score of 78 joints was compared with other scores using reduced joint counts (7, 14, 28 and 44 ultrasound joint scores) at different time points on adalimumab treatment. They found high correlation between the reduced joint scores and the 78 joint score at all examination time points (Hammer et al., 2010). In addition, a number of studies have shown that scanning MCP joints alone in RA patients is sufficient to demonstrate changes in synovial thickening and vascularity with anti-TNF or corticosteroid treatment (Larché et al., 2010; Seymour, 2012; Taylor et al., 2004). This supports our strategy to scan only the 10 MCP joints during anti-TNF treatment to evaluate its effects on synovial thickening and vascularity.

In PsA, arthritis of the hands may affect the proximal interphalangeal (PIP) and the distal interphalangeal joints (DIP) as well as the MCPs and thus scanning the MCPs may not be sufficient to reflect overall PsA disease activity, especially in some patients who may have predominantly DIP involvement. The transducer available for use in this study limited our ability to scan the DIP and PIP joints because of its broad width, thus we only scanned the MCP joints in the PsA patients. Several studies have demonstrated the utility in scanning the MCP joints of PsA patients (Milosavljevic et al., 2005; Weiner et al., 2008; Wiell et al., 2007). The sensitivity of ultrasound in PsA has been found to be highest for the detection of synovitis in the MCP joints than in the DIP or PIP joints and ultrasound has been shown to have highest concordance with MRI for the detection of synovitis at the MCP joints (Weiner et al., 2008; Wiell et al., 2007).
4.3.2 PDUS methodology – image acquisition and scoring of enthesitis in AS cohort

Enthesitis is a hallmark feature of spondyloarthritis and although inflammation can occur at any site, the entheses of the lower limbs are most commonly involved. A number of systems for scoring ultrasonographic features of enthesitis have been proposed, some incorporating only grey scale changes and some also incorporating the presence of power Doppler signal (Alcalde et al., 2007; Balint et al., 2002; D'Agostino et al., 2009; de Miguel et al., 2009). The scoring system used in this study was the Glasgow Ultrasonographic Enthesitis Scoring System (GUESS). This was selected as it used a limited series of easily reproducible fixed reference points and established normal parameters to derive a total numerical score. This score which allows the grading of enthesitis involvement has been proposed to be useful in the evaluation of treatment efficacy, whereas other enthesitis scoring systems have been devised for diagnostic purposes but would probably not be sensitive enough to study changes longitudinally (d'Agostino, 2010). As this scoring system is limited to grey scale ultrasonographic changes of enthesitis only, we used a separate semi-quantitative power Doppler scoring system as described by D’Agostino et al (D'Agostino et al., 2009).

4.3.3 Relationships between ultrasound parameters and clinical measures of disease activity

In RA patients, the semi-quantitative and quantitative ultrasound scores for synovial thickening and vascularity were closely correlated with numbers of swollen joints determined by clinical examination. However, only the semi-quantitative and quantitative ultrasound scores of synovial vascularity correlated closely with DAS28ESR and DAS28CRP scores, thus confirming the validity of PDUS as a biomarker of synovitis. Synovial thickening as determined by grey scale ultrasound may represent fibrous tissue or active synovitis and cannot distinguish between the two, thus when used alone it does not demonstrate a close relationship with clinical assessments of disease activity, which is in agreement with other studies (Qvistgaard et al., 2001). In contrast, as power Doppler ultrasound is able to detect slow flow in small blood vessels it is able to detect changes in the vascularity of the synovium, thus power Doppler signal can be used to identify synovial swelling with active synovitis and ultrasound vascularity parameters correlate closely with disease activity scores. This is in agreement with a number of other studies (Backhaus et al., 1999; Ellegaard et al.,
2009; Larché et al., 2010; Szkudlarek et al., 2001; Szkudlarek et al., 2006; Walther et al., 2001; Walther et al., 2002) and confirms the validity of the PDUS methodology used in this study.

The semi-quantitative and quantitative markers of synovial thickening and vascularity did not correlate with numbers of tender joints determined by clinical examination which is also in agreement with other studies (Backhaus et al., 1999; Ellegaard et al., 2009). Assessment of tender joint counts by clinical examination is highly subjective and dependent on a number of factors including the pressure applied by the assessor, as well as physical and psychological patient factors determining pain perception. While joints with active synovitis may score positively for tenderness, other changes such as joint deformities, involvement of periarticular structures, and presence of joint effusions or coexistent fibromyalgia may also contribute to joint tenderness.

There were no correlations between the ultrasound parameters of synovial thickness and vascularity and ESR or CRP levels. Although these inflammatory markers can correlate with disease activity and radiographic progression in RA, up to 40% of RA patients can have normal levels of CRP and ESR (Pincus and Sokka, 2009). Although some ultrasound studies have reported positive correlations between power Doppler signal and CRP in RA patients, these have generally involved scans of larger joints such as wrists or knees (Ellegaard et al., 2009; Gullick et al., 2010). A recent study involving scans of the MCP joints only did not detect significant correlations with ESR or CRP levels in RA patients, which is in agreement with the findings of our study (Larché et al., 2010).

The finding that numbers of swollen joints by clinical examination correlate closely with ultrasound measures of synovial thickening and vascularity, whereas numbers of tender joints by examination do not correlate with these ultrasound measures may be useful in clinical practice. Although tender joint counts are weighed more heavily in the DAS score calculation, studies have shown that it is the numbers of swollen joints that make a greater contribution to the physician’s assessment and perception of patient disease activity (Studenic et al., 2012). This observation together with the findings from our study may suggest that patients with predominantly tender joints may derive greater benefit from an increase in their analgesic medication, rather than initiation of further anti-inflammatory disease modifying therapy such as anti-TNF. In contrast, patients with greater numbers of swollen joints on
examination may derive greater benefit from treatment with anti-TNF agents in order to reduce synovitis and prevent long-term joint damage.

In the PsA group, there was no correlation between the semi-quantitative and quantitative measures of synovial thickening or vascularity and numbers of swollen or tender joints on examination, ESR or CRP levels. Swollen joints or tender joints on clinical examination in PsA may reflect not only synovial thickening and inflammation, but also involvement of periarticular structures, such as tenosynovitis which may explain the lack of correlation with ultrasound measures of synovial thickening or vascularity. One study did find a positive correlation between numbers of swollen joints on examination and ultrasound indices, but this incorporated scoring of tenosynovitis and joint effusion in addition to synovial hypertrophy and power Doppler signal, whereas our study did not (Milosavljevic et al., 2005). As discussed in the previous chapter, due to the limitations of the PsARC score only being able to assess change in arthritis activity over time, rather than giving a score of current activity as the DAS28 score does, it has not been possible to investigate correlations between PsARC scores and the ultrasound endpoints. The lack of correlation between the ultrasound parameters and ESR and CRP levels may be explained by the fact that ESR and CRP are only elevated in less than half of all patients with PsA and do not always reflect arthritis activity. In addition, synovial thickening and vascularity assessment carried out by PDUS may only reflect one aspect of PsA pathology as it does not take into account extra-synovial involvement (De Agustin JJ, 2012; Milosavljevic et al., 2005). The small number of patients in this group may be an alternative explanation for the lack of correlation between the semi-quantitative and quantitative measures of synovial thickening or vascularity and clinical indices of disease activity.

In the AS group, there were no significant correlations between total GUESS score of entheses at baseline and baseline BASDAI, BASFI, BASMI scores, or CRP and ESR levels. This may be due to the small number of patients in this study or more likely, the fact that the Bath indices used to assess disease activity in AS mainly aim to evaluate axial involvement and do not specifically address peripheral enthesitis. Only one of the six items in the BASDAI questionnaire refers to enthesitis, whereas the BASMI assessment is limited to evaluating spinal involvement only (van Tubergen and Landewe, 2009). As discussed in the previous chapter, ESR and CRP levels are elevated in less than half of all AS patients even in
the presence of active disease (van Tubergen and Landewe, 2009). A number of other studies of enthesitis scoring systems have also failed to show correlations between the presence of enthesitis on ultrasound and clinical assessments of disease activity (Alcalde et al., 2007; Aydin et al., 2010; Balint et al., 2002; Borman et al., 2006; D'Agostino et al., 2011).

4.3.4 Changes in PDUS parameters with anti-TNF treatment

4.3.4.1 RA patients

Ultrasound measures of synovial thickening and vascularity demonstrated a significant improvement with anti-TNF treatment in this cohort, which in agreement with a number of other studies (Albrecht et al., 2008; Ellegaard et al., 2009; Filippucci et al., 2006; Hammer et al., 2010; Iagnocco et al., 2008; Naredo et al., 2008a; Ribbens et al., 2003; Taylor et al., 2004). To our knowledge, this is the first longitudinal ultrasound study using both semi-quantitative and quantitative ultrasound measures of synovial thickening and vascularity to characterise longitudinally changes in the MCP joints of patients with RA during anti-TNF treatment. Most other studies have only studied changes in ultrasound parameters with anti-TNF treatment by comparing baseline measurements to one time point on treatment, which has included 6 weeks, 18 weeks and 12 months and have used either semi-quantitative or quantitative measures and focused on either synovial thickening or vascularity (Ellegaard et al., 2009; Iagnocco et al., 2008; Taylor et al., 2004). Thus in this study, we were able to assess quantitatively the evolution of changes in ultrasound parameters over multiple time points during the first 12 weeks on anti-TNF treatment.

Evaluation of changes in the quantitative ultrasound scores for synovial thickening within the RA cohort as a whole revealed significant improvement after 4 and 12 weeks on treatment, whereas the semi-quantitative and quantitative scores of synovial vascularity improved significantly after 1 week on treatment, with further improvements after 4 and 12 weeks. This suggests that synovial thickening and synovial vascularity exhibit differing kinetics of improvement with anti-TNF treatment, with synovial vascularity showing an earlier and more marked improvement with treatment compared to synovial thickening. Power Doppler signal reflects vascularisation of the pannus in RA joints, which is the target tissue of anti-TNF. A number of studies have demonstrated that presence of power Doppler signal on ultrasound correlates well with histological changes of synovitis and synovial membrane microvascular density (Walther et al., 2001; Walther et al., 2002). Some of the key mechanisms of action of
anti-TNF therapies include reduction of neovascularisation and angiogenesis through reduced expression of vascular endothelial growth factor (VEGF) in the synovium (Paleolog et al., 1998), as well as a reduction in synovial tissue expression of inflammatory cytokines, chemokines and adhesion molecules and reduced synovial inflammatory cell infiltration and leucocyte trafficking to RA joints (Tak et al., 1996; Taylor et al., 2000; Wong et al., 2008). All these effects may explain the rapid, early response and reduction of power Doppler signal with anti-TNF treatment. It has been proposed that the finding of power Doppler signal in rheumatoid joints may be considered a measurement of response to anti-TNF therapy independent of clinical and grey scale ultrasound changes (Naredo et al., 2008a). The persistence of power Doppler signal in rheumatoid joints has been linked to radiographic progression of disease, whereas treatment with anti-TNF has been shown to abolish this relationship by reduction in synovial vascularisation (Naredo et al., 2008a; Taylor et al., 2004). As synovial thickening detected by grey scale ultrasonography represents an overview of the amount of swollen tissue around the joint without distinguishing between fibrous tissue and active synovitis, this may explain the slower response in the ultrasound measures of synovial thickening with anti-TNF treatment.

When the RA cohort was divided into responders and non-responders to anti-TNF treatment, clear differences were evident in the change of the semi-quantitative and quantitative scores for synovial thickening and synovial vascularity with time on treatment. Responders showed significant improvements in synovial vascularity at 1, 4 and 12 weeks on treatment, whereas non-responders did not demonstrate significant changes in any of the synovial vascularity parameters with treatment. Responder patients also showed significant improvement in the ultrasound parameters for synovial thickening, whereas non-responders did not show significant changes in synovial thickening with treatment. Comparison between responders and non-responders in the absolute change from baseline in all the ultrasound endpoints showed that the semi-quantitative and quantitative measures of synovial vascularity were better able to demonstrate significant differences between responders and non-responders, rather than synovial thickening. Other studies using PDUS have also demonstrated the ability of ultrasound endpoints to discriminate between two treatment groups (Larché et al., 2010; Seymour, 2012; Taylor et al., 2004). The ability of PDUS to differentiate between treatment groups and its rapid kinetics of change with treatment adds to its value as an early, objective marker of therapeutic response. This, combined with the greater sensitivity of PDUS than
clinical endpoints in evaluating RA disease activity suggests that incorporating PDUS as an endpoint in clinical studies may allow reduction of patient numbers and length of trials designed to give early indications of therapeutic efficacy.

In this cohort of RA patients, responders to anti-TNF treatment showed a trend to having greater power Doppler signal at baseline compared to the non-responder patients, although these differences were not statistically significant. It may be inferred that patients with higher baseline power Doppler signal may be more responsive to anti-TNF, although confirmation in larger cohorts would be required. Anti-TNF treatment has been shown to abolish the positive relationship between baseline vascular signal on ultrasound and progression of joint damage. Thus it has been suggested that patients with the highest baseline disease activity, as assessed by power Doppler may derive the greatest benefit from anti-TNF treatment (Taylor et al., 2004). Ellegaard et al scanned the wrist joint of RA patients before and 1 year after treatment with anti-TNF evaluating quantitatively synovial power Doppler signal. They found that baseline power Doppler activity in contrast to DAS28 score, CRP or swollen joint counts was able to predict which patients will remain on anti-TNF 1 year after initiating therapy (Ellegaard et al., 2009).

### 4.3.4.2 PsA patients

The semi-quantitative and quantitative parameters of synovial thickening on ultrasound decreased significantly at 12 weeks on anti-TNF treatment, with similar kinetics of response to anti-TNF as in the RA group. This is in agreement with the only other study which has examined the changes in ultrasonographic measures of synovial thickening and vascularity in a small group of PsA patients treated with infliximab (De Agustin JJ, 2012). As in RA, anti-TNF has been shown to lead to deactivation of the endothelium in PsA and to cause a reduction in neoangiogenesis leading to a reduction in the inflammatory cell infiltrate and clinical improvement (Goedkoop et al., 2004a). This may underlie the improvement in synovial thickening with treatment.

There was low power Doppler activity in the MCP joints of this PsA cohort even at baseline prior to the start of anti-TNF and consequently no significant changes in synovial vascularity were seen with treatment. One of the reasons for this may be that joints other than the MCPs in this group had more active synovitis but this was not detected as these joints were not
scanned. A reduction in power Doppler signal with anti-TNF treatment has been reported in
the knee joints affected by PsA as well as in another study which scanned the clinically most
affected joints of patients with PsA including knees, MCPs, PIPs, DIPs and wrists (De
Agustin JJ, 2012; Fiocco et al., 2005). Thus perhaps due to the small number of patients in
our cohort and the fact that we scanned only the MCP joints, rather than scanning the most
symptomatic joints may explain why the PsA group in this study had low power Doppler
activity at baseline.

### 4.3.4.3 AS patients

We observed a high frequency of abnormal findings detected by grey scale and power
Doppler ultrasound in the lower limb entheses in this cohort of AS patients, despite the
majority of patients being clinically asymptomatic, as reported previously (Alcalde et al.,
2007; Balint et al., 2002; D'Agostino et al., 2003; d'Agostino, 2010; D'Agostino et al., 2009;
Naredo et al., 2010). The most frequent site of involvement in this cohort was the distal
patellar ligament. Tendon thickening and presence of power Doppler signal at the enthesis
insertion were the commonest types of pathology seen, which have been proposed to be signs
of acute entheseal inflammation. In the AS cohort there was a trend towards a reduction in
total GUESS score after 12 weeks on anti-TNF treatment, but this did not reach statistical
significance. Specifically, there was no significant improvement in tendon thickening with
anti-TNF treatment which is a sign of acute inflammation on grey scale ultrasound. However,
there was a marked reduction in total power Doppler score after 12 weeks on treatment in the
whole group, suggesting that PDUS of entheses can be incorporated as a complementary
method in the overall assessment of disease activity and monitoring of treatment response in
AS patients. Few studies have investigated the changes in entheseal pathology by ultrasound
with anti-TNF treatment. Tendon thickening is generally considered a sign of acute
inflammation and as such it would be expected that there would be an improvement with
anti-TNF treatment, although it may be that 12 weeks was insufficiently long for this to be
detected. In a large multi-centre study investigating enthesitis changes by PDUS in response
to anti-TNF therapy, there were significant improvements in entheses thickening,
hypoechochogenicity and bursitis after 6 months of treatment (Naredo et al., 2010) D’Agostino
et al reported two cases of Achilles enthesitis characterised by tendon thickening and
presence of power Doppler signal with improvements in both ultrasound features after 14
weeks of infliximab treatment (D'Agostino et al., 2002). As we only evaluated entheseal
changes over 12 weeks, this period of time on treatment may have been insufficient to detect marked changes.

As described, there were no correlations between GUESS score or power Doppler score of entheses at baseline and the clinical or laboratory markers of AS disease activity. However, PDUS changes of entheses appear to be responsive to therapy, which may suggest that PDUS abnormalities of entheses may be markers of AS disease activity, which are independent of the conventional clinical and laboratory measures.

4.3.5 Limitations of 2-dimensional grey scale and power Doppler ultrasonography and future perspectives

The current study has certain limitations with respect to the ultrasonography investigations and these include the lack of a control group, its open-label design and small number of patients in each group, especially in the PsA and AS groups. However this is the first longitudinal study which incorporates comprehensive semi-quantitative and quantitative measures of synovial thickening and vascularity to characterise changes in the MCP joints in RA and PsA patients during 12 weeks on anti-TNF treatment.

Scanning in the RA group was limited to the MCP joints which are invariably involved in RA, but the PIP joints which are also commonly involved were not evaluated. Scanning of the joints in PsA patients was limited to the MCP joints and the focus was on changes within the synovium with treatment, without evaluating extra-synovial abnormalities characteristic of PsA, such as enthesitis, paratenonitis bony abnormalities such as periosteal reaction. Other common sites of disease involvement such as the DIP, PIP joints, feet or knees were not evaluated. This may have under-estimated the changes in synovitis with therapy in the PsA group.

In the AS patients, the intra-observer reliability of the ultrasound image acquisition and scoring of the entheses was not performed to assess reliability and reproducibility and scanning was limited to the lower limb entheses.

One of the main limitations of ultrasound is that this technique is operator dependent. The study set up aimed to minimise variability in image acquisition by: i) having one sonographer scan all patients at each visit (DH), ii) by performing each scan according to a strict protocol and iii) by using the same ultrasound machine and settings throughout the study. Intra-
observer variability in image acquisition and in image scoring showed excellent agreement in the assessment of synovial thickening and vascularity of MCP joints. Despite this, standardisation of image acquisition may be further improved by the use of three-dimensional (3D) ultrasound. This technique generates volumetric images containing the entire power Doppler signal within the acoustic window representing the summation of a virtually infinite number of conventional two-dimensional (2D) images. The images can be obtained within a few seconds, even by an unskilled operator due to the automatic sweep of the transducer which would minimise further any margin of error. 3D ultrasound has been shown to be responsive and repeatable in monitoring changes in disease activity of RA patients treated with biological therapy (Albrecht et al., 2008; Meenagh et al., 2007; Naredo et al., 2013). Certainly, for use in multicentre studies or open-label studies, 3D ultrasound would improve inter-observer reliability.

In spondyloarthritis, 3D ultrasound has also been shown to improve inter-reader reliability compared to 2D ultrasound in the scanning of Achilles tendons (Iagnocco, 2009).

Ultrasound elastography is a method to assess the mechanical properties of tissue by identifying changes in tissue elasticity. This technique has been widely used in non-musculoskeletal applications but its ability to characterise changes in tendons is starting to be evaluated and the technique may in the future complement ultrasound examinations of tendinopathy and entheses in patients with spondyloarthropathy (Coates et al., 2012).

The development and validation of a scoring system for synovitis in RA and PsA patients and for enthesitis in AS patients by the EULAR/OMERACT Ultrasound Task Force in the future will allow standardisation of the evaluation of synovial and entheseal pathology by ultrasound and allow comparisons to be made between different studies examining the effect of therapeutic interventions on various ultrasound parameters.

4.4 Conclusion

The study has demonstrated that the wide range of semi-quantitative and quantitative measures of synovial thickening and vascularity are reproducible and capable of detecting treatment effects with anti-TNF in RA and PsA patients. The correlation of semi-quantitative and quantitative measures of synovial vascularity with DAS28 score confirmed the validity of PDUS as a biomarker of synovitis in RA. Synovial thickening and power Doppler activity in RA patients improved with anti-TNF treatment and this effect was more marked in
responders than non-responders. Synovial thickening and synovial vascularity showed different kinetics of change with treatment; with power Doppler activity improving within a week of anti-TNF treatment, thus suggesting that it may serve as an early marker of treatment response. Similarly, power Doppler signal within the entheses improved in AS patients with anti-TNF treatment. In view of these results, the subsequent objective of this thesis was to investigate if the morphological changes in the synovial joints or entheses during anti-TNF treatment reflected underlying immunopathological changes during therapy, in particular changes in the frequency of circulating Th1 and Th17 cells.

In the RA cohort, despite the small numbers of patients, the ultrasound parameters of synovial thickening and vascularity were able to discriminate between anti-TNF responders and non-responders. RA patients who responded to anti-TNF tended to have higher baseline power Doppler activity than non-responders, which may indicate that PDUS could be used to stratify patient selection for anti-inflammatory treatment by detecting those patients with potentially reversible joint inflammation at baseline. This may be important for patient selection in clinical trials where there has been concern about the introduction of bias through the recruitment of patients with equivocal clinical swelling. The ability of ultrasound endpoints to show significant changes over short treatment duration in a small number of patients supports the use of PDUS in early phase clinical studies, where its use may aid in reducing the numbers of patients and length of trials through its ability to provide an early signal of treatment effectiveness.
Chapter 5. Quantitative assessment of the frequency of circulating IL17 and IFNγ-producing cells by Enzyme-Linked Immunospot assay during anti-TNF therapy

5.1 Introduction

The work presented in this chapter characterised the changes in the frequency of IL17 and IFNγ-producing cells in the peripheral blood of patients with inflammatory arthritis longitudinally over the first 12 weeks of anti-tumour necrosis factor α (anti-TNF) treatment using the Enzyme-Linked ImmunoSpot (ELISpot) technique. The objective of the work in this chapter was to determine whether anti-TNF treatment induced changes in the frequency of circulating IL17 and IFNγ-producing cells as suggested by work in animal models of RA (Notley et al., 2008) and in preliminary clinical studies (Aerts et al., 2010; Alzabin et al., 2012) and to determine if these changes occurred in different disease phenotypes.

This is the first study to use the ELISpot technique to characterise longitudinally the changes in IL17 and IFNγ-secreting peripheral blood mononuclear cells during anti-TNF therapy. The study also compares for the first time these changes in patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA) and evaluates the magnitude and kinetics of change of these cell types during anti-TNF treatment across the three disease groups.

5.1.1 The ELISpot technique and its applications

The ELISpot assay, originally based on the sandwich Enzyme-Linked Immunosorbent Assay (ELISA), has been developed for the quantitation of individual cells secreting a specific cytokine in response to a stimulus. The first description of the ELISpot assay was published over 25 years ago and it was originally developed to detect antibody-secreting B cells (Czerkinsky et al., 1983). Subsequently, the protocol was modified for the detection of T cells secreting cytokines and ELISpot is now widely used to evaluate both CD4+ and CD8+ T cells responding to an antigenic or mitogenic stimulus (Lehmann and Zhang, 2012). ELISpot has been applied to study immune responses in various diseases including infections, cancer,
allergies and autoimmune diseases. It has become a standard tool in evaluating T-cell reactivity in clinical trials of vaccines and other forms of immunotherapy across a range of diseases including various cancers, human immunodeficiency virus (HIV) and Hepatitis C amongst others (Lehmann and Zhang, 2012). It is also evolving from a research tool to a clinical assay as recent Phase I and II studies of cancer vaccines tested in various malignancies have suggested that ELISpot may be a useful assay to predict clinical benefit after therapeutic immune modulation (Slota et al., 2011). A further example of the use of the ELISpot technique clinically is the T-cell based IFN-γ assay for diagnostic purposes to detect active or latent tuberculosis infection by measuring T cells responding to antigens from Mycobacterium tuberculosis (Soysal and Bakir, 2011).

5.1.2 Advantages and limitations of the ELISpot assay

The key advantages of the ELISpot assay are that it is quantitative, highly sensitive and can provide an objective analysis of cellular function (Karlsson et al., 2003; Lehmann and Zhang, 2012). It is amenable to high throughput analysis and can be used for the evaluation of cellular immune responses in large cohorts of individuals during the course of clinical studies or during vaccine trials. Multiple studies have demonstrated that ELISpot can be standardised to meet rigorous performance standards (Janetzki et al., 2005; Lehmann and Zhang, 2012; Slota et al., 2011).

The ELISpot technique has also been shown to be very sensitive and to perform better than intracellular cytokine staining (ICS) for the detection of low level responses, as it allows the detection of secreted cytokines at the single cell level and it can detect cytokine-secreting cells with a frequency as low as 1 cell in 200,000 (Karlsson et al., 2003). Both ICS and ELISpot are single cell assays providing information on cellular frequencies. However, intracellular cytokine staining measures the accumulated intracellular cytokine, while ELISpot measures the released cytokine. The latter may be more biologically relevant as cytokine production can be post-translationally regulated so although the cytokine is synthesised by the cell it may not be then released to exert its effector function. In addition, cells in the ICS assays do undergo pharmacological manipulation with a secretion inhibitor to promote cytokine accumulation within the cells, whereas the cells in the ELISpot assay are not pharmacologically treated (Cox et al., 2006; Lehmann and Zhang, 2012). A comparison of ELISpot and ICS assays is presented in Table 5.1. As ICS allows phenotypic
characterisation of cytokine-producing cells, it thus provides complementary information to the ELISpot technique.

As the cytokines in the ELISpot assay adhere to a solid phase immediately after production, they are thus prevented from being consumed by the producing cell or other cells in the culture. In contrast, the information obtained from ELISA of secreted cytokines, measured in culture supernatants, may be less biologically relevant as concomitant consumption of the relevant cytokine or effects of simultaneously produced antagonists may influence the total amount of cytokine measured (Cox et al., 2006; Lehmann and Zhang, 2012). A comparison between ELISpot and ELISA assays is presented in Table 5.2.

The ELISpot assay is thus particularly suited to evaluating the changes in circulating IL17-producing cells with anti-TNF treatment in the present study as the frequency of IL17-producing cells in peripheral blood is low. In addition, the ELISpot provides an objective analysis of cytokine production and it allows comparison between patients and different time points in the context of a longitudinal study (Karlsson et al., 2003; Lehmann and Zhang, 2012). As ELISpot, intra-cellular flow cytometry, cytometric bead array and ELISA would provide complementary information; together the results from all the techniques would provide a better overall picture of the changes in Th1 and Th17 cells seen with anti-TNF treatment in this study.

The limitation of the ELISpot assay is that it has an inherent limit of detection in that when cells produce abundant amount of cytokine the wells can become saturated and the number of spot forming cells cannot be accurately quantified (Janetzki et al., 2005; Lehmann, 2005). In the context of our study, this was a limitation of the IFNγ ELISpot assay in some of the patients with abundant IFNγ production, but this was not the case for IL17-producing cells because of their lower frequency. The ELISpot assay also does not provide phenotypic information on the cytokine-producing cells. Another challenge is that ELISpot assays can be subject to operator-dependent variability, however studies have shown that ELISpot assays can provide reproducible results among different laboratories even in the hands of inexperienced users when the assay procedure and data analysis is standardised.
Table 5.1 A comparison of the characteristics and application of ELISpot and intracellular cytokine staining

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ELISpot</th>
<th>Intracellular Cytokine Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution</td>
<td>Allows determination of cytokine production at single cell level</td>
<td>Allows determination of cytokine production at single cell level</td>
</tr>
<tr>
<td>Type of cytokine measured</td>
<td>Secreted cytokine</td>
<td>Intracellular cytokine measured</td>
</tr>
<tr>
<td></td>
<td>No pharmacological manipulation of cells needed to measure cytokine</td>
<td>Cells need pharmacological manipulation (e.g. Brefeldin A to retain cytokine inside cell) to allow cytokine detection</td>
</tr>
<tr>
<td>Cytokine secretion kinetics</td>
<td>Cells alive and cytokine continuously captured so technique is independent of cytokine secretion kinetics</td>
<td>Cells fixed and cytokine measured at one specific time point only</td>
</tr>
<tr>
<td>Sensitivity of technique</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Lowest detection limit</td>
<td>No inherent lowest detection limit, excellent at monitoring low frequency immune responses</td>
<td>Better at measuring high frequency immune responses</td>
</tr>
<tr>
<td>Phenotypic analysis of cytokine producing cells</td>
<td>Not directly possible</td>
<td>Directly possible</td>
</tr>
<tr>
<td>Primary application</td>
<td>Longitudinal monitoring of cytokine responses to an intervention (treatment or vaccination); definition of positive or negative responses; detection of low frequency immune responses</td>
<td>Discrimination and phenotypic analysis of cytokine-producing cells</td>
</tr>
</tbody>
</table>
Table 5.2  A comparison of the characteristics and application of ELISpot and ELISA

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ELISpot</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution</td>
<td>Allows determination of cytokine production at single cell level</td>
<td>Measures total secreted cytokine, cannot obtain per cell productivity information, how many cells secrete cytokine of interest or rate of secretion</td>
</tr>
<tr>
<td>Type of cytokine measured</td>
<td>Secreted cytokine immediately adheres to solid phase</td>
<td>Soluble secreted cytokine measured which may be subject to consumption or influenced by simultaneously produced antagonists</td>
</tr>
<tr>
<td>Cytokine secretion kinetics</td>
<td>Cell alive and cytokine continuously captured; can provide information on how many cells produce cytokine of interest and at what rate</td>
<td>Cannot obtain per cell cytokine productivity information</td>
</tr>
<tr>
<td>Sensitivity of technique</td>
<td>High</td>
<td>Moderate</td>
</tr>
</tbody>
</table>
(Zhang et al., 2009). In the present study, variability was minimised as I performed all the cryopreservation and thawing of PBMCs from the study patients, all the ELISpot assays for the study samples and reading of the ELISpot plates according to strict protocols in order to minimise any variability (Janetzki et al., 2005; Lehmann, 2005).

5.1.3 The use of ELISpot assays in rheumatology

There are only a few studies that have made use of the ELISpot technique to date in rheumatology. Schotte et al used ELISpot assays for TNFα, IL1, IL6, IL10 and IFNγ to investigate changes in pro-inflammatory cytokine-secreting peripheral blood mononuclear cells in patients with RA after long-term treatment with etanercept (Schotte et al., 2004). Berg at al used IFNγ ELISpot to characterise changes in the spontaneous production of IFNγ by PBMCs after 4 and 8 weeks of treatment with etanercept in RA patients (Berg et al., 2001). Another small study used ELISpot assays to characterise differing patterns of cytokine secretion from peripheral blood mononuclear cells (PBMCs) and synovial fluid mononuclear cells (SFMC) in patients with new onset synovitis versus patients with chronic RA (Kanik et al., 1998). ELISpot assays for IFNγ and IL4 have also been used to determine numbers of cytokine-producing cells in synovial fluid and peripheral blood from patients with different types of inflammatory arthritis in comparison with healthy controls (Ronnelid J, 1998).

To our knowledge, there is only one study in rheumatology thus far which has studied the frequency of IL17 and IFNγ-producing peripheral blood mononuclear cells in patients with RA and healthy controls using ELISpot assays and this study investigated the frequency of these cells in response to stimulation with citrullinated proteins (Steendam et al., 2012).

Studies investigating changes in the frequency of IL17 or IFNγ-producing peripheral blood mononuclear cells with anti-TNF treatment have utilised either intracellular cytokine staining or measured IL17 or IFNγ levels in serum or cell culture supernatants from stimulated cells. These studies have also mainly concentrated on one type of inflammatory arthritis and in the main have investigated the change in these cell types at a single time point on anti-TNF treatment compared to baseline, rather than longitudinally.
5.2 Results

5.2.1 Intra and inter-assay variability of IL17 and IFN\(\gamma\) ELISpot assays

All patient samples from each of the time points on the study were analysed concurrently and on the same plate, both in the IL17 and IFN\(\gamma\) assays and I performed all the ELISpot assays, in order to minimise inter-assay variability. The main factors that may lead to poor intra-assay variability in the ELISpot procedure are the cryopreservation and thawing method of PBMCs as cell viability must be maintained >95% and also the consistency of the technician pipetting technique (Cox et al., 2006; Slota et al., 2011). In order to minimise the intra-assay variability in testing the patient samples, I carried out the isolation, cryopreservation and thawing of PBMCs and subsequently their preparation for use in the IL17 and IFN\(\gamma\) ELISpot assays.

To express the precision or reproducibility of immunoassays, the coefficient of variability (CV) is reported and both inter-assay and intra-assay CV values are determined (Advani et al., 2008; Samri et al., 2006; Slota et al., 2011). CV is a dimensionless number defined as the standard deviation of a set of measurements divided by the mean of the set. The inter-assay CV is an expression of the plate to plate consistency between assays run at different times and CV values <15% indicate excellent inter-assay consistency. The intra-assay CV reflect variability within each experiment and CV values <10% indicate excellent intra-assay consistency.

After setting up and optimising the IL17 and IFN\(\gamma\) ELISpot techniques, I tested the inter-assay variability in order to determine the consistency between assays carried out at different times. PBMCs from a test patient were set up in triplicates for each stimulation condition and evaluated in the IL17 and IFN\(\gamma\) ELISpot assays as described in Sections 2.3.5.2 and 2.3.5.3. The reproducibility was evaluated by calculating the coefficient of variation across 5 different experiments performed at different times. The results for IL17 and IFN\(\gamma\) ELISpot, presented graphically in Figure 5.1 A and B and summarised in Tables 5.3 and 5.4, show that there is excellent reproducibility for all the stimulation conditions used for both the IL17 and IFN\(\gamma\) ELISpot assays and the CV values are all <15%.

To assess the intra-assay variability of the IL17 and IFN\(\gamma\) ELISpot assays, PBMCs from a test patient were set up in triplicates for each of the stimulation conditions and tested 10 times in
Figure 5.1  Determination of IL17 and IFNγ ELISpot inter-assay variability

PBMCs from a control patient’s samples were tested in the IL17 and IFNγ ELISpot assays and reproducibility was evaluated by comparing the total number of IL17 spSFC/10^6 (A) and IFNγ spSFC/10^6 (B) across 5 different experiments performed at different times. 200,000 PBMCs patient were seeded in triplicate per stimulation condition (media only as unstimulated control; anti-CD3 antibody 1μg/ml; anti-CD3 antibody 0.25 μg/ml; 50ng/ml PMA and 500ng Ionomycin (PI); 1μg/ml PHA as positive control). The results are expressed as mean number of spot forming cells per million PBMCs, error bars show SEM. spSFC/10^6, specific spot-forming cells per million PBMCs
Table 5.3  Summary of inter-assay variability results for each stimulation condition for IL17 ELISpot assay

<table>
<thead>
<tr>
<th>Stimulation condition</th>
<th>%CV value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 1.0µg/ml</td>
<td>7.86</td>
</tr>
<tr>
<td>PMA 50ng/ml and Ionomycin 500ng/ml</td>
<td>12.44</td>
</tr>
<tr>
<td>PHA 1µg/ml</td>
<td>6.81</td>
</tr>
</tbody>
</table>

PMA, Phorbol 12-Myristate 13-Acetate; PHA, phytohaemagglutinin

Table 5.4  Summary of inter-assay variability results for each stimulation condition for IFNγ ELISpot assay

<table>
<thead>
<tr>
<th>Stimulation condition</th>
<th>%CV value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 0.25µg/ml</td>
<td>6.78</td>
</tr>
<tr>
<td>PMA 50ng/ml and Ionomycin 500ng/ml</td>
<td>14.38</td>
</tr>
<tr>
<td>PHA 1µg/ml</td>
<td>14.44</td>
</tr>
</tbody>
</table>

PMA, Phorbol 12-Myristate 13-Acetate; PHA, phytohaemagglutinin
the same experiment as described in Section 2.3.5.1 and the CV was calculated. The intra-assay CV values for IL17 and IFN\(\gamma\) ELISpot show that the intra-assay CV is <10%, indicating excellent reproducibility (Tables 5.5 and 5.6).

5.2.2 The role of CD4+ cells in IL17 and IFN\(\gamma\) cytokine production in the ELISpot assay

To determine the relative contribution of CD4+ cells to IL17 and IFN\(\gamma\) production in the ELISpot assays, PBMCs depleted of CD4+ cells using magnetic beads were tested in 3 patients with RA. Whole PBMCs, as well as a sample of the same patients’ PBMCs after CD4+ cell depletion were set up in the IL-17 and IFN\(\gamma\) ELISpot as described in Section 2.3.5. The effectiveness of the depletion and purity of the depleted fraction was verified by FACS and this confirmed that the remaining cell population was effectively depleted of CD4+ cells (Figure 5.2A). The depletion of CD4+ cells from the PBMC population significantly attenuated IL17 responses, demonstrating that the majority of IL17 was produced by CD4+ cells (Figures 5.2 B and C). In contrast, CD4+ cell depletion only reduced IFN\(\gamma\) responses slightly, showing that the majority of IFN\(\gamma\) production was driven by non-CD4+ cells (Figures 5.2D and E).

5.2.3 Frequency of IL17 and IFN\(\gamma\)-producing cells in peripheral blood of healthy controls and patients with inflammatory arthritis

A comparison between healthy controls and patients with RA, AS and PsA in numbers of IL17-producing peripheral blood mononuclear cells at baseline prior to anti-TNF initiation is shown in Figure 5.3A. Compared to healthy controls, RA, AS and PsA patients showed a trend towards having higher frequencies of IL17-producing cells in peripheral blood, although the differences were not statistically significant (healthy controls mean±SEM 282±85 spSFC/10^6 versus RA patients mean±SEM 466±56 spSFC/10^6, \(p=0.13\); AS patients mean±SEM 432±122 spSFC/10^6 versus healthy controls \(p=0.51\); PsA patients mean±SEM 450±98 spSFC/10^6 versus healthy controls \(p=0.23\)). Patients with RA and AS also had greater numbers of IFN\(\gamma\)-producing peripheral blood mononuclear cells compared to healthy controls (healthy controls mean±SEM 831±206 spSFC/10^6 versus RA patients mean±SEM
Table 5.5  
**Summary of intra-assay variability results for each stimulation condition for IL17 ELISpot assay**

<table>
<thead>
<tr>
<th>Stimulation condition</th>
<th>%CV value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1 1µg/ml</td>
<td>8.64</td>
</tr>
<tr>
<td>CD3 0.25µg/ml</td>
<td>9.11</td>
</tr>
<tr>
<td>PMA 50ng/ml and Ionomycin 500ng/ml</td>
<td>8.13</td>
</tr>
<tr>
<td>PHA 1µg/ml</td>
<td>6.62</td>
</tr>
</tbody>
</table>

PMA, Phorbol 12-Myristate 13-Acetate; PHA, phytohaemagglutinin

Table 5.6  
**Summary of intra-assay variability results for each stimulation condition for IFNγ ELISpot assay**

<table>
<thead>
<tr>
<th>Stimulation condition</th>
<th>%CV value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 0.25mg/ml</td>
<td>4.54</td>
</tr>
<tr>
<td>PMA 50ng/ml and Ionomycin 500ng/ml</td>
<td>8.73</td>
</tr>
<tr>
<td>PHA 1mg/ml</td>
<td>2.06</td>
</tr>
</tbody>
</table>

PMA, Phorbol 12-Myristate 13-Acetate; PHA, phytohaemagglutinin
Figure 5.2  The effect of CD4+ cell depletion on IL-17 and IFNγ responses by ELISpot

Cells were stained with CD4-PE pre and post magnetic labelling to assess the effectiveness of CD4+ depletion by flow cytometry (A). Whole PBMCs prior to depletion contain CD4+ T cells (left histogram) whilst after depletion of CD4+ T cells with magnetic beads, there are
virtually no CD4+ T cells present in this representative patient (right histogram). The frequency of IL17-producing peripheral blood mononuclear cells is markedly attenuated when CD4+ cells are depleted from the PBMC population (B). The effects of depletion are shown in 3 representative patients. 200,000 PBMCs prior to and after the depletion from each patient were set up in the IL17 ELISpot assay and stimulated with 1μg/ml anti-CD3 antibody (OKT3 clone). Results are expressed as number of IL17 specific spot forming cells per million PBMCs. Representative experimental wells of the IL17 ELISpot assay from one patient are shown demonstrating that after depletion of CD4+ cells the number of IL17-positive spots per well is attenuated (C). Each spot represents one cytokine-producing cell. The numbers under each well indicate the total number of IL17-positive spots in that well. The frequency of IFNγ-producing peripheral blood mononuclear cells is only slightly attenuated when CD4+ cells are depleted from the PBMC population (D). The effects of the depletion are shown in 3 representative patients. 200,000 PBMCs prior to and after the depletion from each patient were set up in the IFNγ ELISpot assay and stimulated with 0.25μg/ml anti-CD3 antibody (OKT3 clone). Results are expressed as number of IFNγ specific spot forming cells per million PBMCs. Representative experimental wells of the IFNγ ELISpot assay of one patient are shown demonstrating that after depletion of CD4+ cells the number of IFNγ-positive spots per well is only slightly attenuated. Each spot represents one cytokine-producing cell. The numbers under each well indicate the total number of IFNγ-positive spots in that well.

Abbreviation: spSFC/10⁶, specific spot-forming cells per million PBMCs.
Figure 5.3  Comparison of numbers of IL17 and IFNγ producing cells in peripheral blood of healthy controls versus patients with inflammatory arthritis

The numbers of IL17-producing (A) and IFNγ-producing (B) peripheral blood mononuclear cells at baseline between healthy controls (n=9) and patients with rheumatoid arthritis (n=25), ankylosing spondylitis (n=15) and psoriatic arthritis (n=8) were compared. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1µg/ml anti-CD3 antibody (OKT3 clone) in IL17 ELISpot and 0.25 µg/ml anti-CD3 antibody (OKT3 clone) in IFNγ ELISpot for 20 hours and the numbers of cytokine-producing cells were enumerated. Numbers above each time point show mean±SEM spot forming cells per 10^6 PBMCs.

Bars represent mean±SEM, differences between groups assessed using Mann Whitney U test spSFC/10^6, specific spot-forming cells per million PBMCs.
1001±121 spSFC/10⁶, p=0.43; AS patients mean±SEM 1011±129 spSFC/10⁶ versus healthy controls p=0.54) (Figure 5.3B).

5.2.4 Changes in the frequency of IL17 and IFNγ-producing peripheral blood mononuclear cells during anti-TNF treatment

5.2.4.1 Rheumatoid arthritis cohort

There was an increase in IL17-producing cells with treatment compared to baseline, and the increase at week 1 (baseline mean±SD 466.4±277.6 spSFC/10⁶ versus week 1 mean±SD 776.5±533 spSFC/10⁶, p=0.02) and week 12 (baseline mean±SD 466.4±277.6 spSFC/10⁶ versus week 12 mean±SD 759.8±510 spSFC/10⁶, p=0.003) was statistically significant (Figure 5.4A).

The RA cohort comprised patients treated with either etanercept or adalimumab. When the cohort was divided according to type of anti-TNF treatment, the pattern of increase in IL17-producing peripheral blood mononuclear cells with time on treatment was the same, suggesting that this increase did not differ between anti-TNF treatment types (Figures 5.4B and 5.4C).

The same pattern of increase in IL17-producing peripheral blood mononuclear cells with anti-TNF treatment was observed in both responders and non-responders in the RA group (Figures 5.5A and B). A comparison between anti-TNF responders and non-responders in the numbers of IL17-producing cells at each time point during anti-TNF treatment showed no significant differences between the two groups at any of the time points (Figure 5.5C).

The IL17 ELISpot wells of a representative patient with RA illustrating the increase in number of IL17 positive spots with time on anti-TNF treatment are shown in Figure 5.6.

The numbers of IFNγ-producing peripheral blood mononuclear cells at baseline and at each time point on treatment showed more variability between patients (Figure 5.7A). There was a trend towards a gradual increase in the numbers of IFNγ-producing peripheral blood mononuclear cells with time on anti-TNF treatment in the whole RA cohort (Figure 5.7A) and the increase at 1 week was statistically significant (baseline mean±SD 877± 468
**Figure 5.4** Changes in numbers of IL17-producing cells in the peripheral blood of patients with rheumatoid arthritis over 12 weeks on treatment with anti-TNF

Changes in numbers of IL17-producing peripheral blood mononuclear cells with time on anti-TNF treatment are shown for the whole RA cohort (n=25) (A), in RA patients treated with etanercept (n=18) (B) and with adalimumab (n=7) (C). 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1μg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and the numbers of cytokine-producing cells were enumerated. Bars represent mean±SEM. *p<0.05, **p<0.01 versus baseline visit by Wilcoxon matched pairs test.

spSFC/10^6, specific spot-forming cells per million PBMCs.
Figure 5.5  Comparison between rheumatoid arthritis anti-TNF responder and non-responder patients in the changes in numbers of IL17 producing cells in peripheral blood with time on anti-TNF treatment

Changes in numbers of IL17-producing peripheral blood mononuclear cells with time on anti-TNF are shown for treatment responders (n=16) (A) and treatment non-responders (n=9) (B) as determined by improvement in DAS28 score >1.2 from baseline or <1.2 from baseline respectively. Bars represent mean±SEM. No significant differences were found versus baseline visit by Wilcoxon matched pairs test for within-group comparisons with time on treatment. Changes in numbers of IL17-producing peripheral blood mononuclear cells at each time point on treatment for responders and non-responders to anti-TNF therapy were directly compared (C). Bars represent mean±SEM. No significant differences were found between responders versus non-responders evaluated by Mann Whitney U test. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1μg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10^6, specific spot-forming cells per million PBMCs
Figure 5.6  Representative ELISpot wells of a patient with rheumatoid arthritis showing increasing frequency of IL17 producing cells with time on anti-TNF treatment

ELISpot wells of a representative patient with rheumatoid arthritis showing increasing frequency of IL17-producing cells during anti-TNF treatment from baseline to week 12 on treatment. 200,000 PBMCs per time point were seeded in triplicate and stimulated with 1µg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and numbers of IL17 cytokine-producing cells determined by ELISpot. Each spot represents one cytokine-producing cell. The numbers under each well indicate the number of spot forming cells per well.
Figure 5.7 Changes in numbers of IFNγ-producing cells in the peripheral blood of patients with rheumatoid arthritis over 12 weeks on treatment with anti-TNF

Changes in numbers of IFNγ-producing peripheral blood mononuclear cells during anti-TNF treatment are shown for the whole RA cohort (n=25) (A), for RA patients treated with etanercept (n=18) (B) and for RA patients treated with adalimumab (n=7) (C). Bars represent mean±SEM. *p<0.05, versus baseline visit by Wilcoxon matched pairs test. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 0.25 μg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10⁶, specific spot-forming cells per million PBMCs
spSFC/10^6 versus week 1 mean±SD 1283±635 spSFC/10^6, p=0.04), but the increase at 4 weeks (p=0.39) and at 12 weeks (p=0.95) did not reach statistical significance. When the RA cohort was divided according to anti-TNF treatment type, the pattern of increase in IFNγ-producing cells during anti-TNF treatment was the same with both etanercept and adalimumab (Figures 5.7B and C).

Both responders and non-responders to anti-TNF treatment in the RA cohort showed a trend towards an increase in the frequency of IFNγ-producing peripheral blood mononuclear cells with time on treatment (Figures 5.8 A and B). A comparison between responder and non-responder patients in the number of IFNγ-producing cells at each time point on treatment showed no significant differences between the two groups at any of the time points on treatment (Figure 5.8C).

IFNγ ELISpot wells of a representative patient with RA, illustrating the increase in number of IFNγ positive spots with time on anti-TNF treatment are shown in Figure 5.9.

A comparison between responders and non-responders to anti-TNF treatment in baseline numbers of IL17-producing peripheral blood mononuclear cells demonstrated that non-responders showed a trend towards higher frequencies of IL17-producing cells at baseline compared to responders (responders mean±SEM 428±48 spSFC/10^6 versus non-responders 543±129 spSFC/10^6, p=0.69) (Figure 5.10A). Similarly, non-responders to treatment also showed a trend towards having higher numbers of IFNγ-producing cells at baseline compared to responders (responders mean±SEM 826±140 spSFC/10^6 versus non-responders mean±SEM 1172±217 spSFC/10^6, p=0.15) (Figure 5.10B). However, these differences between responders and non-responders in numbers of IL17-and IFNγ-producing cells at baseline were not statistically significant.

The RA cohort comprised patients with varying disease duration, with mean±SD disease duration of 10.60±9.15 years (range 2 to 40 years). Rheumatoid arthritis patients with disease duration less than 3 years (n=7 patients) showed a trend towards having greater numbers of IL17 and IFNγ-producing cells at baseline compared to patients with disease duration over 3 years (n=18 patients) (Figure 5.11A and B). Patients with disease duration less than 3 years had significantly higher numbers of IL17-producing peripheral blood mononuclear cells prior to anti-TNF treatment initiation compared to patients with longer RA disease duration.
Figure 5.8  Comparison between rheumatoid arthritis anti-TNF responder and non-responder patients in the changes in numbers of IFNγ-producing cells in peripheral blood with time on anti-TNF treatment

Changes in numbers of IFNγ-producing peripheral blood mononuclear cells during anti-TNF are shown for treatment responders (n=16) (A) and treatment non-responders (n=9) (B) as determined by improvement in DAS28 score >1.2 from baseline or <1.2 from baseline respectively. Bars represent mean±SEM. No significant differences were found versus baseline visit by Wilcoxon matched pairs test for within-group comparisons with time on treatment. Changes in numbers of IFNγ-producing peripheral blood mononuclear cells at each time point on treatment for responders and non-responders to anti-TNF therapy were directly compared (C). Bars represent mean±SEM. No significant differences were found between responders versus non-responders evaluated by Mann Whitney U test. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 0.25 µg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10^6, specific spot-forming cells per million PBMCs.
Figure 5.9  Representative ELISpot wells of a patient with rheumatoid arthritis showing increasing frequency of IFNγ-producing cells with time on anti-TNF treatment

ELISpot wells of a representative patient with rheumatoid arthritis showing increasing frequency of IFNγ-producing cells with time on anti-TNF treatment from baseline to week 12 on treatment. 200,000 PBMCs per time point were seeded in triplicate and stimulated with 0.25μg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and numbers of IFNγ cytokine-producing cells determined by ELISpot. Each spot represents one cytokine-producing cell. The numbers under each well indicate the number of spot forming cells per well.
Figure 5.10  Comparison between rheumatoid arthritis anti-TNF responder and non-responder patients in numbers of IL17 and IFNγ-producing cells in peripheral blood at baseline prior to anti-TNF treatment initiation

The numbers of IL17-producing cells at baseline (A) and IFNγ-producing cells at baseline (B) were compared between anti-TNF responders (n=16) and non-responder patients (n=9) (response defined as improvement in DAS28 score >1.2 from baseline to 12 weeks) and healthy controls (n=9). 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1μg/ml anti-CD3 antibody (OKT3 clone) in IL17 ELISpot and 0.25 μg/ml anti-CD3 antibody (OKT3 clone) in IFNγ ELISpot for 20 hours and the numbers of cytokine-producing cells were enumerated. Numbers above each time point show mean±SEM spot forming cells per 10⁶ PBMCs. Bars represent mean±SEM. Differences between responders versus non-responders were evaluated by Mann Whitney U test. spSFC/10⁶, specific spot-forming cells per million PBMCs.
Figure 5.11  Differences in baseline numbers of IL17 and IFNγ–producing cells in the peripheral blood of patients with rheumatoid arthritis depending on disease duration

The numbers of IL17-producing peripheral blood mononuclear cells (A) and IFNγ-producing peripheral blood mononuclear cells (B) and the changes in DAS28ESR were compared between RA patients with early disease (< 3 years n=7) and RA patients with long-standing disease (>3 years, n=18) at baseline and at each time point on treatment. Bars represent mean±SEM. No significant differences were seen between groups with different disease duration at each time point on treatment using Mann Whitney U test. spSFC/10^6, specific spot-forming cells per million PBMCs
(p=0.007). Both patients with shorter and longer RA disease duration showed a trend towards a similar increase in IL17-producing cells with time on anti-TNF treatment (Figure 5.11A). Although there was also a trend towards RA patients with shorter disease duration (<3 years) to having greater numbers of IFNγ-producing cells at baseline compared to patients with longer disease duration (>3 years), this difference was not statistically significant (p=0.22) (Figure 5.11B). However, both RA patients with shorter and longer disease duration showed a trend towards an increase in IFNγ-producing cells over time with anti-TNF treatment.

There were no significant differences in the DAS28ESR scores at baseline or the DAS28ESR scores attained at each time point on treatment between RA patients with disease duration less than 3 years and those patients with disease duration more than 3 years, showing that their disease activity prior to treatment and their response to anti-TNF treatment was not significantly different (Figure 5.11C).

5.2.4.2 Ankylosing spondylitis cohort

In the AS cohort as a whole, there was a significant increase in numbers of IL17-producing peripheral blood mononuclear cells after 12 weeks of anti-TNF treatment compared to baseline (baseline mean±SD 432±474 spSFC/10⁶ versus week 12 mean±SD 651±532 spSFC/10⁶, p=0.04) (Figure 5.12A). There was also a trend towards an increase in IL17-producing cells at weeks 1 and 4 on treatment compared to baseline, but these changes did not reach statistical significance (baseline mean±SD 432±474 spSFC/10⁶ versus week 1 mean±SD 623±473 spSFC/10⁶, p=0.30; baseline versus week 4 mean±SD 583±458 spSFC/10⁶, p=0.20). Similarly, there was a trend towards an increase in IFNγ-producing cells after 12 weeks on anti-TNF treatment in the AS cohort as a whole, but this increase was not statistically significant (baseline mean±SD 1011±428 spSFC/10⁶ versus week 12 mean±SD 1249±408 spSFC/10⁶, p=0.30) (Figure 5.12B).

Both AS responders and non-responders to anti-TNF treatment showed a trend towards an increase in IL17 and IFNγ-producing peripheral blood mononuclear cells during anti-TNF treatment, although the increases did not reach statistical significance at any of the time points (Figure 5.13A and B and Figure 5.14A and B). A direct comparison between responders and non-responders in the numbers of IL17-producing cells at each time point on anti-TNF treatment showed there were no significant differences between the two groups.
Figure 5.12 Changes in numbers of IL17 and IFNγ-producing cells in the peripheral blood of patients with ankylosing spondylitis over 12 weeks on treatment with anti-TNF

The changes in numbers of IL17-producing (A) and IFNγ-producing (B) peripheral blood mononuclear cells with time on anti-TNF treatment in the whole RA cohort (n=25) are demonstrated. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1μg/ml anti-CD3 antibody (OKT3 clone) in IL17 ELISpot and 0.25 μg/ml anti-CD3 antibody (OKT3 clone) in IFNγ ELISpot for 20 hours and the numbers of cytokine-producing cells were enumerated. Bars represent mean±SEM. *p<0.05, versus baseline visit by Wilcoxon matched pairs test. spSFC/10⁶, specific spot-forming cells per million PBMCs
Figure 5.13 Comparison between ankylosing spondylitis anti-TNF responder and non-responder patients in the changes in numbers of IL17-producing cells in peripheral blood with time on anti-TNF treatment

Changes in numbers of IL17-producing peripheral blood mononuclear cells with time on anti-TNF are shown for treatment responders (n=11) (A) and treatment non-responders (n=4) (B) as determined by >50% improvement in BASDAI score or <50% improvement in BASDAI score from baseline respectively. Bars represent mean±SEM. No significant differences were found versus baseline visit by Wilcoxon matched pairs test for within-group comparisons with time on treatment. Changes in numbers of IL17-producing peripheral blood mononuclear cells at each time point on treatment for responders and non-responders to anti-TNF therapy were directly compared (C). Bars represent mean±SEM. No significant differences were found between responders versus non-responders evaluated by Mann Whitney U test. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated...
with 1 μg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10^6, specific spot-forming cells per million PBMCs.
Figure 5.14  Comparison between ankylosing spondylitis anti-TNF responder and non-responder patients in the changes in numbers of IFNγ producing cells in peripheral blood with time on anti-TNF treatment

Changes in numbers of IFNγ-producing peripheral blood mononuclear cells with time on anti-TNF are shown for treatment responders (n=11) (A) and treatment non-responders (n=4) (B) as determined by >50% improvement in BASDAI score or <50% improvement in BASDAI score from baseline respectively. Bars represent mean±SEM. No significant differences were found versus baseline visit by Wilcoxon matched pairs test for within-group comparisons with time on treatment. Changes in numbers of IFNγ-producing peripheral blood mononuclear cells at each time point on treatment for responders and non-responders to anti-TNF therapy were directly compared (C). Bars represent mean±SEM. No significant
differences were found between responders versus non-responders evaluated by Mann Whitney U test. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 0.25 μg/ml anti-CD3 antibody (OKT3 clone) for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10⁶, specific spot-forming cells per million PBMCs.
A comparison was made between responders and non-responders to anti-TNF treatment in the numbers of IFNγ-producing peripheral blood mononuclear cells at each time point on treatment and this did not demonstrate a significant difference between the two groups (Figure 5.14C).

There was a trend towards responders to anti-TNF treatment to have higher baseline numbers of IL17-producing peripheral blood mononuclear cells compared to non-responders (responders mean±SEM 521±515 spSFC/10^6 versus non-responders mean±SEM 191±78 spSFC/10^6, p=0.29) as shown in Figures 5.15A, but these differences did not reach statistical significance. Similarly, anti-TNF responders showed a trend towards higher baseline numbers of IFNγ-producing cells compared to non-responders (responders mean±SEM 1039±158 spSFC/10^6 versus non-responders mean±SEM 443±14 spSFC/10^6, p=0.83), but this was not statistically significant (Figure 5.15B).

The AS cohort comprised patients with varying disease duration; disease duration mean±SD was 10.87±10.65 years (range 1-34 years). In view of the differences observed in baseline numbers of IL17 and IFNγ-producing cells between patients with differing RA disease duration, differences in baseline numbers of IL17 and IFNγ-producing cells in AS patients of differing disease duration were also sought. AS patients with disease duration less than 5 years (n=7 patients) showed a trend towards having higher baseline numbers of IL17 and IFNγ-producing peripheral blood mononuclear cells than patients with disease duration greater than 5 years (n=8 patients), although these differences were not statistically significant (Figures 5.16A and B). There was a trend towards an increase in numbers of IL17 and IFNγ-producing cells with time on anti-TNF treatment irrespective of disease duration. There were no significant differences in the mean percentage change of total BASDAI score from baseline between the AS patients with shorter or longer disease duration (Figure 5.16C). Patients with shorter disease duration however did show a trend towards a greater percentage decrease in BASDAI score from baseline to 12 weeks (mean±SD= -74.5±22.48%) compared to patients with longer disease duration (mean±SD= -55.46±22.25%), but this difference was not statistically significant (p=0.12).
Figure 5.15 Comparison between ankylosing spondylitis anti-TNF responder and non-responder patients in the numbers of IL17 and IFNγ-producing cells in peripheral blood at baseline

The numbers of IL17-producing cells at baseline (A) and IFNγ-producing cells at baseline (B) were compared between anti-TNF responders (n=11) and non-responder patients (n=4) (response defined as improvement in BASDAI score >50% from baseline to 12 weeks) and healthy controls (n=9). 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1μg/ml anti-CD3 antibody (OKT3 clone) in IL17 ELISpot and 0.25 μg/ml anti-CD3 antibody (OKT3 clone) in IFNγ ELISpot for 20 hours and the numbers of cytokine-producing cells were enumerated. Numbers above each time point show mean±SEM spot forming cells per 10⁶ PBMCs. Bars represent mean±SEM. Differences between responders versus non-responders were evaluated by Mann Whitney U test. spSFC/10⁶, specific spot-forming cells per million PBMCs.
Figure 5.16  Differences in baseline numbers of IL17 and IFNγ-producing cells in the peripheral blood of patients with ankylosing spondylitis depending on disease duration

The numbers of IL17-producing peripheral blood mononuclear cells (A) and IFNγ-producing peripheral blood mononuclear cells (B) and the changes in BASDAI score were compared between AS patients with early disease (< 5 years, n=7) and AS patients with long-standing disease (>5 years, n=8) at baseline and at each time point on treatment. Bars represent mean±SEM. No significant differences were seen between groups with differing disease duration at each time point on treatment evaluated by Mann Whitney U test. spSFC/10^6, specific spot-forming cells per million PBMCs.
5.2.4.3 Psoriatic arthritis cohort

The changes in numbers of IL17 and IFNγ-producing peripheral blood mononuclear cells during anti-TNF treatment in the PsA group are presented in Figures 5.17A and B respectively. There was a trend towards an increase in numbers of IL17-producing cells at week 4 (baseline mean±SD 450±276 spSFC/10^6 versus week 4 mean±SD 744±444 spSFC/10^6, p=0.16) and week 12 (baseline mean±SD 450±276 spSFC/10^6 versus week 12 mean±SD 609±373 spSFC/10^6, p=0.48) on anti-TNF treatment compared to baseline, but these increases were not statistically significant (Figure 5.17A). There was also a trend towards an increase in numbers of IFNγ-producing cells at week 4 (baseline mean±SD 896±599 spSFC/10^6 versus week 4 mean±SD 1274±878 spSFC/10^6, p=0.38) and week 12 (baseline mean±SD 896±599 spSFC/10^6 versus week 12 mean±SD 1476±801 spSFC/10^6, p=0.15) of anti-TNF treatment compared to baseline, but these increases did not reach statistical significance (Figure 5.17B). Due to the small number of patients in the PsA group, it was not possible to determine if there were any differences between anti-TNF responder and non-responder patients in how the frequency of IL17 and IFNγ-producing cells changes during treatment.

5.2.5 Comparison of the time course of change in the numbers of IL17 and IFNγ-producing cells with anti-TNF treatment across the three disease groups

The numbers of IL17 and IFNγ-producing cells increased from baseline during anti-TNF treatment in all three types of inflammatory arthritis (Figure 5.18). The pattern of the increase in numbers of IL17 and IFNγ-producing cells with anti-TNF treatment was similar across the three disease types. There were no significant differences between disease types in numbers of IL17 and IFNγ-producing cells at any of the time points on treatment.

As there were no significant differences between the three types of inflammatory arthritis in the kinetics or magnitude of change in numbers of IL17 and IFNγ-producing cells with anti-TNF treatment, the pattern of change of these two cell types was determined in the whole study cohort (Figures 5.19 and 5.20). Combining the patients in all three disease groups showed there were significant increases in numbers of IL17-producing peripheral blood mononuclear cells at 1 week (p=0.04), 4 weeks (p=0.03) and 12 weeks (p=0.0008) after anti-
Figure 5.17 Changes in numbers of IL17 and IFNγ-producing cells in the peripheral blood of patients with psoriatic arthritis over 12 weeks on treatment with anti-TNF

Changes in numbers of IL17-producing peripheral blood mononuclear cells (A) and IFNγ-producing cells (B) with time on anti-TNF treatment are shown in the PsA cohort (n=8). Bars represent mean±SEM. No significant differences were found versus baseline visit by Wilcoxon matched pairs test for within-group comparisons with time on treatment. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1µg/ml anti-CD3 antibody (OKT3 clone) in IL17 ELISpot and 0.25 µg/ml anti-CD3 antibody (OKT3 clone) in IFNγ ELISpot for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10⁶, specific spot-forming cells per million PBMCs.
Figure 5.18  Comparison between disease groups in the changes in numbers of IL17 and IFNγ-producing cells in the peripheral blood with time on anti-TNF treatment

The changes in the numbers of IL17-producing (A) and IFNγ-producing (B) peripheral blood mononuclear cells with time on anti-TNF treatment between the three disease groups were compared. Bars represent mean±SEM. No significant differences were seen between disease groups at each time point on treatment by Mann Whitney U test. 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1μg/ml anti-CD3 antibody (OKT3 clone) in IL17 ELISpot and 0.25 μg/ml anti-CD3 antibody (OKT3 clone) in IFNγ ELISpot for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10⁶, specific spot-forming cells per million PBMCs.
Figure 5.19 Changes in numbers of IL17-producing cells in all study patients treated with anti-TNF

Changes in numbers of IL17-producing peripheral blood mononuclear cells with time on anti-TNF treatment are shown in all study patients (n=48) (A), in study patients treated with etanercept (n=25) (B) and with adalimumab (n=23) (C), in study patients who were...
responders to anti-TNF treatment (D) and those were non-responders at 12 weeks (E). 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 1\(\mu\)g/ml anti-CD3 antibody (OKT3 clone) for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10^6, specific spot-forming cells per million PBMCs. Bars represent mean±SEM. *p<0.05, **p<0.01, ***p<0.0005 versus baseline visit by Wilcoxon matched pairs test. spSFC/10^6, specific spot-forming cells per million PBMCs.
Figure 5.20 Changes in numbers of IFN-γ-producing cells in all study patients treated with anti-TNF

Changes in numbers of IFN-γ-producing peripheral blood mononuclear cells with time on anti-TNF treatment are shown in all study patients (n=48) (A), in study patients treated with etanercept (n=25) (B) and with adalimumab (n=23) (C), in study patients who were

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Responders

Non-Responders
responders to anti-TNF treatment (D) and those were non-responders at 12 weeks (E). 200,000 PBMCs were seeded in triplicate in each experiment and stimulated with 0.25 μg/ml anti-CD3 antibody (OKT3 clone) in IFNγ ELISpot for 20 hours and the numbers of cytokine-producing cells were enumerated. spSFC/10⁶, specific spot-forming cells per million PBMCs. Bars represent mean±SEM. No significant differences were seen at any of the time points on treatment versus the baseline visit by Wilcoxon matched pairs test. spSFC/10⁶, specific spot-forming cells per million PBMCs.
TNF treatment was initiated compared to baseline (Figure 5.19A). The pattern of change in IL17-producing cells during anti-TNF treatment was the same when the study cohort was divided according to treatment type (etanercept or adalimumab, Figure 5.19B and C) and according to response status at 12 weeks (Figures 5.19D and E). This suggests that neither the type of anti-TNF agent used nor the patient’s response to treatment were factors influencing the pattern of change in IL17-producing cells during anti-TNF treatment.

Combining all the patients in the three disease groups also showed that there was a trend towards an increase in numbers of IFNγ-producing cells during anti-TNF treatment, although this increase was not statistically significant at any of the time points (Figure 5.20A). The pattern of change in IFNγ-producing cells with time on anti-TNF treatment was also similar when the study cohort was divided according to treatment type (etanercept or adalimumab, Figure 5.20B and C) and according to response status at 12 weeks (Figures 5.20D and E).

5.3 Discussion

5.3.1 Frequencies of IL17 and IFNγ-producing peripheral blood mononuclear cells in patients with inflammatory arthritis compared to healthy controls

The frequencies of IL17-producing cells in the peripheral blood of patients with RA, AS or PsA compared to healthy controls have not been previously studied using the IL17 ELISpot assay. Comparison of the frequency of IL17-producing peripheral blood mononuclear cells between healthy controls and patients with RA, AS and PsA revealed a trend towards an increase in this cell type in the patient groups compared to healthy controls. Other studies have used intra-cellular cytokine staining of stimulated PBMCs to determine frequencies of CD4+IL17+ cells in peripheral blood or measured secreted IL17 by ELISA in culture supernatants from stimulated PBMCs. These studies have also reported increased frequencies of CD4+IL17+ cells in the peripheral blood and higher IL17 levels in cell supernatants from stimulated PBMCs of RA, AS and PsA patients compared to healthy controls or osteoarthritis patients, supporting a role of this cell type in the pathogenesis of these diseases (Chen et al., 2011; Gullick et al., 2010; Jandus et al., 2008; Leipe et al., 2010; Shen et al., 2009; van Hamburg et al., 2011; Xueyi et al., 2012). Increased CD4+IL17+ cell frequencies have been shown both in RA patients with early disease who are treatment naïve and in patients with
established disease treated with various therapeutic regimens (Leipe et al., 2010; van Hamburg et al., 2011).

There was also a trend towards RA and AS patients having increased numbers of IFNγ-producing peripheral blood mononuclear cells at baseline compared to healthy controls, although this difference did not reach statistical significance and there appeared to be more individual variation between patients in the level of IFNγ production by PBMCs. Further investigation of this would be needed in a larger cohort of patients to be able to conclude whether there indeed is a significant difference between RA and AS patients and healthy controls in the frequency of IFNγ-producing peripheral blood mononuclear cells. Studies investigating differences in IFNγ-producing peripheral blood mononuclear cells between RA patients and healthy controls have reported differing results. Early studies in RA patients reported that patients with high inflammatory activity showed reduced IFNγ production from PBMCs stimulated with phytohemagglutinin (PHA) and concanavalin (ConA) compared to healthy controls. In contrast, patients with low inflammatory activity showed comparable IFNγ production compared to healthy controls (Seitz et al., 1987). However, this study evaluated inflammatory activity based on the level of ESR and duration of morning stiffness and the patients in the study were treated with gold salts or penicillamine which are now rarely used, making it difficult to make direct comparison with the findings from our study (Seitz et al., 1987). Increased IFNγ production by stimulated PBMCs from patients with RA compared to healthy controls has also been reported with an IFNγ ELISpot assay (Ronnelid J, 1998). In contrast, Berg et al did not detect significant differences between RA patients and healthy controls in the spontaneous production of IFNγ by PBMCs using ELISpot (Berg et al., 2001). In another study, patients with new onset synovitis of less than a year duration were found to have increased numbers of IFNγ-secreting peripheral blood mononuclear cells detected by ELISpot compared to patients with chronic RA (Kanik et al., 1998). More recent studies which have utilised intra-cellular cytokine staining and ELISA to evaluate frequency of IFNγ-producing cells and IL17 levels respectively have also reported differing results with respect to the frequency of IFNγ-producing cells in RA, AS and PsA patients compared to healthy controls. Baeten et al reported a decreased percentage of T cells positive for IFNγ in patients with spondyloarthritis and RA compared to healthy controls as evaluated by flow cytometry (Baeten et al., 2001). Jandus et al have reported increased frequency of IFNγ-secreting CD4+ cells in AS and PsA patients compared to healthy controls, but no difference
in frequencies of CD4+IFNγ+ cells between RA patients and healthy controls (Jandus et al., 2008). Other studies have also failed to detect significant differences in the frequency of CD4+IFNγ+ cells between patients with RA, AS or PsA and healthy controls (Gullick et al., 2010; Leipe et al., 2010; Yamada et al., 2008).

The differences in functional assays used to assess the frequency of IFNγ-producing cells in the different studies and the greater sensitivity of the IL17 and IFNγ ELISpot assays in comparison with flow cytometry techniques and ELISA may explain the discrepancy between our findings and these studies. IL17-producing cells are present in small numbers in peripheral blood hence IL17 ELISpot which is more suited to studying low level responses than intra-cellular cytokine staining or ELISA would be perhaps more likely to detect small but significant differences in the frequency of this cell type. Other possible explanations for the differences in the results may be due to the nonhomogeneous patient cohorts used in other studies, which have included patients with huge variations in disease duration and different immunomodulatory treatments.

5.3.2 Contribution of CD4+ cells producing IL17 and IFNγ in patients with RA

Studies have shown that IL17 can be produced by a number of cell types including CD4+ T cells, γδT cells, invariant natural killer T cells and mast cells (Miossec et al., 2009; van den Berg and Miossec, 2009). As the ELISpot assay does not provide information on the phenotypic characteristics of cytokine-producing cells, the contribution of CD4+ cells to IL17 and IFNγ production by peripheral blood mononuclear cells as assessed by ELISpot was evaluated. Comparison of IL17 and IFNγ secretion by whole PBMCs and PBMCs depleted of CD4+ cells from test RA patients revealed that CD4+ cell depletion significantly reduced the frequency of IL17-producing cells, whereas there was only a minimal reduction in the frequency of IFNγ-producing cells. The results confirm that CD4+ cells are the main producers of IL17 in the peripheral blood of RA patients, whereas non-CD4+ cells are the main producers of IFNγ. The phenotypes of IL17 and IFNγ-producing cells and their change during anti-TNF treatment were evaluated further using intra-cellular cytokine staining as described in Chapter 6. The phenotype of IL17-producing cells in the peripheral blood of RA patients has been studied by other investigators using flow cytometry and the majority of the cells producing IL17 in peripheral blood have been shown to be CD4+T cells, whereas
the main cell types producing IFNγ in peripheral blood include CD8+T cells and natural killer T cells (Jandus et al., 2008; Leipe et al., 2010; Shen et al., 2009).

5.3.3 Changes in the frequency of circulating IL17 and IFNγ-producing cells with anti-TNF treatment in inflammatory arthritis

Anti-TNF treatment induced a significant increase in IL17-producing peripheral blood mononuclear cells in RA patients at 1 and 12 weeks after treatment initiation. In AS patients, anti-TNF treatment caused a gradual increase in IL17-producing cells as early as 1 week after treatment initiation and this increase was statistically significant at 12 weeks compared to baseline levels. In PsA patients, there was also a trend towards an increase in IL17-producing peripheral blood mononuclear cells with anti-TNF treatment at 12 weeks.

There was significant increase in IFNγ-producing cells at 1 week after anti-TNF initiation in the RA cohort compared to baseline and a trend towards an increase at 4 and 12 weeks on treatment compared to baseline. In AS and PsA patients, there was a trend towards an increase in IFNγ-producing cells at 4 and 12 weeks after treatment initiation.

These changes in IL17 and IFNγ-producing peripheral blood mononuclear cells with anti-TNF treatment were observed in patients treated with both adalimumab and those treated with etanercept and the pattern of change of the frequency of the cells during treatment was similar with those two treatments. Adalimumab and etanercept differ in structure; adalimumab is a humanised monoclonal antibody, whereas etanercept is a soluble p75 receptor fusion protein linked to the Fc portion of human IgG. Although they both effectively neutralise soluble TNF and this is their main pharmacological mechanism of action, they differ with respect to their ability to engage transmembrane TNF, ability to fix complement and bind Fc receptors to form complexes. They also differ in that etanercept can also bind members of the lymphotoxin family in addition to TNF (Taylor, 2010; Wong et al., 2008). Despite these differences, both agents appear to act similarly in increasing IL17 and IFNγ-producing peripheral blood mononuclear cells, suggesting that this is a class effect of anti-TNF agents, rather than a treatment-specific effect. The increase in IL17 and IFNγ-producing cells with anti-TNF treatment occurred in patients with RA, AS and PsA despite these diseases differing in pathogenesis. This suggests that this effect of anti-TNF treatment is also not disease-specific.
This is the first study to evaluate changes in both IL17 and IFNγ-producing peripheral blood mononuclear cells with anti-TNF treatment longitudinally using the ELISpot assay. This technique is particularly suited to use in longitudinal studies as it is reproducible and objective and its high sensitivity in studying low level immune responses makes it particularly suitable to studying IL17-producing cells. The IFNγ ELISpot assay has been used in two other studies to assess the changes in the frequency of IFNγ-producing cells in the peripheral blood of RA patients treated with etanercept. Berg et al reported a significant increase in the frequency of IFNγ-producing PBMCs at 4 weeks after initiation of etanercept treatment compared to baseline in a group of RA patients, but no significant differences at 8 weeks compared to baseline (Berg et al., 2001). Schotte et al however reported a decrease in the numbers of IFNγ-producing cells at 9 months after etanercept was initiated compared to pre-treatment levels (Schotte et al., 2004). The differences between the study by Berg et al and our study is that they assessed spontaneous IFNγ production by PBMCs, whereas we assessed IFNγ production in response to anti-CD3 stimulation and our study did not include a time point at 8 weeks after treatment initiation. The study by Schotte et al assessed long-term changes in response to etanercept treatment by evaluating changes in IFNγ-producing cells at 9 months on treatment compared to baseline, whereas our study addressed changes in the short and medium term after anti-TNF initiation.

Other studies evaluating changes in IL17 or IFNγ producing cells with anti-TNF treatment have utilised intracellular cytokine staining or measured IL17 or IFNγ levels in serum or cell culture supernatants from stimulated T cells or PBMCs. These studies have also mainly concentrated on one type of inflammatory arthritis and mostly investigated the change in cytokine-producing cells at a single time point on anti-TNF treatment compared to baseline, rather than longitudinally. An increase in the percentage of CD4+IL17+ cells and CD4+IFNγ+ cells evaluated by flow cytometry with anti-TNF treatment has been reported in RA patients (Alzabin et al., 2012; Maurice et al., 1999). Increased IL17 and IFNγ production by cultured PBMCs from RA patients after initiation of anti-TNF has also been reported (Aerts et al., 2010; Alzabin et al., 2012; Nissinen et al., 2004). Bose et al reported an increase in IL17 and IFNγ production by cultured PBMCs from PsA patients 1 month after anti-TNF initiation (Bosè et al., 2011). In AS patients, 12 weeks of etanercept treatment has been shown to induce a significant increase in percentages of CD4+IFNγ+ cells and CD8+IFNγ+ cells in the peripheral blood (Zou et al., 2003). These studies will be discussed in more detail...
in the subsequent chapter in light of the changes in IL17 and IFNγ-producing cells observed by flow cytometry in our study.

5.3.4 Baseline differences in numbers of IL17 and IFNγ-producing cells in anti-TNF treatment responder and non-responders patients

There was a trend towards higher baseline numbers of IL17 and IFNγ-producing cells in rheumatoid arthritis patients who were non-responders to anti-TNF compared to responders to treatment. These differences did not reach statistical significance and would need confirmation in larger patient cohorts. However, if confirmed, this finding may suggest that differences in baseline numbers of IL17-producing cells could predict poor response to anti-TNF therapy. Based on this observation, relationships between the immunological changes in IL17 and IFNγ-producing cells and clinical and ultrasonographic parameters of treatment response with time on anti-TNF were explored. As discussed further in Chapter 8, this investigation of the relationships between baseline numbers of IL17-producing cells in RA patients and response to anti-TNF therapy evaluated clinically by DAS28 score or improvement in ultrasound parameters further supports the observation that higher baseline numbers of IL17-producing cells may be indicative of poor treatment response.

Only two other studies have investigated whether IL17 production prior to anti-TNF initiation would be predictive of response to treatment in RA patients. One study using intra-cellular cytokine staining reported that there was a significant inverse correlation between the percentage of Th17 cells in peripheral blood prior to anti-TNF initiation and DAS28 score attained at 4 weeks after therapy (Alzabin et al., 2012). In another study, high serum IL17 levels in RA patients prior to anti-TNF initiation were found to be a significant predictor of therapeutic non-response at 6 months on treatment (Chen et al., 2011). If higher baseline Th17 cells or IL17 levels are indeed confirmed in large patient cohorts to be predictive of poor treatment response to anti-TNF therapy, this has the potential to be useful clinically as a predictive marker of response, as currently there are no biomarkers available that can be used prior to treatment initiation to determine whether a patient will respond to anti-TNF treatment. Being able to determine this at baseline prior to anti-TNF initiation would perhaps allow patients less likely to respond to anti-TNF to be treated with alternative biologic agents.
and this may be the first step towards being able to tailor RA treatment according to individual patient disease immunological profiles.

In contrast to the trend towards higher baseline IL17 and IFNγ levels in RA non-responders in this study, in AS patients there was a trend towards non-responders to anti-TNF to show lower baseline levels of IL17 and IFNγ-producing cells compared to responders. As the number of AS non-responders was very small in this cohort, firm conclusions cannot be made based on this observation and confirmation is needed in larger patient cohorts. This potential difference between RA and AS non-responders may be due to these two types of inflammatory arthritis having differing disease pathogenesis. Whereas in RA there are distinct features of autoimmunity with involvement of T and B cell autoreactivity, AS has been postulated to possess features more suggestive of autoinflammation due to the presence of abnormal innate immune responses in its pathogenesis (Ambarus C, 2012; Lories and Baeten, 2009)

Only one other study has reported on potential differences in baseline Th17 cell related cytokines between anti-TNF responder and non-responder ankylosing spondylitis patients. Xueyi et al reported significantly higher baseline levels of IL6, IL17 and IL23 in anti-TNF non-responder AS patients than in responders (Xueyi et al., 2012). However, the differences with our study are that anti-TNF response in the study by Xueyi et al was assessed at 6 months after treatment initiation and response was defined as at least 20% improvement from baseline according to ASAS (Assessment in Spondyloarthritis International Society) response criteria. In contrast, we assessed response as 50% improvement in BASDAI score at 3 months according to ASAS guidelines for assessment of anti-TNF responses in AS patients. These differences in the definition of treatment response, the duration of treatment, as well as the small number of patients in our study may account for the different results.

Despite the potential differences between anti-TNF responders and non-responders in baseline numbers of IL17-producing cells in our study, anti-TNF treatment in both responder and non-responder patients increased IL17 and IFNγ-producing peripheral blood mononuclear cells in a similar way. This suggests that the increase in these cell types with anti-TNF treatment may not be associated with treatment response. Although the change in these cell types with anti-TNF treatment has been reported by a number of studies as discussed, only three studies have studied the differences between anti-TNF responder and
non-responder patients with respect to the change in frequency of IL17 and IFNγ-producing cells during anti-TNF therapy (Chen et al., 2011; Nissinen et al., 2004; Xueyi et al., 2012). Chen et al reported an increase in the frequency of CD4+IL17+ cells by flow cytometry and an increase in serum IL17 levels after 6 months of anti-TNF treatment in RA non-responders, whereas CD4+IL17+ cells and serum IL17 levels decreased in anti-TNF responders at 6 months (Chen et al., 2011) In contrast, Nissinen et al reported a significant increase in the percentage of CD3+ T cells in RA responders two weeks after infliximab infusion compared to non-responders, although they did not characterise the phenotype of the CD3+ T cells further (Nissinen et al., 2004). In AS patients, Xueyi et al reported a significant decrease in the frequencies of CD4+IL17+ cells in anti-TNF responders and a significant increase in non-responders. However, these are all small individual studies and confirmation would be needed in large patient groups, with standardised assessments of treatment response and evaluation of changes in IL17 and IFNγ-producing cells with the same methodologies at identical time points on treatment.

5.3.5 Effect of RA and AS disease duration on numbers of IL17 and IFNγ-producing cells at baseline

When the RA cohort was divided based on disease duration, patients with shorter disease duration (less than 3 years) were found to have significantly higher baseline numbers of IL17-producing cells than patients with longer disease duration (> 3 years), despite both groups of patients showing a similar increase in IL17-producing cells with time on anti-TNF therapy and no differences in their response to treatment. Similarly, there was a trend towards RA patients of shorter disease duration (less than 3 years) to have greater numbers of IFNγ-producing cells at baseline compared to patients with longer disease duration (more than 3 years). In the AS group, patients with shorter disease duration (less than 5 years) showed a trend towards having higher baseline numbers of IL17 and IFNγ-producing cells compared to patients with longer disease duration (more than 5 years).

Three years was the distinction used between patients with shorter or longer RA disease duration because in the RA cohort there was a group of patients with disease duration range from 2 to 3 years after diagnosis (n=7) and a group of patients with longer disease duration ranging from 6 to 40 years post diagnosis (n=18). Similarly, in the AS group, there was a group of patients with disease duration ranging from 1 to 5 years (n=7) and a group of
patients with disease duration ranging from 12 to 34 years (n=8). There is no widely accepted ‘definition’ of early RA. Historically, in the literature, early RA was considered if disease duration was less than 5 years, however this varies widely and more typically early RA is defined as 2 or 3 years post diagnosis (Scott, 2007). The difficulty in defining ‘early RA’ is that at presentation, patients with RA may already have evidence of erosive changes or joint damage, showing that even so called ‘early RA’ may already represent a chronic established disease process.

In view of the lack of an accepted definition of ‘early RA’ studies are not directly comparable as they include patients of differing disease duration and differing treatment regimens with only a few studies evaluating changes in treatment-naïve RA patients. Kanik et al used ELISpot assays to compare patterns of cytokine secretion in patients with new onset synovitis of less than a year and patients with chronic RA (Kanik et al., 1998). They found that patients with new onset synovitis had increased numbers of PBMCs secreting IL2 and IFNγ, whereas patients with chronic RA had increased numbers of PBMCs secreting IL6, IL10 and TNFα (Kanik et al., 1998). It has been shown that patients with early undifferentiated inflammatory arthritis in whom RA subsequently develops had a higher expression of IL17 in synovial fluid compared with patients with early inflammatory arthritis in whom RA did not develop (Raza et al., 2005). CD4+ and CD8+ T cells have also been found to be increased significantly in the peripheral blood of RA patients with disease duration less than a year, compared to patients with late RA who had similar levels to healthy controls (Coulthard et al., 2012). A study in patients with early RA who were treatment-naïve identified that these patients had increased CD4+IL17+ T cells in peripheral blood compared to healthy controls (van Hamburg et al., 2011). In coculture experiments with rheumatoid arthritis synovial fibroblasts (RASF), Th17 cells but not Th1 cells were shown to be potent inducers of the inflammatory cytokines IL6, IL8 and tissue destructive matrix metalloproteinases. In addition, activation of RASF by Th17 cells provided a Th17 cell polarising environment in a feedback loop, resulting in strikingly increased expression of IL17. These findings confirm the pathogenic role of Th17 cells and implicate this cell type in playing a major role in the development of persistent arthritis (van Hamburg et al., 2011). Synovial membrane IL17 expression has also been found to be predictive of joint damage in patients with RA, especially in the earlier stages of RA when IL17 may have a greater contribution towards joint damage progression (Kirkham et al., 2006). Further insights into the different roles of Th1 and Th17 cells in arthritis initiation and pathogenesis have emerged from some experimental animal models. Some animal
models of arthritis are dependent on Th1/IFNγ (Doodes et al., 2008), whilst others are more Th17/IL17 dependent (Nakae et al., 2003) or can switch from TNFα dependency in the early phases of disease to Th17/IL17 dependency in the later stages (Koenders et al., 2006). This also supports the possibility that RA T helper cell subsets may play different roles in the different stages of disease.

Taken together with our findings, these studies suggest that the pathogenesis of early and late RA may differ, supporting a role for IL17-producing cells in the early stages of RA and implicating them in the development of persistent arthritis.

5.4 Conclusion

The work presented in this chapter characterised the changes in the frequency of circulating IL17 and IFNγ-producing cells of patients with inflammatory arthritis during anti-TNF treatment using the ELISpot assay. This is the first study to characterise longitudinally the changes in these cell types during anti-TNF therapy using the ELISpot assay in patients with RA, AS and PsA. The IL17 and IFNγ ELISpot assays were both highly reproducible with low intra and inter-assay variability, confirming the technique and results obtained were robust and reproducible. The results showed that anti-TNF treatment induced an increase in IL17-producing cells in RA and AS patients and a similar trend towards an increase in IL17-producing cells was observed in PsA patients. There was also a significant increase in IFNγ-producing cells with anti-TNF treatment in RA patients and a trend to an increase in IFNγ-producing cells in patients with AS and PsA. These changes did not differ between type of anti-TNF treatment (etanercept or adalimumab), patient responder status or type of inflammatory arthritis, suggesting this may be a class effect of anti-TNF agents, rather than a treatment or disease-specific effect. Numbers of IL17-producing cells prior to anti-TNF initiation showed a trend to be higher in RA patients who were subsequently anti-TNF non-responders compared to responders. In contrast in AS patients, numbers of IL17-producing cells prior to anti-TNF initiation showed a trend to be higher in patients who were subsequently anti-TNF responders compared to non-responders. Another interesting observation to emerge from this study was that RA patients with shorter disease duration (less than 3 years) had significantly higher numbers of IL17-producing peripheral blood mononuclear cells at baseline compared to patients with longer disease duration, implicating IL17-producing cells in early RA disease pathogenesis and possibly in the development of
persistent arthritis. Confirmation of these findings are needed in larger patient cohorts, as well as an evaluation of the changes in these cell types over a broader range of time points on treatment. This will also aid in the confirmation whether these changes in IL17 and IFNγ-producing cells are indeed a class effect of anti-TNF agents and whether their change with treatment is linked to treatment response.
Chapter 6. Changes in the frequency and phenotype of peripheral blood CD4+IL17+ and CD4+IFNγ+ T cells during anti-TNF treatment

6.1 Introduction

The work presented in this chapter describes the use of flow cytometry to evaluate changes in the frequency of peripheral blood CD4+IL17+ cells (Th17 cells) and CD4+IFNγ+ cells (Th1 cells) longitudinally during anti-TNF treatment in patients with rheumatoid arthritis (RA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA). Changes in the phenotype of Th17 cells during anti-TNF treatment were also investigated.

The objective of the work in this chapter was to determine changes in the frequency of circulating Th1 and Th17 cells during anti-TNF treatment thus complementing and confirming the findings obtained using IL17 and IFNγ ELISpot assays and additionally providing information on the phenotype of IL17-producing T helper cells in the peripheral blood of patients with inflammatory arthritis.

This is the first study to evaluate and compare changes in the frequency of circulating Th17 and Th1 cells in patients with three different types of inflammatory arthritis; RA, AS and PsA and to also examine whether anti-TNF therapy induces phenotypic changes in circulating Th17 cells. The results of the ELISpot and flow cytometry analyses would thus provide a basis to investigate immune correlates of anti-TNF treatment response.

6.1.1 Frequency of peripheral blood Th17 and Th1 cells in patients with RA, AS and PsA

Th17 cells are a third effector arm of CD4+T helper cells and complement the function of Th1 cells, with both cell types implicated in the pathogenesis of inflammatory arthritis (Cope, 2008b). A number of studies have assessed Th17 and Th1 cell frequencies in various rheumatic diseases but some discrepancies exist in the results especially in patients with RA.

Although the vast majority of studies have reported increased frequency of Th17 cells in the peripheral blood of patients with RA (Gullick et al., 2010; Leipe et al., 2010; Shen et al., 2009; van Hamburg et al., 2011), other studies have failed to detect significant differences...
between RA patients and healthy controls (Jandus et al., 2008; Yamada et al., 2008). These studies have used intracellular cytokine staining to determine frequencies of CD4+IL17+ cells in peripheral blood and ELISA techniques to measure IL17 levels in cell culture supernatants from stimulated PBMCs. Using the IL17 ELISpot assay in our study (Chapter 5), there was a trend towards an increased frequency of IL17-producing peripheral blood mononuclear cells in RA patients compared to healthy controls. To seek further confirmation of this trend we analysed frequencies of CD4+IL17+ cells in RA patients at baseline and compared this to frequencies in healthy controls using flow cytometry.

A number of studies investigating differences in IFNγ-producing peripheral blood mononuclear cells (PBMC) between RA patients and healthy controls have also reported conflicting results. A few studies have reported reduced IFNγ production from PBMCs and reduced frequencies of CD4+IFNγ+ cells by flow cytometry in RA patients (Baeten et al., 2001; Nissinen et al., 2004; Schotte et al., 2004). Other studies have reported that patients with varying RA inflammatory activity had differing amounts of IFNγ production by stimulated PBMCs and that patients with new onset synovitis had increased frequency of IFNγ-secreting PBMC compared to patients with chronic RA (Kanik et al., 1998; Seitz et al., 1987). Other studies have failed to detect significant differences between RA patients and healthy controls in the frequency of peripheral blood Th1 cells (Berg et al., 2001; Gullick et al., 2010; Leipe et al., 2010; Shen et al., 2009; Yamada et al., 2008). One study using IFNγ ELISpot reported increased IFNγ production by stimulated PBMCs from RA patients compared to healthy controls (Ronnelid J, 1998). In view of these discrepancies and the trend towards increased frequency of IFNγ-producing peripheral blood mononuclear cells in RA patients at baseline detected using the IFNγ ELISpot assay in our study, we also sought to compare frequencies of Th1 cells using intra-cellular cytokine staining.

Increased frequencies of Th17 cells in the peripheral blood of patients with AS and PsA compared to healthy controls have been reported by a number of studies (Jandus et al., 2008; Leipe et al., 2010; Shen et al., 2009). However, studies investigating the frequency of Th1 cells in patients with AS or PsA compared to healthy controls have yielded conflicting results, with some studies reporting an increase in circulating Th1 cells in patients with AS or PsA, whereas other studies report a decrease compared to healthy controls (Baeten et al., 2001; Jandus et al., 2008; Leipe et al., 2010). In view of the trend towards an increase in the
frequency of IFNγ-producing peripheral blood mononuclear cells seen by IFNγ ELISpot in our study, we also set out to compare the frequencies of Th1 and Th17 cells at baseline in patients with AS and PsA compared to healthy controls using flow cytometry.

6.1.2 Changes in frequency of peripheral blood Th1 and Th17 cells with anti-TNF treatment

Work by our group in the collagen-induced arthritis model of RA (Williams et al., 1992) demonstrated that anti-TNF therapy ameliorates arthritis by decreasing numbers of Th1 and Th17 cells in arthritic joints, but paradoxically leads to an increase in Th1 and Th17 cells in the draining lymph nodes (Notley et al., 2008).

Studies investigating the changes in the frequency of peripheral blood Th17 cells with anti-TNF treatment in patients have utilised either intracellular cytokine staining of PBMCs or ELISA techniques to measure IL17 in cell culture supernatants and have reported an increase in IL17-producing peripheral blood mononuclear cells during anti-TNF treatment in patients with RA, AS and PsA (Aerts et al., 2010; Alzabin et al., 2012; Bosè et al., 2011; Chen et al., 2011; Xueyi et al., 2012). Increased frequency of IFNγ-producing cells in the peripheral blood with anti-TNF therapy has also been reported in patients with RA and AS (Berg et al., 2001; Bosè et al., 2011; Maurice et al., 1999; Nissinen et al., 2004; Zou et al., 2003). In contrast, one study has reported a decrease in IFNγ-producing cells with anti-TNF treatment in RA patients using ELISpot assays (Schotte et al., 2004). However, each of these studies has investigated the changes in the frequency of Th17 or Th1 cells with anti-TNF therapy in one type of inflammatory arthritis only and almost all of the studies have investigated the changes in cytokine-producing cells at a single time point on anti-TNF treatment compared to baseline, rather than longitudinally. In this study, I have used flow cytometry to characterise changes in the frequency of circulating Th1 and Th17 cells during anti-TNF treatment longitudinally in patients with RA, AS and PsA and have also investigated differences between treatment responders and non-responders and between patients on different types of anti-TNF agents with respect to the changes in the frequency of these cell types with therapy. Only two other studies have investigated whether the change in the frequency of Th17 cells with anti-TNF treatment was associated with treatment response; one study was carried out in RA patients and the other study in patients with AS patients (Chen et al., 2011; Xueyi et al., 2012).
6.1.3 Phenotype of circulating Th17 cells in inflammatory arthritis and changes in Th17 cell phenotype with anti-TNF treatment

A number of characteristic cell surface markers of human Th17 cells have been described although no single surface marker is specific for Th17 cells (Annunziato et al., 2012). The vast majority of Th17 cells have been identified within the memory (CD45RO) CD4 population (Acosta-Rodriguez et al., 2007a; Annunziato et al., 2007; Shen et al., 2009). Th17 cells have a high expression of the chemokine receptor, CCR6 which allows homing of these cells to inflamed tissues (Acosta-Rodriguez et al., 2007b). Its ligand, CCL20 is produced in chronically inflamed tissues in response to IL17 by several cell types allowing further attraction and recruitment of Th17 cells (Hirota et al., 2007; Pene et al., 2008; Tanida et al., 2009). Th17 cells also frequently express the chemokine receptor, CCR4, although other cell types, including Th2 and regulatory T cells can also express CCR4 (Acosta-Rodriguez et al., 2007b). Other characteristic cell surface markers of human Th17 cells include the IL23 receptor (IL23R), CD161 and IL12 cytokine receptor (IL12R) (Annunziato et al., 2012). Both CD4+ and CD8+ T helper cells are capable of producing IL17 and have been implicated in the pathogenesis of a number of inflammatory and infective conditions (Korn et al., 2009). However, the vast majority of IL17-positive T helper cells has been described within the CD4+ population in patients with inflammatory arthritis (Shen et al., 2009). CD4+ T cells expressing both IL17 and IFNγ have also been observed in peripheral blood and synovial fluid of patients with immune-mediated inflammatory conditions such as RA (Church et al., 2010; Gullick et al., 2010; Shen et al., 2009).

Although a number of studies have described the phenotypic characteristics of Th17 cells in patients with inflammatory arthritis compared with healthy controls (Jandus et al., 2008; Shen et al., 2009), there are no studies which have investigated longitudinally if anti-TNF treatment induces phenotypic changes in circulating Th17 cells. One study compared the migration-related phenotype of Th17 cells in the peripheral blood of RA patients and found that patients who were in disease remission with anti-TNF had significantly lower expression of CCR6 than patients with active disease (Aerts et al., 2010).

Thus, in addition to characterising longitudinally the changes in the frequency of Th17 cells with anti-TNF treatment in RA, AS and PsA patients, we also studied whether there were any changes in the expression of surface markers CD45RO and CCR6 on Th17 cells with anti-TNF therapy. This allowed us to determine if anti-TNF treatment induced changes in the memory phenotype or migration-related phenotype of Th17 cells.
6.2 Results

6.2.1 Comparison of frequency and phenotype of CD4+ and CD8+ cells producing IL17 and IFNγ between healthy controls and patients with inflammatory arthritis

Using flow cytometry the frequency of CD4+ and CD8+ T cells producing IL17 and IFNγ as well as the surface phenotype of IL17-producing cells within the peripheral blood was characterised in the disease groups compared to healthy controls. There was a trend towards a higher percentage of CD4+IL17+ cells in the peripheral blood of patients with RA (mean±SD 0.79±0.38%) and PsA (mean±SD 0.6±0.35%) at baseline compared to healthy controls (mean±SD 0.56±0.38%). AS patients had a significantly higher percentage of CD4+IL17+ cells at baseline (mean±SD 1.1±0.52%) compared to healthy controls (Figure 6.1). This shows that CD4+IL17+ cell (Th17 cells) frequencies tended to be higher in patients with inflammatory arthritis compared to healthy controls.

Patients with RA (mean±SD 12.93±10.06%) and AS (mean±SD 12.60±6.70%) also showed a trend towards having a higher frequency of CD4+IFNγ+ cells at baseline compared to healthy controls (mean±SD 7.4±2.63%), although these differences were not statistically significant (Figure 6.2A). There were no differences in the frequency of CD4+IFNγ+ cells between healthy controls and patients with PsA (Figure 6.2A).

There were no significant differences in the percentages of CD8+IFNγ+ cells between healthy controls and any of the disease groups (Figure 6.2B).

Compared with healthy controls, a significantly greater percentage of CD4+IL17+ cells in the patients with inflammatory arthritis expressed CD45RO, showing that a greater proportion of the Th17 cells in these patients reside within the memory T cell pool (Figure 6.3).

A significantly greater percentage of the CD4+IL17+ cells in patients with RA, AS and PsA also expressed the Th17 cell homing receptor, CCR6 compared to healthy controls (Figure 6.4).

Further phenotypic analysis by FACS showed that the vast majority of the IL17+ T cells resided within the CD4+ population and that the frequency of CD8+IL17+ cells was
Figure 6.1  Comparison between healthy controls and disease groups in frequency of CD4+IL17+ cells in peripheral blood

Frequency of interleukin-17 (IL17)-positive CD4+ T cells in healthy controls (n=9) and patients with rheumatoid arthritis (RA) (n=25), ankylosing spondylitis (AS) (n=15) and psoriatic arthritis (PsA) (n=8) (A). Bars show mean±SEM, *p<0.05, comparison between patient groups made using Mann Whitney U test. Typical examples of intracellular staining for IL17 in healthy controls and patients with RA, AS and PsA are shown (B). Gated on
CD4+ cells, CD4+IL17+ cells were identified based on fluorescence minus one and unstimulated controls.
Figure 6.2  Comparison between healthy controls and disease groups in frequency of CD4+ILIFNγ+ and CD8+IFNγ+ cells in peripheral blood

Frequency of interferon-gamma (IFNγ)-positive CD4+ T cells (A) and CD8+IFNγ+ cells (B) in healthy controls (n=9) and patients with rheumatoid arthritis (RA) (n=25), ankylosing spondylitis (AS) (n=15) and psoriatic arthritis (PsA) (n=8). Bars show mean±SEM, comparison between patient groups made using Mann Whitney U test. Typical examples of
CD4+IFNγ+ intracellular staining in healthy controls and patients with RA, AS and PsA (B). Gated on CD4+ cells, CD4+IFNγ+ cells were identified based on fluorescence minus one and unstimulated controls.
Figure 6.3  Comparison between healthy controls and disease groups in the expression of memory phenotype of CD4+IL17+ cells in peripheral blood

Comparison between healthy controls and patients with RA, AS and PsA in the expression of memory phenotype of CD4+IL17+ cells in peripheral blood (A). Bars show mean±SEM, comparison between patient groups made using Mann Whitney U test, *p<0.05, ** p<0.001. Typical examples of CD45RO cell surface staining on CD4+IL17+ cells is shown in healthy controls and patients with RA, AS and PsA (B).
controls and patients with RA, AS and PsA (B). Gated on CD4+IL17+ cells, CD45RO positivity determined using fluorescence minus one controls.
Figure 6.4 Comparison between healthy controls and disease groups in the expression of CCR6 on CD4+IL17+ cells in peripheral blood

Comparison between healthy controls and patients with RA, AS and PsA in the expression of CCR6 on CD4+IL17+ cells in peripheral blood (A). Bars show mean±SEM, comparison
between patient groups made using Mann Whitney U test, *p<0.05, ** p<0.001. Typical examples of CCR6 cell surface staining on CD4+IL17+ cells is shown in healthy controls and patients with RA, AS and PsA (B). Gated on CD4+IL17+cells, CCR6 positivity determined using fluorescence minus one controls.
negligible (Figure 6.5A and B). Analysis to determine the frequency of CD4+ or CD8+ cells coexpressing IL17 and IFNγ showed that double producers of IL17 and IFNγ (CD4+IL17+IFNγ+ cells or CD8+IL17+IFNγ+ cells) were infrequent (Figure 6.5 A and B). The vast majority of CD4+IL17+ cells expressed CD45RO showing that Th17 cells reside within the memory T cell pool and also a very high proportion of CD4+IL17+ cells expressed CCR6 on their surface (Figure 6.5C and D).

6.2.2 Changes in the frequency of circulating CD4+IL17+ and CD4+IFNγ+ cells in RA patients during anti-TNF treatment

There was a significant increase in the frequency of Th17 cells at 12 weeks after anti-TNF initiation in the RA group as a whole (Figure 6.6A). When the RA group was divided according to type of anti-TNF treatment, the trend towards an increase in Th17 cells at 12 weeks after anti-TNF initiation was similar both with etanercept and adalimumab (Figure 6.6 B and C).

Dividing the RA cohort according to response to anti-TNF treatment also revealed that both responders and non-responders to treatment showed an increase in Th17 cells during anti-TNF treatment (Figure 6.7A and B). There were no significant differences between responder and non-responder patients in the frequency of Th17 cells at 4 or 12 weeks on treatment (Figure 6.7C). However, non-responders showed a trend towards having a higher percentage of Th17 cells at baseline than non-responders which is discussed in more detail below.

Analysis of the change in the expression of memory marker CD45RO (Figure 6.8A and B) or the chemokine receptor CCR6 (Figure 6.8C and D) on the surface of Th17 cells during anti-TNF treatment in the RA group, revealed that there were no significant changes in the expression of either marker in the whole RA group. There were also no significant differences in the expression of either marker between responders and non-responders to treatment.

There was a significant increase in the frequency of CD4+IFNγ+ cells (Th1 cells) 12 weeks after anti-TNF initiation in the RA group as a whole (Figure 6.9A). When the cohort was
Figure 6.5  Phenotype of CD4+IL17+ cells in a representative RA patient

Phenotype of CD4+IL17+ cells is shown in a representative RA patient. Gated on CD4+ cells, (A) shows the presence of CD4+IL17+ cells in peripheral blood, with minimal frequencies of CD4+IL17+IFNγ+ cells and minimal frequencies of CD8+IL17+ cells (B) or CD8+IL17+IFNγ+ cells. Gated on CD4+IL17+ cells, the majority of these cells are shown to express CD45RO demonstrating that they reside within the memory T cell pool (C) and the majority of these cells also express chemokine receptor CCR6 (D).
Figure 6.6 Changes in the frequency of CD4+IL17+ cells in the peripheral blood of RA patients during anti-TNF treatment

Changes in the frequency of circulating CD4+IL17+ cells in the peripheral blood of RA patients during anti-TNF treatment in the cohort as a whole (n=25) (A) and in the cohort
divided according to anti-TNF treatment type, etanercept (n=18) (B) and adalimumab (n=7) (C). Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test, *p<0.05. Dot plots showing the changes in the frequency of CD4+IL17+ cells during anti-TNF treatment in a representative RA patient (D).
Figure 6.7 Comparison of the changes in frequency of CD4+IL17+ cells in the peripheral blood with time on anti-TNF treatment between anti-TNF responder and non-responder patients within the RA cohort

Changes in the frequency of CD4+IL17+ cells in peripheral blood during anti-TNF treatment in anti-TNF responder (n=16) (A) and non-responder patients (n=9) (B) within the RA cohort and a comparison between responders and non-responders is shown in C. Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test; comparison between groups made using Mann Whitney U test, *p<0.05.
Figure 6.8  Changes in the surface phenotype of CD4+IL17+ cells in the peripheral blood of RA patients with time on anti-TNF treatment

Changes in the surface phenotype of CD4+IL17+ cells in the peripheral blood of RA patients during anti-TNF treatment. Changes in the expression of CD45RO on CD4+IL17+ cells during anti-TNF treatment in the whole RA cohort (n=25) (A) and a comparison between anti-TNF responder (n=16) and non-responder patients (n=9) (B). Changes in the expression of CCR6 on CD4+IL17+ cells during anti-TNF treatment in the whole RA cohort (C) and a comparison between anti-TNF responder and non-responder patients (D). Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test; comparison between groups made using Mann Whitney U test.
Figure 6.9  Changes in the frequency of CD4+IFNγ+ cells in the peripheral blood of RA patients with time on anti-TNF treatment

Changes in the frequency of CD4+IFNγ+ cells in peripheral blood of RA patients during anti-TNF treatment in the RA cohort (n=25) (A) and when cohort divided according to anti-TNF treatment type, etanercept (n=18) (B) or adalimumab (n=9) (C). Bars show mean±SEM,
comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test, *$p<0.05$. 
divided according to anti-TNF treatment type, both etanercept and adalimumab exhibited the same pattern of increase in Th1 cells 12 weeks after anti-TNF initiation (Figure 6.9B and C).

Responders to anti-TNF treatment demonstrated a significant increase in Th1 cells at 4 and at 12 weeks on treatment, whereas there was no significant change in the frequency of this cell type in the non-responders group (Figure 6.10A and B). As shown in Figure 6.10C, there were no significant differences between RA responders and non-responders in the frequency of Th1 cells at 4 or 12 weeks on treatment. However, there was trend towards RA non-responders to have a higher frequency of Th1 cells at baseline compared to responders, which is discussed in more detail below.

There were no significant changes in the frequency of CD8+IFNγ+ cells during anti-TNF treatment in the RA group as a whole (Figure 6.11A) and there were no significant differences between anti-TNF responders and non-responders in the percentages of CD8+IFNγ+ cells at each time point on treatment (Figure 6.11B).

Non-responders to anti-TNF treatment in the RA cohort exhibited a tendency to having a higher frequency of Th17 cells (responders mean±SD 0.8±0.5% versus non-responders mean±SD 1.0±0.5%, p=0.3) and Th1 cells (responders mean±SD 10.6±8.3% versus non-responders 17.1±11.9%, p=0.1) at baseline compared to responders to treatment, although the differences were not statistically significant (Figure 6.12).

Similar to the findings using the ELISpot assay, RA patients with shorter disease duration (<3 years) were found to have greater percentages of Th17 and Th1 cells at baseline and subsequently at each time point on treatment compared to RA patients with longer disease duration (>3 years) (Figure 6.13A and B). The trend towards an increase in Th17 and Th1 cells with anti-TNF treatment at 12 weeks was seen in both groups, irrespective of disease duration.
Figure 6.10  Comparison of changes in frequency of CD4+IFNγ+ cells in the peripheral blood with time on anti-TNF treatment between anti-TNF responder and non-responder patients within the RA cohort

Changes in the frequency of CD4+IFNγ+ cells in peripheral blood during anti-TNF treatment in anti-TNF responder (n=16) (A) and non-responder patients (n=9) (B) within the RA cohort and as a direct comparison between responders and non-responders (C). Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test; comparison between groups made using Mann Whitney U test, *p<0.05.
Figure 6.11  Changes in the frequency of CD8+IFNγ+ cells in the peripheral blood of RA patients with time on anti-TNF treatment

Changes in the frequency of circulating CD8+IFNγ+ cells in the peripheral blood of RA patients during anti-TNF treatment in the cohort as a whole (n=25) (A) and in anti-TNF responders (n=16) compared to non-responders (n=9) (B). Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test; comparison between groups made using Mann Whitney U test.
Figure 6.12  Comparison between rheumatoid arthritis anti-TNF responder and non-responder patients in percentages of CD4+IL17+ and CD4+IFNγ+ cells in peripheral blood at baseline prior to anti-TNF treatment initiation

Comparison between RA anti-TNF responder (n=16) and non-responder (n=9) patients in percentages of CD4+IL17+ (A) and CD4+IFNγ+ cells (B) in peripheral blood at baseline prior to anti-TNF treatment initiation. Bars show mean±SEM, comparison between groups made using Mann Whitney U test.
Figure 6.13 Differences in baseline frequencies of CD4+IL17+ and CD4+IFNγ+ cells in the peripheral blood of patients with rheumatoid arthritis depending on disease duration

Comparison of the frequency of CD4+IL17+ (A) and CD4+IFNγ+ cells (B) in the peripheral blood and their changes during anti-TNF treatment in the rheumatoid arthritis cohort according to disease duration. Bars show mean±SEM, comparison between groups made using Mann Whitney U test, *p<0.05.
6.2.3 Changes in frequency of circulating CD4+IL17+, CD4+IFNγ+ and CD8+IFNγ+ cells in AS patients during anti-TNF treatment

There was a significant increase in CD4+IL17+ cell frequencies in AS patients 12 weeks after anti-TNF initiation (Figure 6.14A). Responders to anti-TNF treatment showed a trend towards having higher percentages of Th17 cells at baseline and at each time point on treatment compared to non-responders, although these differences were not statistically significant (Figure 6.14B). However, both responders and non-responders showed a trend towards an increase in the frequency of CD4+IL17+ cells with time on anti-TNF.

Similarly to the RA group, there were no significant changes in the expression of CD45RO or CCR6 on the surface of CD4+IL17+ cells during anti-TNF treatment within the AS group (data not shown). In the AS cohort, as in the RA cohort, the vast majority of peripheral blood IL17+ T helper cells resided within the CD4+ cell population, with a minimal frequency of CD8+IL17+ cells and minimal frequency of cells expressing both IL17 and IFNγ.

There was an increase in the frequency of CD4+IFNγ+ cells during anti-TNF treatment in the AS group (Figure 6.14C) and this was observed both in responders and non-responders to treatment (Figure 6.14D). In contrast to the RA group, the percentages of CD8+IFNγ+ cells in the AS group increased significantly at 4 and 12 weeks after anti-TNF initiation (Figure 6.14E). This pattern occurred both in responders and non-responders to treatment, with no significant differences in the frequency of CD8+IFNγ+ cells between responder and non-responder groups at any of the time points on treatment (Figure 6.14F).

Responders to treatment showed a trend towards having a higher percentage of CD4+IL17+ cells at baseline (mean±SD 1.2±0.5%) compared to non-responders (mean±SD 0.8±0.6%) (Figure 6.15A). However there were no marked differences in the frequency of CD4+IFNγ+ cells between responders (mean±SD 12.2±7.1%) and non-responders (mean±SD 13.8±6.7%) (Figure 6.15B).

To determine whether differences were apparent in the baseline frequency of CD4+IL17+ and CD4+IFNγ+ cells depending on AS disease duration, the frequencies of these cell types were compared between AS patients with disease duration <5 years and those with disease
Figure 6.14  Changes in frequencies of CD4+IL17+, CD4+IFNγ+ cells and CD8+IFNγ+ cells in the peripheral blood of patients with ankylosing spondylitis over 12 weeks on treatment with anti-TNF
Changes in frequencies of CD4+IL17+ cells (A) in whole AS cohort (n=15) and comparison between responders (n=11) and non-responders (n=4) (B) in the change in frequency with anti-TNF treatment. Changes in frequencies of CD4+IFNγ+ (C) in whole AS cohort and comparison between responders and non-responders (D) in the change in frequency with anti-TNF treatment. Changes in frequencies of CD8+IFNγ+cells (E) in whole AS cohort and comparison between responders and non-responders (F) in the change in frequency with anti-TNF treatment. Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test; comparison between groups made using Mann Whitney U test, *p<0.05.
Figure 6.15  Comparison between ankylosing spondylitis anti-TNF responder and non-responder patients in the frequency of CD4+IL17+ and CD4+IFNγ+ cells in peripheral blood at baseline

Comparison between AS anti-TNF responder (n=11) and non-responder (n=4) patients in percentages of CD4+IL17+ (A) and CD4+IFNγ+ cells (B) in peripheral blood at baseline prior to anti-TNF treatment initiation. Bars show mean±SEM, comparison between groups made using Mann Whitney U test.
duration >5 years (Figure 6.16). Both patients with shorter and those with longer disease duration showed a trend towards an increase in the frequency of these two cell types with anti-TNF treatment. Patients with shorter disease duration tended to have higher percentages of CD4+IL17+ and CD4+IFNγ+ cells at baseline compared to patients with longer disease duration (Figure 6.16A and B).

6.2.4 Changes in frequency of circulating CD4+IL17+, CD4+IFNγ+ and CD8+IFNγ+ cells in PsA patients during anti-TNF treatment

In the PsA group as a whole, there was an increase in the frequency of CD4+IL17+ and CD4+IFNγ+ cells with anti-TNF treatment, with the increase at 4 weeks compared to baseline reaching statistical significance (Figure 6.17A and B). Similarly to the AS group, there was also a significant increase in the percentages of CD8+IFNγ+ cells at 12 weeks after anti-TNF initiation compared to baseline (Figure 6.17C).

Similarly to the AS and RA cohorts, in patients with PsA, the vast majority of IL17+ T cells in peripheral blood were CD4+ cells, with a minimal frequency of CD8+IL17+ cells and minimal frequency of cells expressing both IL17 and IFNγ. There were also no significant changes in the expression of CD45RO or CCR6 on the surface of CD4+IL17+ cells during anti-TNF treatment within the PsA group (data not shown).

6.2.5 Changes in the frequency of circulating CD4+IL17+ and CD4+IFNγ+ cells during anti-TNF treatment in all study patients

Examining the time course of the change in frequency of CD4+IL17+ and CD4+IFNγ+ cells in all study patients revealed significant increases in each cell type with anti-TNF treatment at 4 weeks and 12 weeks compared to baseline (Figure 6.18), with the increase in CD4+IL17+ cells at 12 weeks being most marked (p<0.0001).
Figure 6.16  Differences in baseline frequencies of CD4+IL17+ and CD4+IFNγ+ cells in the peripheral blood of patients with ankylosing spondylitis depending on disease duration

Comparison of the frequency of CD4+IL17+ (A) and CD4+IFNγ+ cells (B) in the peripheral blood and their changes during anti-TNF treatment in ankylosing spondylitis cohort according to disease duration. Bars show mean±SEM, comparison between groups made using Mann Whitney U test.
Figure 6.17 Changes in frequency of CD4+IL17+, CD4+IFNγ+ and CD8+IFNγ+ cells in the peripheral blood of patients with psoriatic arthritis over 12 weeks on treatment with anti-TNF

Changes in the frequency of CD4+IL17+ (A), CD4+IFNγ+ (B) and CD8+IFNγ+ (C) cells in the peripheral blood of patients with psoriatic arthritis (n=8) during 12 weeks of treatment with anti-TNF. Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test, *p<0.05.
Figure 6.18  Changes in the frequency of CD4+IL17+ and CD4+IFN\(\gamma\)+ cells in all study patients treated with anti-TNF

Changes in the frequency of CD4+IL17+ (A), CD4+IFN\(\gamma\)+ (B) cells in the peripheral blood of all study patients during 12 weeks of treatment with anti-TNF. Bars show mean±SEM, comparison of time points on treatment versus baseline made using Wilcoxon matched pairs test, *p<0.05, **p<0.001, ***p<0.0005, ****p<0.0001.
6.3 Discussion

6.3.1 Increased frequency of peripheral blood Th17 and Th1 cells in patients with inflammatory arthritis

Comparison of the frequency of peripheral blood Th17 cells between healthy controls and patients with inflammatory arthritis revealed that patients with RA, AS and PsA all showed a trend towards increased percentages of Th17 cells, with AS patients having a significantly greater percentage than healthy controls. These findings are in agreement with the results obtained using the IL17 ELISpot assay (Chapter 5), which also revealed that all three disease groups showed a trend towards increased frequency of IL17-producing peripheral blood mononuclear cells. Furthermore, using the IL17 ELISpot assay in an experiment where PBMCs were depleted of CD4+ T cells, we showed that depletion of CD4+ cells almost completely abolished IL17 responses, showing that peripheral blood CD4+ cells are the main producers of IL17. Thus, the results using flow cytometry to assess the frequency of CD4+ cells with positive intracellular staining for IL17, together with the ELISpot technique which is a functional cell-based assay measuring secreted cytokine, convincingly demonstrate that patients with inflammatory arthritis have increased frequencies of circulating Th17 cells.

A number of other studies have also demonstrated increased frequencies of Th17 cells in the peripheral blood of patients with AS, RA or PsA by flow cytometry, or increased IL17 production by ELISA of cell culture supernatants from these disease groups compared to healthy controls (Chen et al., 2011; Gullick et al., 2010; Jandus et al., 2008; Leipe et al., 2010; Shen et al., 2009; Xueyi et al., 2012). While it seems there is agreement in the literature that patients with seronegative spondyloarthritis (AS or PsA) have increased numbers of circulating Th17 cells in the peripheral blood, there are a few studies that have failed to show an increased frequency of Th17 cells or increased IL17 production by simulated PBMCs from RA patients compared to healthy controls (Aerts et al., 2010; Church et al., 2010; Jandus et al., 2008; Yamada et al., 2008). These differences may reflect the heterogeneity of the RA disease process, as all the studies have included patients with varying disease duration, treatment regimes and disease activity. In our study, all the patients had very active RA (with DAS28 scores at least >5.1), as these patients are due to start anti-TNF treatment, whereas disease activity is highly variable in all the other studies. In our study, we have also assessed the frequency of Th17 cells prior to anti-TNF initiation, whereas other studies have included patients on various treatment combinations, also including patients on various
biologic therapies. While no longitudinal studies have been carried out to date comparing the effects of various treatments on the frequencies of Th17 cells in RA, Yamada et al reported that patients taking methotrexate had a higher frequency of Th17 cells, although they could not attribute this directly to treatment (Yamada et al., 2008). Two of the studies which failed to detect a significant difference in the frequency of peripheral blood Th17 cells in RA patients compared to controls had only 10 RA patients in each group. There is marked variability in the frequency of Th17 cells between patients, which in addition to the small patient numbers in these studies may explain the failure to detect a significant difference (Aerts et al., 2010; Jandus et al., 2008). Despite these discrepancies in the frequency of circulating Th17 cells in RA patients compared to healthy controls, studies which have compared the frequencies of Th17 cells between synovial fluid, synovial tissue and peripheral blood in RA patients agree that Th17 cells are enriched in the synovial fluid or tissue compared to peripheral blood (Church et al., 2010; Gullick et al., 2010; Shahrara et al., 2008), thus supporting a role for Th17 cells in RA disease pathogenesis.

Using intracellular cytokine staining we found that there was also a trend towards RA and AS patients to have higher percentages of peripheral blood Th1 cells compared to healthy controls. This trend was not obvious in patients with PsA, however this may have been due to the small number of patients in this cohort and due to the large variability in Th1 cell frequencies between patients. There was no significant difference in the frequency of peripheral blood CD8+IFN$\gamma$+ cells between disease groups and healthy controls. An increased frequency of IFN$\gamma$-producing PBMC in RA and AS patients compared to healthy controls was also observed using the IFN$\gamma$ ELISpot assay in our study. Other studies investigating differences in IFN$\gamma$-producing peripheral blood mononuclear cells between RA and AS patients and healthy controls have reported conflicting results. A few studies have reported reduced IFN$\gamma$ production from PBMCs and reduced frequencies of CD4+IFN$\gamma$+ cells by flow cytometry in RA and AS patients (Baeten et al., 2001; Nissinen et al., 2004; Schotte et al., 2004). Other studies have failed to detect significant differences between RA patients and healthy controls in the frequency of peripheral blood Th1 cells (Berg et al., 2001; Gullick et al., 2010; Leipe et al., 2010; Shen et al., 2009; Yamada et al., 2008). One study using IFN$\gamma$ ELISpot reported increased IFN$\gamma$ production by stimulated PBMCs from RA patients compared to healthy controls (Ronnelid J, 1998). The different results obtained by these studies could also arise from the heterogeneous patient populations in each one, with
patients of varying disease duration, disease activity and treatment regimens. In support of this, two studies have found that patients with differing RA inflammatory activity had differing amounts of IFNγ production by stimulated PBMCs. Patients with new onset synovitis and very active disease were found to have increased frequency of IFNγ-secreting peripheral blood mononuclear cells compared to patients with chronic RA (Kanik et al., 1998; Seitz et al., 1987). Another possible explanation for the differing results obtained in the frequency of Th1 cells in RA and AS patients compared to healthy controls, is that chronic exposure to high TNFα concentrations has been shown to deactivate T cells directly in vitro and to reduce cytokine production (Cope et al., 1994). This could account for the downregulation of T-cell-derived IFNγ. However, the recent change in the treatment paradigm of inflammatory arthritis aims for early initiation of disease modifying agents to aggressively suppress disease activity. Thus, these drug treatments may influence cytokine balance and by reducing inflammation may explain why more recent studies investigating the frequency of peripheral blood IFNγ-producing cells have either observed no significant differences or increased frequencies of Th1 cells in patients with RA and AS.

6.3.2 Phenotype of peripheral blood Th17 cells in patients with inflammatory arthritis and changes during anti-TNF treatment

The vast majority of peripheral blood IL17-positive T cells were found to reside within the CD4+ T cell population, with negligent amounts of CD8+IL17+ cells detected in patients with RA, AS and PsA, which is in agreement with other studies (Jandus et al., 2008; Leipe et al., 2010; Shen et al., 2009). IL17 producing CD4+ T cells have been reported to be capable of coexpressing the archetypical Th1 cytokine IFNγ (Acosta-Rodriguez et al., 2007b). However, we found that there was also a very low frequency of CD4+ or CD8+ cells producing IL17 and IFNγ simultaneously in the peripheral blood of patients in all three disease groups. While other studies have also reported a low frequency of circulating CD4+ cells coexpressing IL17 and IFNγ in some RA and AS patients, these coexpressing cells have been shown to be further enriched in synovial fluid (Gullick et al., 2010; Leipe et al., 2010; Shen et al., 2009).

Th17 were also found to have a high expression of CD45RO, showing that these cells reside within the memory T cell pool and to have a high expression of CCR6 on their surface, as shown by other investigators (Jandus et al., 2008; Leipe et al., 2010; Shen et al., 2009).
However, we also found that in comparison with healthy controls, patients with RA, AS and PsA had a significantly higher expression of CD45RO and CCR6 on CD4+IL17+ cells. The increased expression of CD45RO and CCR6 on Th17 cells in patients compared to healthy controls has been reported by a few other studies (Homey et al., 2000; Jandus et al., 2008; Leipe et al., 2010; Matsui et al., 2001) and suggests that Th17 cells in disease groups are highly differentiated. The increased expression of the chemokine receptor CCR6 may facilitate the homing of these cells to inflamed tissues (Hirota et al., 2007; Matsui et al., 2001). It has been reported that RA patients in disease remission treated with anti-TNF have significantly lower CCR6 expression than anti-TNF treated patients who still had active disease, although the change in CCR6 expression was not studied longitudinally (Aerts et al., 2010). One of the mechanisms of action of anti-TNF agents is a reduction in leucocyte trafficking to inflamed joints through decreased expression of synovial adhesion molecules and chemokine receptors (Tak et al., 1996; Taylor et al., 2000). It may therefore be hypothesised that anti-TNF treatment may induce a reduction in CCR6 expression to reduce the influx of Th17 cells into joints. Therefore, in our study, we investigated if anti-TNF treatment induced a change in Th17 cell phenotype during the 12 weeks on treatment. We found that CD4+ T cells remained the main producers of IL17 in peripheral blood and that the frequency of CD8+IL17+ cells did not alter, nor did the frequency of CD4+ or CD8+ coexpressing IL17 and IFNγ. Although at baseline patients had a higher expression of CD45RO and CCR6 on Th17 cells compared to healthy controls, the expression of these two surface markers did not alter during 12 weeks on treatment. There was also no difference between anti-TNF responders and non-responders in the change in the expression of CD45RO or CCR6 with time on treatment. One study investigated the distribution of T cells among subsets of central memory CCR7+CD45RA- and effector memory CCR7-CD45RA- and CCR7-CD45RA+ cells before and 1 month after anti-TNF treatment and found no major changes in the percentages of circulating memory T cell subsets in patients with psoriasis or inflammatory bowel disease during treatment (Bosè et al., 2011).

We found no significant changes in the phenotype of Th17 cells during anti-TNF treatment in this study. However, further studies investigating the phenotype of Th17 cells over a longer time on treatment would be needed to exclude the possibility that changes in phenotypic expression may occur over a longer period on treatment or to confirm that there is no change also at later time points.
6.3.3 Increase in peripheral blood Th17 cells during anti-TNF treatment

Using intra-cellular cytokine staining we found a significant increase in Th17 cells 12 weeks after anti-TNF initiation in patients with RA, AS and PsA. These findings support our results obtained using the IL17 ELISpot assay, which also showed that there was a significant increase in the frequency of IL17-producing peripheral blood mononuclear cells 12 weeks after anti-TNF initiation in patients with RA and AS and patients with PsA showed a trend towards an increase at 4 and 12 weeks on treatment.

The increase in Th17 cells with anti-TNF treatment shown using flow cytometry and ELISpot was observed in patients treated with adalimumab and those treated with etanercept and the pattern of change in Th17 cells with treatment was the same in the three disease groups, suggesting that this increase in Th17 cells may be a class effect of anti-TNF agents, independent of disease type or anti-TNF agent type. A number of other studies have also reported an increase in IL17-producing cells with anti-TNF treatment, although the studies differ in the type of inflammatory arthritis studied, methods for assessing changes in IL17-producing cells and time points at which the changes were assessed after treatment initiation (Aerts et al., 2010; Alzabin et al., 2012; Bosè et al., 2011). Only one of these studies has investigated the change in frequency of Th17 cells during anti-TNF treatment longitudinally by comparing changes at 4 and then 8-12 weeks after treatment initiation to baseline in RA patients (Alzabin et al., 2012). None of these other studies have investigated the changes in Th17 cells with anti-TNF treatment in three different types of inflammatory arthritis at the same time points.

In a study of 18 patients with RA, carried out by our group, the percentage of CD4+IL17+ cells in peripheral blood was shown to increase significantly at 4 and then at 8-12 weeks on anti-TNF treatment (Alzabin et al., 2012). This work showed that there was a trend towards increased IL17 production in cell supernatants from stimulated PBMCs at 4 and 8-12 weeks after anti-TNF treatment was initiated compared to baseline. There was also a trend towards a progressive increase in the levels of mRNA encoding the Th17-specific transcription factor RORγT in PBMCs at 4 and 8-12 weeks after anti-TNF initiation (Alzabin et al., 2012).

In a cross-sectional study, an increased frequency of circulating Th17 cells was found in RA patients treated with anti-TNF agents compared to healthy controls or RA patients not on anti-TNF therapy and this increase was found to occur irrespective of RA disease activity (Aerts et al., 2010). This study also found that IL17 production by cultured PBMCs increased
significantly 12 weeks after anti-TNF initiation compared to baseline in a group of 8 RA patients (Aerts et al., 2010).

A significant increase in IL17 production by stimulated PBMCs from patients with psoriasis and PsA has been reported 1 month after anti-TNF was commenced (Bosè et al., 2011).

We found that the time course of increase in Th17 cells with anti-TNF treatment did not differ between anti-TNF responders and non-responders in the RA and AS groups, suggesting that this increase may occur independently of treatment response. This is in agreement with the results of Aerts et al who in a cross-sectional study found that RA patients on anti-TNF therapy had increased circulating Th17 cells irrespective of whether they were in disease remission or still had active disease in comparison with RA patients not on biological therapy (Aerts et al., 2010). However, another study showed that after 6 months of anti-TNF treatment in RA patients, mean circulating frequencies of Th17 cells and serum IL17 levels decreased in responders but increased in non-responders (Chen et al., 2011). The conclusions of this study may differ from ours, as they assessed changes in Th17 cells with treatment as well as treatment response at 6 months compared to baseline, whereas we made these assessments at 3 months. No other studies to date have investigated whether this change in the frequency of Th17 cells with anti-TNF treatment could be linked to treatment response or non-response in RA patients.

We observed a trend towards non-responders to anti-TNF therapy in the RA cohort to have a higher frequency of IL17-producing cells at baseline compared to responders and this was determined by both intra-cellular cytokine staining and IL17 ELISpot. This raises the possibility that the numbers of circulating Th17 cells at baseline could predict poor response to anti-TNF therapy in RA patients. This hypothesis is explored in our study further by relating the frequencies of baseline IL17-producing peripheral blood mononuclear cells to DAS28 response and improvement in ultrasound parameters of disease activity as discussed in Chapter 8. Baseline IL17 levels in cell supernatants from cultured PBMCs of RA patients have been reported to be a significant predictor of therapeutic response using logistic regression analysis (Chen et al., 2011). IL17 targeting biological agents are now in development and early data in RA patients show promising results (Genovese et al., 2012). Confirmation of these findings is needed in large patient cohorts, as well as the investigation of whether these patients with higher baseline IL17 levels may have more Th17 driven
disease and whether they may benefit from anti-IL17 biologic therapy rather than anti-TNF therapy.

Although in the AS cohort, there was no significant difference between anti-TNF responders and non-responders in the pattern of Th17 cell increase with anti-TNF treatment, AS responders showed a trend to having higher frequencies of Th17 cells at baseline compared to non-responders. This finding is consistent with our results using the IL17 ELISpot assay as discussed in Chapter 5. This observation is in contrast to the findings in RA patients where there was a trend towards higher baseline frequency of Th17 cells in non-responders to anti-TNF. This observation also differs from the findings of Xueyi et al who reported significantly higher baseline levels of IL6, IL17 and IL23 in anti-TNF non-responder AS patients than in responders (Xueyi et al., 2012). However, due to the small number of patients in the AS group, firm conclusions cannot be made and investigation of this using larger cohorts of patients would be needed.

### 6.3.4 Increase in peripheral blood Th1 cells during anti-TNF treatment

Using intracellular cytokine staining we found a significant increase in the frequency of peripheral blood Th1 cells 12 weeks after anti-TNF initiation in RA patients. In patients with AS and PsA, there was a significant increase in Th1 cells at week 4 with a trend towards a further increase at week 12 on treatment. These findings are in agreement with our results obtained using the IFNγ ELISpot assay, which also showed an increase in IFNγ-producing peripheral blood mononuclear cells during anti-TNF treatment. Similar to the increases seen in Th17 cells with anti-TNF treatment, the same pattern of increase in Th1 cells occurred in patients treated with adalimumab and etanercept and also in all three disease groups, suggesting that the increase in circulating Th1 cells may be a class effect of anti-TNF, rather than dependent on the type of anti-TNF agent or disease process.

In recent years, the studies evaluating the changes in the frequency of circulating T helper cells with anti-TNF treatment in inflammatory arthritis using flow cytometry have focussed on changes in either Th17 or Th1 cells. Only one other study, apart from ours has investigated simultaneously the changes in IL17 and IFNγ production by stimulated PBMCs from RA patients at baseline and then at 3 months after anti-TNF initiation (Aerts et al., 2010). In this small study of 8 RA patients, in addition to the significant increase in IL17, a
significant increase in IFN\(\gamma\) production was also reported 3 months after anti-TNF initiation. Two further studies also support our findings of an increase in the frequency of peripheral blood CD4+IFN\(\gamma\)+ cells with anti-TNF treatment in RA patients (Maurice et al., 1999; Nissinen et al., 2004). Maurice et al found a significant increase in the number of circulating CD4+IFN\(\gamma\)+ cells 3 days after infliximab infusion in RA patients compared to pre-treatment levels (Maurice et al., 1999). Nissinen et al found a significant increase in IFN\(\gamma\) secretion by stimulated PBMCs from RA patients 2 weeks and 6 weeks after infliximab infusion (Nissinen et al., 2004).

In patients with AS, 12 weeks of etanercept treatment was found to induce a significant increase in the number of CD4+IFN\(\gamma\)+ cells compared to pre-treatment levels using flow cytometry (Zou et al., 2003). Another study showed that treatment with infliximab induced a significant and persistent increase in the percentage of CD4+IFN\(\gamma\)+ cells in the peripheral blood of patients with spondyloarthritis as early as day 7 after infliximab infusion and this increase was maintained at day 84 after treatment initiation (Baeten et al., 2001). In patients with PsA, a significant increase in IFN\(\gamma\) production by stimulated PBMCs has been reported 1 month after anti-TNF was commenced (Bosè et al., 2011).

### 6.3.5 Increase in peripheral blood CD8+IFN\(\gamma\)+ cells during anti-TNF treatment in patients with AS and PsA

We found no significant changes in the frequency of circulating CD8+IFN\(\gamma\)+ cells during anti-TNF treatment in patients with RA. This finding is in agreement with the results of another study in RA which also failed to detect a significant increase in the frequency of CD8+IFN\(\gamma\)+ cells with infliximab treatment (Maurice et al., 1999). In contrast, there was a significant increase in the frequency of peripheral blood CD8+IFN\(\gamma\)+ cells at 4 and 8 weeks on treatment in AS patients and at 12 weeks in PsA patients after anti-TNF initiation. Two other studies in AS patients have also reported a significant increase in CD8+IFN\(\gamma\)+ cells with anti-TNF treatment in AS patients, thus supporting our results (Baeten et al., 2001; Zou et al., 2003).

Whereas anti-TNF therapy appears to increase the frequency of peripheral blood Th1 and Th17 cells similarly in patients with RA, AS and PsA, it appears to have differing effects on
CD8+IFNγ+ cells between RA patients and patients with seronegative spondyloarthritis. This may point to the differences in the pathogenesis of RA and spondyloarthropathy as discussed in Chapter 1. Whereas RA is characterised by distinct features of autoimmunity with involvement of T and B cell autoreactivity, AS has been postulated to possess features more suggestive of autoinflammation due to the presence of abnormal innate immune responses in its pathogenesis (Ambarus C, 2012; Lories and Baeten, 2009). CD8+ T cells recognise pathogen peptides presented by MHC Class I complexes on antigen presenting cells. Since a Class I MHC association with AS is the dominant genetic susceptibility element, CD8+IFNγ+ cells restricted by HLA-B27 may be involved in AS disease pathogenesis alongside Th1 and Th17 cells (Inman and El-Gabalawy, 2009).

6.3.6 Effect of RA disease duration on frequency of circulating Th17 and Th1 cells at baseline

Using flow cytometry we have shown that RA patients with shorter disease duration (less than 3 years) showed a trend towards a higher frequency of Th17 cells at baseline compared to patients with longer disease duration (longer than 3 years). Also RA patients with shorter disease duration had significantly greater Th1 cells at baseline compared to patients with longer disease duration. These findings are in agreement with our results obtained using the IL17 and IFNγ ELISpot assays and the possible significance of these findings have been discussed in Chapter 5.

6.4 Conclusion

The data presented in this chapter show the findings obtained using flow cytometry to characterise the frequency and phenotype of peripheral blood Th17 and Th1 cells in patients with RA, AS and PsA compared to healthy controls, as well as longitudinal changes in the frequency of these cell types during anti-TNF therapy. Patients with inflammatory arthritis showed a trend towards having higher frequencies of circulating Th17 cells compared to healthy controls. CD4+ T cells were shown to be the main producers of IL17 in peripheral blood and Th17 cells in the disease groups had a significantly higher expression of CD45RO, a marker of memory cells, as well as a higher expression of CCR6, a chemokine receptor with a role in the homing of Th17 cells to inflamed tissues. Anti-TNF treatment induced a
significant increase in the frequency of Th17 and Th1 cells in patients with RA, AS and PsA and this increase was evident in anti-TNF responders and non-responders. The time course of increase in the frequency of Th1 and Th17 cells was the same with etanercept and adalimumab and in all three disease phenotypes suggesting this may be a class effect of anti-TNF agents. Anti-TNF treatment also induced a significant increase in the frequency of CD8+IFNγ+ cells in patients with AS and PsA, but not in patients with RA, supporting the differing contribution of this cell type to the pathogenesis of RA and spondyloarthritis.

Confirmation of these findings is needed in larger patient cohorts followed up over a longer period of time in order to identify whether the changes in the frequency of Th1 and Th17 cells seen are a transient and relatively short term phenomenon of anti-TNF therapy, or whether they persist. The effects of anti-TNF treatment on Th1 and Th17 cells were investigated in the peripheral blood of patients in this study which does not always accurately reflect events at the site of disease activity. Investigations of the changes in the frequency of Th1 and Th17 cells, as well as their related chemokine receptors with anti-TNF treatment in synovial tissue from RA patients and skin biopsies from PsA patients would provide a more complete picture. Studying changes in the expression of Th17 and Th1 cell cytokine receptors, as well as cell activation markers with treatment would also provide additional information on the phenotype and functionality of these cells.

The results presented in this chapter are in agreement with the results obtained using the IL17 and IFNγ ELISpot assays thus adding strength to our findings. With the use of two different but complementary techniques, intracellular cytokine staining and ELISpot, we show convincingly that anti-TNF treatment induces an increase in Th1 and Th17 cells and that this effect occurs in patients with RA, AS and PsA and appears to be a class effect of anti-TNF agents. The results obtained using these two techniques provide a basis to further investigate relationships between changes in immunological parameters with anti-TNF treatment and response to treatment evaluated using clinical measures and power Doppler ultrasonography, in order to evaluate the immune correlates of treatment response or failure.
Chapter 7. Changes in serum cytokines and chemokines during anti-TNF treatment in patients with inflammatory arthritis

7.1 Introduction

The work presented in this chapter investigated changes in a panel of serum cytokines and chemokines longitudinally during anti-TNF therapy in patients with RA, AS and PsA. Using the ELISpot and intracellular cytokine staining assays (Chapters 5 and 6, respectively), it was shown that anti-TNF therapy led to an increase in circulating Th1 and Th17 cells in patients with RA, AS and PsA. Thus, the objective of the work in this chapter was to quantitate changes in serum cytokines and chemokines during anti-TNF treatment using multiplex cytokine assays and ELISA in order to further characterise the effects of anti-TNF therapy on the Th1 and Th17 cell pathways, as well as on other pro-inflammatory cytokines, chemokines and TNFα homeostasis. The specific focus was on IL12 and IL23, which are cytokines involved in the differentiation of Th1 and Th17 cells respectively; IL17 and IFNγ as the signature cytokines produced by these cell types, as well as a range of other inflammatory cytokines and chemokines. Based on previous studies, as well as the known mechanisms of action of anti-TNF therapy, the work in this chapter investigated a number of different hypotheses on the effects of anti-TNF therapy on the serum cytokines and chemokines being studied. In view of the observed increase in circulating Th1 and Th17 cells during anti-TNF therapy (Chapters 5 and 6), one of the expected outcomes of the work presented in this chapter was that there would be an associated increase in the serum levels of IL17 and IFNγ during therapy. Work in the collagen-induced arthritis model of RA demonstrated that a possible mechanism underlying the observed expansion of Th1 and Th17 cells during anti-TNF treatment in this animal model was through an increase in the IL12/23p40 subunit shared between IL12 and IL23 (Notley et al., 2008). Thus, another hypothesis of the work in this chapter was that in patients with inflammatory arthritis anti-TNF therapy would lead to an increase in serum levels of IL12, IL23 and their shared subunit, IL12/23p40. Anti-TNF therapy has been shown to reduce trafficking of leukocytes to inflamed joints through reduced expression of chemokines by the synovium (Tak et al., 1996; Taylor et al., 2000), thus it would be expected that this may be reflected in the serum and that
levels of chemokines involved in leukocyte trafficking may decrease during anti-TNF treatment. The effects of anti-TNF therapy on systemic TNFα homeostasis was also determined by investigating changes in serum TNFα and soluble TNF receptors during treatment. Based on previous work investigating TNFα homeostasis in serum samples during anti-TNF therapy in RA patients (Charles et al., 1999), it would be expected that anti-TNF treatment would increase serum levels of TNFα and that levels of TNFRI and TNFRII would be reduced.

In order to be able to simultaneously measure a number of analytes in the serum samples, multiplex cytokine assays (Cytometric Bead Array, BD Biosciences and MesoScale Discovery MultiSpot assay, MSD) were used. IL12, IL23 and IL12/23p40 were measured by sandwich ELISAs (R&D Systems) as these were not available as part of the multiplex assays. Currently, there are two main formats of multiplex cytokine assays available, bead-based (liquid-phase) assays and plate-based (solid-phase) assays. The first method relies on different populations of microbeads with discrete fluorescence characteristics which are distinguished using flow cytometry (Fu, 2008; Varro et al., 2007). In this type of assays, each microbead is pre-coated with capture antibodies specific for the analyte of interest, which are then recognised by specific labelled secondary antibodies. Different bead-based assays exist and these include Luminex-based assays (Luminex®, Flow-Cytomix™ (e-Bioscience) and the BD Cytometric bead array (CBA, BD Biosciences), which was used in this study. The second format of multiplex assays is based on the ELISA principle, in which multiple capture antibodies are scattered at different positions in each well of a microplate (Chowdhury et al., 2009; Fu, 2008). Examples of this type of multiplex assays include fluorescence-based assays (A², Beckman Coulter), chemiliminescence-based assays (Mosaic ELISA by R&D Systems) and an electrochemiluminescence-based assay (MesoScale Discovery, MSD), which was used in this study. Studies on cross-comparisons between different types of multiplex assays investigating differences in their sensitivities or reliabilities are at present limited (Chowdhury et al., 2009; Dabitao et al., 2011).
7.2 Results

7.2.1 Changes in serum cytokines and chemokines during anti-TNF therapy in RA cohort

A panel of serum cytokines comprising IL6, IL10, IL17, IFNγ, TNFα, TNFα Receptor 1 (TNFRI, also known as p55) and TNFα Receptor II (TNFRII, also known as p75) was measured in the RA cohort at baseline and at weeks 1, 4 and 12 on anti-TNF treatment by CBA. In addition, serum IL12p70, IL23 and IL12/23p40 levels were measured at baseline and weeks 1, 4 and 12 on treatment by ELISAs (Tables 7.1 and Table 7.2).

Serum levels of IL10, IL12p70 and IFNγ were below the lower limit of detection in all the study patients, whereas IL17 was detectable in only 4 out of 25 patients and IL23 was detectable in only 7 out of 25 study patients. The cytokines which show a change during anti-TNF treatment are discussed in more detail below.

As part of a collaborative study, the opportunity arose to be able to also use a different type of multiplex cytokine assay to measure serum cytokines and chemokines, the MesoScale Discovery Multi-Spot assay. Thus, another panel of serum cytokines and chemokines comprising IL6, IL8, IL10, IL17, IL12p70, IFNγ, TNFα, thymus-and activation-regulated cytokine (TARC), macrophage inflammatory protein 1β (MIP1β), monocyte chemotactic protein-1 (MCP1), monocyte chemotactic protein-4 (MCP4) and interferon-inducible protein-10 (IP10) was tested at baseline and at weeks 1 and 12 on anti-TNF treatment using the MesoScale Discovery (MSD) Multi-Spot assay system (Tables 7.3 and 7.4). Due to time and funding constraints, the use of the MSD assay was limited to the RA cohort. There were no significant changes in serum IL17 or IL10 levels at weeks 1 or 12 compared to baseline. There was a trend towards an increase in serum IL12p70 levels with anti-TNF treatment in this patient group, but this was not statistically significant. The cytokines and chemokines which show a change during anti-TNF treatment are discussed further below.

Interleukin-6. IL6 is central to the pathogenesis of inflammatory arthritis (Chapter 1). It is produced by monocytes, synovial fibroblasts, B cells and T cells and functions to induce B cell proliferation and antibody production; T cell proliferation, differentiation and
Table 7.1 Summary of changes in serum cytokines during 12 weeks of anti-TNF treatment in the RA cohort

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Baseline Concentration (pg/ml)</th>
<th>1 week Concentration (pg/ml)</th>
<th>4 weeks Concentration (pg/ml)</th>
<th>12 weeks Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
<td>p-value</td>
</tr>
<tr>
<td>IL6</td>
<td>15.85±23.22</td>
<td>9.17±11.16</td>
<td>14.67±28.23</td>
<td>7.99±6.31</td>
</tr>
<tr>
<td>IL10</td>
<td>BLD</td>
<td>BLD</td>
<td>BLD</td>
<td>BLD</td>
</tr>
<tr>
<td>IL17</td>
<td>23.36±6.24</td>
<td>25.28±10.27</td>
<td>23.91±3.53</td>
<td>20.00±0.00</td>
</tr>
<tr>
<td>IL12p70</td>
<td>BLD</td>
<td>BLD</td>
<td>BLD</td>
<td>BLD</td>
</tr>
<tr>
<td>IL23</td>
<td>101.60±32.19</td>
<td>84.26±35.13</td>
<td>88.21±25.05</td>
<td>82.08±30.31</td>
</tr>
<tr>
<td>IL12/IL23p40</td>
<td>54.51±57.39</td>
<td>101.10±61.97</td>
<td>104.90±70.20</td>
<td>107.20±109.60</td>
</tr>
<tr>
<td>IFNγ</td>
<td>BLD</td>
<td>BLD</td>
<td>BLD</td>
<td>BLD</td>
</tr>
<tr>
<td>TNFα</td>
<td>20.80±21.07</td>
<td>54.75±38.27</td>
<td>51.14±35.29</td>
<td>44.53±23.60</td>
</tr>
<tr>
<td>TNFRI</td>
<td>1232.00±586.50</td>
<td>1302.00±583.90</td>
<td>1284.00±609.40</td>
<td>1263±688.20</td>
</tr>
<tr>
<td>TNFRII</td>
<td>4186.00±1997.00</td>
<td>2616.00±1220.50</td>
<td>2950.00±2147.00</td>
<td>3798.00±3234.00</td>
</tr>
</tbody>
</table>

All cytokines (excluding IL12, IL23 and IL12/23p40) measured in serum using cytometric bead array (CBA). IL12, 23, IL12/23p40 levels in serum determined by ELISA. Abbreviations: IL, interleukin; IFNγ, interferon gamma; TNFα, tumour necrosis factor α, TNFRI, tumour necrosis factor α receptor I; TNFRII, tumour necrosis factor α receptor II; BLD, below limit of detection. Time points on treatment compared versus baseline using Wilcoxon matched pairs test; *p<0.05; **p<0.005.
Table 7.2  Summary of changes in serum cytokines during 12 weeks of anti-TNF treatment in anti-TNF responders and non-responders from RA cohort

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Baseline Concentration (pg/ml)</th>
<th>Week 1 Concentration (pg/ml)</th>
<th>p-value</th>
<th>Week 4 Concentration (pg/ml)</th>
<th>p-value</th>
<th>Week 12 Concentration (pg/ml)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>10.46±6.25</td>
<td>7.42±2.98</td>
<td>ns</td>
<td>7.07±2.57</td>
<td>ns</td>
<td>6.97±2.51</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>26.59±36.63</td>
<td>12.00±18.05</td>
<td>ns</td>
<td>26.26±43.95</td>
<td>ns</td>
<td>54.58±126.80</td>
<td>ns</td>
</tr>
<tr>
<td>IL12/23p40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>93.79±56.08</td>
<td>94.47±57.20</td>
<td>ns</td>
<td>97.85±63.31</td>
<td>ns</td>
<td>111.60±135.10</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>95.79±63.10</td>
<td>113.40±73.10</td>
<td>ns</td>
<td>118.10±84.64</td>
<td>ns</td>
<td>100.20±54.37</td>
<td>ns</td>
</tr>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>21.50±2.86</td>
<td>46.49±33.06</td>
<td>ns</td>
<td>60.46±44.52</td>
<td>*</td>
<td>48.32±32.15</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>20.00±0.00</td>
<td>64.38±44.69</td>
<td>*</td>
<td>38.72±11.77</td>
<td>*</td>
<td>40.73±12.54</td>
<td>ns</td>
</tr>
<tr>
<td>TNFRI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>1129.00±631.20</td>
<td>1255.00±615.60</td>
<td>ns</td>
<td>1209.00±717.00</td>
<td>ns</td>
<td>1135.00±771.70</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>1415.00±475.50</td>
<td>1405.00±547.20</td>
<td>ns</td>
<td>1409±374.00</td>
<td>ns</td>
<td>1470.00±503.10</td>
<td>ns</td>
</tr>
<tr>
<td>TNFRII</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responders</td>
<td>3921.00±2137.00</td>
<td>2767.00±139.00</td>
<td>ns</td>
<td>3131.00±2508.00</td>
<td>ns</td>
<td>3816.00±3573.00</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>4656.00±1736.00</td>
<td>2291.00±933.40</td>
<td>*</td>
<td>2649.00±1443.00</td>
<td>*</td>
<td>3770.00±2828.00</td>
<td>ns</td>
</tr>
</tbody>
</table>

All cytokines (excluding IL12, IL23 and IL12/23p40) measured in serum using cytometric bead array (CBA). IL12, 23, IL12/23p40 levels in serum determined by ELISA. Abbreviations: IL, interleukin; IFNγ, interferon gamma; TNFα, tumour necrosis factor a, TNFRI, tumour necrosis factor α receptor I; TNFRII, tumour necrosis factor α receptor II. Time points on treatment compared versus baseline using Wilcoxon matched pairs test; *p<0.05
Table 7.3 Summary of changes in serum cytokines measured by MesoScale Discovery Multi-Spot assay (MSD) during 12 weeks of anti-TNF treatment in the RA cohort

<table>
<thead>
<tr>
<th>Cytokine/ chemokine</th>
<th>Baseline Concentration (pg/ml)</th>
<th>1 week Concentration (pg/ml)</th>
<th>p-value</th>
<th>12 weeks Concentration (pg/ml)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>8.95±12.40</td>
<td>3.50±6.15</td>
<td>**</td>
<td>2.70±3.21</td>
<td>***</td>
</tr>
<tr>
<td>IL8</td>
<td>13.06±5.88</td>
<td>9.21±2.36</td>
<td>**</td>
<td>10.58±5.51</td>
<td>*</td>
</tr>
<tr>
<td>IL10</td>
<td>11.04±26.28</td>
<td>11.61±23.11</td>
<td>ns</td>
<td>13.86±23.21</td>
<td>ns</td>
</tr>
<tr>
<td>IL17</td>
<td>0.42±0.25</td>
<td>0.36±0.17</td>
<td>ns</td>
<td>0.39±0.21</td>
<td>ns</td>
</tr>
<tr>
<td>IL12p70</td>
<td>5.01±10.86</td>
<td>6.40±13.90</td>
<td>ns</td>
<td>10.55±18.96</td>
<td>ns</td>
</tr>
<tr>
<td>IFNγ</td>
<td>1.62±1.32</td>
<td>1.84±1.83</td>
<td>ns</td>
<td>2.17±1.59</td>
<td>ns</td>
</tr>
<tr>
<td>TNFα</td>
<td>7.99±2.42</td>
<td>56.00±41.76</td>
<td>**</td>
<td>52.90±34.82</td>
<td>**</td>
</tr>
<tr>
<td>TARC</td>
<td>551.80±411.90</td>
<td>408.60±337.20</td>
<td>*</td>
<td>483.80±317.60</td>
<td>*</td>
</tr>
<tr>
<td>MIP1β</td>
<td>161.30±50.87</td>
<td>123.50±47.82</td>
<td>**</td>
<td>139.8±58.88</td>
<td>ns</td>
</tr>
<tr>
<td>MCP4</td>
<td>315.10±109.80</td>
<td>373.50±143.50</td>
<td>ns</td>
<td>372.70±151.10</td>
<td>ns</td>
</tr>
<tr>
<td>MCP1</td>
<td>512.90±157.30</td>
<td>479.20±164.50</td>
<td>ns</td>
<td>479.50±188.90</td>
<td>ns</td>
</tr>
<tr>
<td>IP10</td>
<td>427.70±292.30</td>
<td>271.70±168.30</td>
<td>*</td>
<td>341.30±293.60</td>
<td>ns</td>
</tr>
</tbody>
</table>

Abbreviations: IL, interleukin; IFNγ, interferon gamma; TNFα, tumour necrosis factor α, TARC, Thymus-And Activation-Regulated Cytokine; MIP1β, Macrophage Inflammatory Protein 1β; MCP1, Monocyte Chemotactic Protein-1; MCP4, Monocyte Chemotactic Protein-4; IP10, Interferon-Inducible Protein-10. Time points on treatment compared versus baseline using Wilcoxon matched pairs test; *p<0.05, **P<0.005
Table 7.4 Summary of changes in serum cytokines measured by MesoScale Discovery Multi-Spot assay (MSD) during 12 weeks of anti-TNF treatment in anti-TNF responders and non-responders from RA cohort

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Baseline Concentration (pg/ml)</th>
<th>Week 1 Concentration (pg/ml)</th>
<th>p-value</th>
<th>Week 12 Concentration (pg/ml)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6 Responders</td>
<td>5.48±4.39</td>
<td>1.51±0.71</td>
<td>**</td>
<td>1.56±1.09</td>
<td>**</td>
</tr>
<tr>
<td>IL6 Non-Responders</td>
<td>13.57±17.78</td>
<td>6.35±9.17</td>
<td>ns</td>
<td>4.54±4.59</td>
<td>*</td>
</tr>
<tr>
<td>IL8 Responders</td>
<td>13.89±7.38</td>
<td>8.93±2.44</td>
<td>**</td>
<td>10.49±5.79</td>
<td>**</td>
</tr>
<tr>
<td>IL8 Non-Responders</td>
<td>11.95±3.01</td>
<td>9.61±2.38</td>
<td>ns</td>
<td>10.71±5.43</td>
<td>ns</td>
</tr>
<tr>
<td>IL10 Responders</td>
<td>6.43±8.72</td>
<td>16.34±28.68</td>
<td>ns</td>
<td>12.31±14.01</td>
<td>ns</td>
</tr>
<tr>
<td>IL10 Non-Responders</td>
<td>18.53±41.70</td>
<td>3.73±1.26</td>
<td>ns</td>
<td>16.18±33.82</td>
<td>ns</td>
</tr>
<tr>
<td>IL17 Responders</td>
<td>0.38±0.21</td>
<td>0.37±0.19</td>
<td>ns</td>
<td>0.38±0.24</td>
<td>ns</td>
</tr>
<tr>
<td>IL17 Non-Responders</td>
<td>0.46±0.29</td>
<td>0.35±0.19</td>
<td>ns</td>
<td>0.39±0.17</td>
<td>ns</td>
</tr>
<tr>
<td>IL12p70 Responders</td>
<td>6.58±13.16</td>
<td>8.72±16.46</td>
<td>ns</td>
<td>14.83±21.82</td>
<td>ns</td>
</tr>
<tr>
<td>IL12p70 Non-Responders</td>
<td>1.38±1.59</td>
<td>0.92±1.03</td>
<td>ns</td>
<td>0.94±0.92</td>
<td>ns</td>
</tr>
<tr>
<td>IFNγ Responders</td>
<td>1.38±1.22</td>
<td>1.41±0.83</td>
<td>ns</td>
<td>2.23±1.71</td>
<td>*</td>
</tr>
<tr>
<td>IFNγ Non-Responders</td>
<td>1.99±1.42</td>
<td>2.46±2.68</td>
<td>ns</td>
<td>2.19±1.36</td>
<td>ns</td>
</tr>
<tr>
<td>TNFα Responders</td>
<td>7.36±1.48</td>
<td>46.47±40.21</td>
<td>*</td>
<td>54.63±37.67</td>
<td>**</td>
</tr>
<tr>
<td>TNFα Non-Responders</td>
<td>8.82±3.18</td>
<td>69.61±43.10</td>
<td>*</td>
<td>50.40±32.29</td>
<td>*</td>
</tr>
<tr>
<td>TARC Responders</td>
<td>479.00±284.70</td>
<td>330.20±219.80</td>
<td>*</td>
<td>461.00±282.00</td>
<td>*</td>
</tr>
<tr>
<td>TARC Non-Responders</td>
<td>623.80±577.70</td>
<td>519.80±493.10</td>
<td>ns</td>
<td>486.60±370.40</td>
<td>ns</td>
</tr>
<tr>
<td>MIP1β Responders</td>
<td>166.90±54.83</td>
<td>126.40±49.85</td>
<td>ns</td>
<td>129.50±41.22</td>
<td>*</td>
</tr>
<tr>
<td>MIP1β Non-Responders</td>
<td>152.40±46.60</td>
<td>118.20±49.08</td>
<td>ns</td>
<td>152.50±76.05</td>
<td>ns</td>
</tr>
<tr>
<td>MCP4 Responders</td>
<td>333.30±132.00</td>
<td>395.60±164.00</td>
<td>ns</td>
<td>436.10±133.90</td>
<td>ns</td>
</tr>
<tr>
<td>MCP4 Non-Responders</td>
<td>286.40±60.00</td>
<td>333.80±100.40</td>
<td>ns</td>
<td>336.70±101.2</td>
<td>ns</td>
</tr>
<tr>
<td>MCP1 Responders</td>
<td>540.10±168.80</td>
<td>487.00±197.20</td>
<td>ns</td>
<td>466.00±258.90</td>
<td>ns</td>
</tr>
<tr>
<td>MCP1 Non-Responders</td>
<td>470.00±138.40</td>
<td>465.00±99.35</td>
<td>ns</td>
<td>444.20±140.00</td>
<td>ns</td>
</tr>
<tr>
<td>IP10 Responders</td>
<td>516.30±321.40</td>
<td>291.60±162.80</td>
<td>*</td>
<td>297.70±196.10</td>
<td>*</td>
</tr>
<tr>
<td>IP10 Non-Responders</td>
<td>287.00±179.80</td>
<td>235.80±191.00</td>
<td>ns</td>
<td>394.50±388.30</td>
<td>ns</td>
</tr>
</tbody>
</table>

Abbreviations: IL, interleukin; IFNγ, interferon gamma; TNFα, tumour necrosis factor α, TARC, Thymus-And Activation-Regulated Cytokine; MIP1β, Macrophage Inflammatory Protein 1β; MCP1, Monocyte Chemotactic Protein-1; MCP4, Monocyte Chemotactic Protein-4; IP10, Interferon-Inducible Protein-10. Time points on treatment compared versus baseline using Wilcoxon matched pairs test; *p<0.05, **P<0.005
cytotoxicity; it induces angiogenesis, the hepatic acute phase response and synergises with other pro-inflammatory cytokines (McInnes and Schett, 2007).

There was a significant reduction in serum IL6 levels (measured using the CBA assay) in the whole RA cohort at weeks 1 and 12 after anti-TNF initiation (Figure 7.1A). Both anti-TNF responders and non-responders demonstrated a decrease in serum IL6 levels at weeks 1 and 12, but this reduction was more marked and sustained in responders (Figure 7.1 B-D). At baseline, serum IL6 levels were higher in patients who did not respond to anti-TNF treatment (mean±SD 26.6±36.6 pg/ml) compared with responders (mean±SD 10.5±6.3 pg/ml), but this difference was not statistically significant (p=0.8, Figure 7.1D).

The marked reduction of serum IL6 during anti-TNF treatment was confirmed using the MSD platform, which also showed a significant reduction in serum IL6 levels in the whole RA cohort at weeks 1 and 12 on treatment (Figure 7.2A). The same changes were seen in responders (Figure 7.2B), whereas non-responders demonstrated a significant reduction in serum IL6 only at week 12 (Figure 7.2C). Comparison between responder and non-responder patients again showed a trend towards non-responders to having higher serum IL6 levels at baseline and at each time point on treatment (Figure 7.2D). The difference in serum IL6 levels at week 12 on treatment between anti-TNF responders and non-responders was statistically significant, with non-responders having higher serum IL6 levels than responders.

**Interleukin-8.** IL8 (also called neutrophil chemotactic factor) induces chemotaxis of neutrophils and other granulocytes (Baggiolini and Clark-Lewis, 1992).

There was a significant reduction in serum IL8 levels in the RA cohort with anti-TNF treatment at week 1 and at week 12 (Figure 7.3A). When the RA cohort was divided according to treatment response, anti-TNF responders demonstrated a significant reduction in serum IL8 levels with treatment, whereas there were no significant changes in non-responders (Figure 7.3B and C).

**Interferon-gamma.** IFNγ has a critical role in the innate and adaptive immune systems. It is produced by CD4+ T helper cells, CD8+ cytotoxic T cells, by natural killer cells and plays a role in the activation of macrophages (Schoenborn and Wilson, 2007).
Figure 7.1  Changes in serum IL6 levels in RA patients during 12 weeks of anti-TNF treatment determined by Cytometric Bead Array (CBA)

Bar charts showing the changes in serum IL6 levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and in anti-TNF responders (n=16) (B) and non-responders (n=9) (C). Comparison between anti-TNF responders and non-responders in changes in serum IL6 levels with anti-TNF treatment (D). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05
Figure 7.2  Changes in serum IL6 levels in RA patients during 12 weeks of anti-TNF treatment determined by MesoScale Discovery Multi-Spot Assay (MSD)

Bar charts showing the changes in serum IL6 levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and in anti-TNF responders (n=16) (B) and non-responders (n=9) (C). Comparison between anti-TNF responders and non-responders in changes in serum IL6 levels with anti-TNF treatment (D). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05, **P<0.005
Figure 7.3  Changes in serum IL8 levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum IL8 levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and in anti-TNF responders (n=16) (B) and non-responders (n=9) (C). Serum IL8 levels measured using MesoScale Discovery MultiSpot Assay system (MSD). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05, **P<0.005.
There was a trend towards a gradual increase in serum IFNγ levels with anti-TNF treatment in the whole RA cohort, although this was not statistically significant (Figure 7.4A). The trend was observed in both anti-TNF responders and non-responders (Figure 7.4B). Non-responders (mean±SD 2.0±1.4 pg/ml) demonstrated a tendency to have higher baseline IFNγ levels than responders (mean±SD 1.3±1.2 pg/ml; p=0.3).

**IP-10.** Interferon gamma inducible protein 10 (IP10) is secreted by a number of cells in response to IFNγ and it has a role in the chemoattraction of monocytes/macrophages and T cells, as well as the promotion of T cell adhesion to endothelial cells (Dufour et al., 2002). IP-10 levels decreased with anti-TNF treatment and the reduction at 1 week compared to baseline was significant in the whole RA cohort (Figure 7.5A). Although both anti-TNF responders and non-responders showed a trend towards a decrease in serum IP-10 levels at 1 week on anti-TNF treatment, the reduction was more marked in responders (Figure 7.5 B and C). At 12 weeks on anti-TNF treatment, there was further decrease in IP10 levels in responders, whereas non-responders showed a trend towards an increase in IP-10 levels (Figure 7.5 B and C).

**IL12/23p40.** IL12 and IL23 are key cytokines involved in the differentiation of Th1 and Th17 cells respectively, which share a common subunit, called p40 (Langrish et al., 2005; Oppmann et al., 2000). There was a trend towards an increase in serum IL12/23p40 levels from baseline in the RA cohort during anti-TNF treatment (baseline mean±SD 54.5±57.4 pg/ml; week 1 mean±SD 101.1±61.9 pg/ml; week 4 mean±SD 104.9±70.2pg/ml; week 12 mean±SD 107.2±109.6pg/ml) (Figure 7.6A). This trend was evident in both anti-TNF responders and non-responders (Figure 7.6B).

**Serum TNFα and soluble TNFReceptors.** TNFα plays a central role in the pathogenesis of inflammatory arthritis, as discussed in Chapter 1. There was a significant increase in serum TNFα levels in the whole RA cohort after 1, 4 and 12 weeks on treatment compared to baseline (Figure 7.7A). This increase occurred both in anti-TNF responders and non-responders and there were no significant differences between the two groups in serum TNFα levels at any of the time points on treatment (Figure 7.7B).
Figure 7.4  Changes in serum IFNγ levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum IFNγ levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and a comparison between anti-TNF responders (n=16) and non-responders (n=9) in the changes in serum IFNγ levels with treatment (B). Serum IFNγ levels measured using MesoScale Discovery MultiSpot Assay system (MSD). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test.
Figure 7.5  Changes in serum IP10 levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum IP10 levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and in anti-TNF responders (n=16) (B) and non-responders (n=9) (C). Serum IP10 levels measured using MesoScale Discovery MultiSpot Assay system (MSD). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05, **p<0.005. IP10, Interferon-Inducible Protein-10.
Figure 7.6 Changes in serum IL12/23p40 levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum IL12/23p40 levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and a comparison between anti-TNF responders (n=16) and non-responders (n=9) in the changes in serum IL12/23p40 levels with treatment (B). Serum IL12/23p40 levels measured using ELISA. Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test.
Figure 7.7 Changes in serum TNFα levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum TNFα levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and a comparison between anti-TNF responders (n=16) and non-responders (n=9) in the changes in serum TNFα levels with treatment (B). Serum TNFα levels measured using Cytometric Bead Array (CBA). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05; **p<0.005. TNFα, Tumour Necrosis Factor alpha
There were no significant changes in serum TNFRI (also called p55) levels during anti-TNF treatment (Table 7.1) in the whole RA cohort or when the cohort was divided according to anti-TNF treatment response (Table 7.2). However, there was a significant decrease in serum TNFRII (also called p75) at 1 week and at 4 weeks on anti-TNF treatment compared to baseline in the whole RA cohort (Figure 7.8A). Levels of TNFRII at 12 weeks were not significantly different from baseline. Both anti-TNF responders and non-responders demonstrated the same pattern of decrease in serum TNFRII levels with treatment and there were no significant differences between the two groups at any of the time points (Figure 7.8B).

Thymus and Activation Regulated Chemokine (TARC, also known as CCL17) is a chemokine which specifically binds and induces chemotaxis in T cells and elicits its effects by interacting with the chemokine receptor CCR4 (Imai et al., 1997). There was a significant decrease in serum TARC levels in the whole RA cohort at weeks 1 and 12 on treatment (Figure 7.9A). The decrease in serum TARC levels with anti-TNF treatment was observed in both anti-TNF responders and non-responders (Figure 7.9B).

Macrophage Inflammatory Protein 1β (MIP1β) is a chemokine which acts as a chemoattractant for natural killer (NK) cells, monocytes and B cells (Bytry RS, 2001). There was a significant decrease in serum MIP1β levels 1 week after anti-TNF initiation in the whole RA cohort and a trend towards a decrease at 12 weeks, although this was not statistically significant (Figure 7.10A). However, anti-TNF responders demonstrated a significant reduction in serum MIP1β levels at 1 and 12 weeks after treatment initiation (Figure 10B). In contrast, anti-TNF non-responders showed a trend towards a decrease in serum MIP1β levels at 1 week on treatment, but this trend was not apparent at 12 weeks (Figure 7.10C). Thus anti-TNF responders demonstrated a more marked and sustained decrease in serum MIP1β levels than non-responders.

7.2.2 Changes in serum cytokines during anti-TNF therapy in AS cohort
A panel of serum cytokines comprising IL6, IL10, IL17, IFNγ, TNFα, TNFRI and TNFRII was tested in the AS cohort at baseline and at weeks 1, 4 and 12 on anti-TNF treatment by CBA and serum IL12p70, IL23 and IL12/23p40 levels were tested at baseline and weeks 1, 4
Figure 7.8  Changes in serum TNFRII levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum TNFRII levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and a comparison between anti-TNF responders (n=16) and non-responders (n=9) in the changes in serum TNFRII levels with treatment (B). Serum TNFα levels measured using Cytometric Bead Array (CBA). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05; **p<0.005. TNFRII, Tumour Necrosis Factor alpha Receptor II (p75).
Figure 7.9  Changes in serum TARC levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum TARC levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and a comparison between anti-TNF responders (n=16) and non-responders (n=9) in the changes in serum TARC levels with treatment (B). Serum TNFα levels measured using MesoScale Discovery MultiSpot Assay system (MSD). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05; **p<0.005. TARC, Thymus-And Activation-Regulated Cytokine.
Figure 7.10  Changes in serum MIP1β levels in RA patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum MIP1β levels during 12 weeks of anti-TNF therapy in whole RA cohort (n=25) (A) and a comparison between anti-TNF responders (n=16) and non-responders (n=9) in the changes in serum MIP1β levels with treatment (B). Serum MIP1β levels measured using MesoScale Discovery MultiSpot Assay system (MSD). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05; **p<0.005. Macrophage Inflammatory Protein 1β.
and 12 on treatment using ELISA (Tables 7.5 and Table 7.6). Serum IL10 and IL17 levels were above the lower limit of detection in less than half of the AS cohort and there were no significant changes in serum levels of these two cytokines during anti-TNF treatment. Serum IL12p70 and IL23 levels were below the lower limit of detection in all study patients. There were no significant changes in serum IL12/23p40 or IFNγ levels during anti-TNF treatment.

There was a significant reduction in serum IL6 levels in the whole AS cohort at weeks 4 and 12 on anti-TNF treatment (Figure 7.11A) and this was also evident in both responders and non-responders to treatment (Figure 7.11B).

There was a significant increase in serum TNFα levels with anti-TNF treatment at weeks 1, 4 and 12 in the whole AS cohort (Figure 7.12A). Both anti-TNF responders and non-responders demonstrated an increase in serum TNFα levels during treatment (Figure 7.12B). There was a reduction in serum TNFRI levels with anti-TNF treatment in the AS group as a whole (Figure 7.13A) and in anti-TNF responder and non-responder patients (Figure 7.13B). There were no significant changes in serum TNFRII levels in the AS cohort (Table 7.5).

7.2.3 Changes in serum cytokines during anti-TNF therapy in PsA cohort

A panel of serum cytokines comprising IL6, IL10, IL17, IFNγ, TNFα, TNFRI and TNFRII was tested in the PsA cohort at baseline and at weeks 1, 4 and 12 on anti-TNF treatment by CBA and serum IL12p70, IL23 and IL12/23p40 levels were tested at baseline and weeks 1, 4 and 12 on treatment by ELISA (Tables 7.7).

Serum IL10, IL17, IL12p70, IL23, IFNγ and TNFα levels were below the lower limit of detection in this cohort of patients.

There were no significant changes in serum IL12/23p40 levels during anti-TNF treatment.

There was a trend towards a reduction in serum IL6 levels in the PsA group, although this reduction did not reach statistical significance at any of the time points, likely due to the small number of patients in this group.
Table 7.5  Summary of changes in serum cytokines during 12 weeks of anti-TNF treatment in the AS cohort

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Baseline Concentration (pg/ml)</th>
<th>1 week Concentration (pg/ml)</th>
<th>p-value</th>
<th>4 weeks Concentration (pg/ml)</th>
<th>p-value</th>
<th>12 weeks Concentration (pg/ml)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>15.84±10.33</td>
<td>10.22±6.43</td>
<td>ns</td>
<td>8.86±3.52</td>
<td>**</td>
<td>8.79±3.90</td>
<td>*</td>
</tr>
<tr>
<td>IL10</td>
<td>12.81±5.27</td>
<td>17.22±9.93</td>
<td>ns</td>
<td>14.75±8.11</td>
<td>ns</td>
<td>10.86±1.60</td>
<td>ns</td>
</tr>
<tr>
<td>IL17</td>
<td>36.37±33.01</td>
<td>39.63±36.11</td>
<td>ns</td>
<td>21.84±3.31</td>
<td>ns</td>
<td>23.43±5.53</td>
<td>ns</td>
</tr>
<tr>
<td>IL12p70</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>IL23</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>IL12/IL23p40</td>
<td>103.2±59.19</td>
<td>84.34±59.43</td>
<td>ns</td>
<td>86.41±54.08</td>
<td>ns</td>
<td>99.50±68.06</td>
<td>ns</td>
</tr>
<tr>
<td>IFNγ</td>
<td>27.12±19.75</td>
<td>37.00±30.59</td>
<td>ns</td>
<td>29.95±24.51</td>
<td>ns</td>
<td>23.01±6.35</td>
<td>ns</td>
</tr>
<tr>
<td>TNFα</td>
<td>24.94±8.69</td>
<td>69.27±44.32</td>
<td>***</td>
<td>61.18±63.59</td>
<td>*</td>
<td>62.36±62.91</td>
<td>*</td>
</tr>
<tr>
<td>TNFRI</td>
<td>1983.00±605.80</td>
<td>1697.00±482.00</td>
<td>*</td>
<td>1689.00±483.20</td>
<td>**</td>
<td>1762.00±470.40</td>
<td>ns</td>
</tr>
<tr>
<td>TNFRII</td>
<td>5526.00±7510.00</td>
<td>5757.00±6308.00</td>
<td>ns</td>
<td>4236.00±3152.00</td>
<td>ns</td>
<td>4236.00±3152.00</td>
<td>ns</td>
</tr>
</tbody>
</table>

All cytokines (excluding IL12, IL23 and IL12/23p40) measured in serum using cytometric bead array (CBA). IL12, 23, IL12/23p40 levels in serum determined by ELISA. Abbreviations: IL, interleukin; IFNγ, interferon gamma; TNFα, tumour necrosis factor α, TNFRI, tumour necrosis factor α receptor I; TNFRII, tumour necrosis factor α receptor II; BLD, below limit of detection. Time points on treatment compared versus baseline using Wilcoxon matched pairs test; *p<0.05; **p<0.005.
## Table 7.6 Summary of changes in serum cytokines during 12 weeks of anti-TNF treatment in anti-TNF responders and non-responders from AS cohort

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Baseline Concentration (pg/ml)</th>
<th>Week 1 Concentration (pg/ml)</th>
<th>p-value</th>
<th>Week 4 Concentration (pg/ml)</th>
<th>p-value</th>
<th>Week 12 Concentration (pg/ml)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6 Responders</td>
<td>15.74±10.13</td>
<td>11.28±6.95</td>
<td>ns</td>
<td>9.21±3.34</td>
<td>*</td>
<td>9.38±4.08</td>
<td>*</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>16.13±12.49</td>
<td>6.68±2.55</td>
<td>ns</td>
<td>7.90±4.34</td>
<td>ns</td>
<td>7.30±3.48</td>
<td>ns</td>
</tr>
<tr>
<td>IL12/23p40 Responders</td>
<td>114.10±63.23</td>
<td>90.92±66.26</td>
<td>ns</td>
<td>92.14±57.28</td>
<td>ns</td>
<td>101.9</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>73.10±37.07</td>
<td>64.60±32.96</td>
<td>ns</td>
<td>70.68±47.59</td>
<td>ns</td>
<td>93.03±59.71</td>
<td>ns</td>
</tr>
<tr>
<td>IFN(\gamma) Responders</td>
<td>31.47±21.25</td>
<td>36.20±34.33</td>
<td>ns</td>
<td>25.25±22.88</td>
<td>ns</td>
<td>20.69±5.28</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>15.15±7.31</td>
<td>39.40±20.51</td>
<td>ns</td>
<td>42.88±27.49</td>
<td>ns</td>
<td>29.38±4.64</td>
<td>ns</td>
</tr>
<tr>
<td>IL17 Responders</td>
<td>24.76±13.31</td>
<td>31.39±24.44</td>
<td>ns</td>
<td>22.53±3.69</td>
<td>ns</td>
<td>21.50±1.90</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>67.33±53.33</td>
<td>68.50±68.59</td>
<td>ns</td>
<td>20.00±1.00</td>
<td>ns</td>
<td>27.93±9.12</td>
<td>ns</td>
</tr>
<tr>
<td>IL10 Responders</td>
<td>13.88±6.10</td>
<td>17.09±11.22</td>
<td>ns</td>
<td>13.27±7.63</td>
<td>ns</td>
<td>10.32±0.50</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>10.40±0.80</td>
<td>17.57±7.22</td>
<td>ns</td>
<td>18.10±9.29</td>
<td>ns</td>
<td>12.08±2.60</td>
<td>ns</td>
</tr>
<tr>
<td>TNF(\alpha) Responders</td>
<td>26.81±9.99</td>
<td>81.44±46.65</td>
<td>**</td>
<td>64.43±76.10</td>
<td>ns</td>
<td>60.78±60.34</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>20.73±1.45</td>
<td>36.83±5.68</td>
<td>ns</td>
<td>53.88±25.10</td>
<td>ns</td>
<td>65.93±78.07</td>
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</tr>
<tr>
<td>TNFRI Responders</td>
<td>1955.00±466.90</td>
<td>1765.00±372.70</td>
<td>ns</td>
<td>1715.00±433.70</td>
<td>ns</td>
<td>1727±319.90</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
<td>2059.00±987.00</td>
<td>1494.00±799.9</td>
<td>ns</td>
<td>1620.00±673.80</td>
<td>ns</td>
<td>1857.00±821.60</td>
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</tr>
<tr>
<td>TNFRII Responders</td>
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<td>-</td>
<td></td>
<td>6327.00±7257.00</td>
<td>ns</td>
<td>3568.00±2811.00</td>
<td>ns</td>
</tr>
<tr>
<td>Non-Responders</td>
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<td>4191.00±2380.00</td>
<td>ns</td>
<td>6000.00±3773.00</td>
<td>ns</td>
<td>3568.00±2811.00</td>
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</tr>
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</table>

All cytokines (excluding IL12, IL23 and IL12/23p40) measured in serum using cytometric bead array (CBA). IL12, 23, IL12/23p40 levels in serum determined by ELISA. Abbreviations: IL, interleukin; IFN\(\gamma\), interferon gamma; TNF\(\alpha\), tumour necrosis factor a,
TNFRI, tumour necrosis factor $\alpha$ receptor I; TNFRII, tumour necrosis factor $\alpha$ receptor II. Time points on treatment compared versus baseline using Wilcoxon matched pairs test; *$p<0.05$
Figure 7.11  Changes in serum IL6 levels in AS patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum IL6 levels during 12 weeks of anti-TNF therapy in whole AS cohort (n=15) (A) and a comparison between anti-TNF responders (n=11) and non-responders (n=4) in the changes in serum IL6 levels with treatment (B). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05, **p<0.005.
Figure 7.12 Changes in serum TNFα levels in AS patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum TNFα levels during 12 weeks of anti-TNF therapy in whole AS cohort (n=15) (A) and a comparison between anti-TNF responders (n=11) and non-responders (n=4) in the changes in serum TNFα levels with treatment (B). Serum TNFα levels measured using Cytometric Bead Array (CBA). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05, **p<0.005.
Figure 7.13 Changes in serum TNFRI levels in AS patients during 12 weeks of anti-TNF treatment

Bar charts showing the changes in serum TNFRI levels during 12 weeks of anti-TNF therapy in whole AS cohort (n=15) (A) and a comparison between anti-TNF responders (n=11) and non-responders (n=4) in the changes in serum TNFRI levels with treatment (B). Serum TNFRI levels measured using Cytometric Bead Array (CBA). Time points on treatment within groups compared using Wilcoxon matched pairs test; comparison between groups using Mann Whitney U test; *p<0.05, **p<0.005. TNFRI, Tumour Necrosis Factor alpha Receptor I (p55).
Table 7.7  Summary of changes in serum cytokines during 12 weeks of anti-TNF treatment in PsA cohort

<table>
<thead>
<tr>
<th>Cytokine/chemokine</th>
<th>Baseline Concentration (pg/ml)</th>
<th>1 week Concentration (pg/ml)</th>
<th>p-value</th>
<th>4 weeks Concentration (pg/ml)</th>
<th>p-value</th>
<th>12 weeks Concentration (pg/ml)</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>IL6</td>
<td>8.56±4.64</td>
<td>5.20±0.49</td>
<td>ns</td>
<td>5.64±1.12</td>
<td>ns</td>
<td>5.55±0.79</td>
<td>ns</td>
</tr>
<tr>
<td>IL10</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>IL17</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>IL12p70</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>IL23</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>IL12/ IL23p40</td>
<td>116.80±100.90</td>
<td>75.89±43.21</td>
<td>ns</td>
<td>113.90±87.69</td>
<td>ns</td>
<td>85.89±48.72</td>
<td>ns</td>
</tr>
<tr>
<td>IFNγ</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>TNFα</td>
<td>BLD</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>TNFRI</td>
<td>1657.00±683.70</td>
<td>1494.00±887.00</td>
<td>ns</td>
<td>1469.00±487.90</td>
<td>ns</td>
<td>1475.00±585.30</td>
<td>*</td>
</tr>
<tr>
<td>TNFRII</td>
<td>4304.00±1155.00</td>
<td>2779.00±1749.00</td>
<td>ns</td>
<td>2837.00±1243</td>
<td>*</td>
<td>3012.00±2022.00</td>
<td>ns</td>
</tr>
</tbody>
</table>

All cytokines (excluding IL12, IL23 and IL12/23p40) measured in serum using cytometric bead array (CBA). IL12, 23, IL12/23p40 levels in serum determined by ELISA. Abbreviations: IL, interleukin; IFNγ, interferon gamma; TNFα, tumour necrosis factor α, TNFRI, tumour necrosis factor α receptor I; TNFRII, tumour necrosis factor α receptor II; BLD, below limit of detection. Time points on treatment compared versus baseline using Wilcoxon matched pairs test; *p<0.05
There was a significant decrease in serum TNFRI levels in the PSA group 12 weeks after anti-TNF initiation and there was also a significant decrease in serum TNFRII levels 4 weeks after anti-TNF initiation.

7.3 Discussion

A panel of serum cytokines and chemokines was tested longitudinally during anti-TNF treatment in patients with RA, AS and PsA in order to evaluate changes in soluble immune markers with treatment and to correlate changes in serum cytokines with the changes observed in Th1 and Th17 cells.

Anti-TNF treatment induced a significant reduction in serum IL6 levels in patients with RA and AS and also a trend towards a reduction in patients with PsA. Anti-TNF therapy also induced an increase in serum TNFα levels in RA and AS patients, but a reduction in serum TNFRI and TNRII levels. In the RA group, serum chemokines IL8, MIP1β, TARC and IP10 decreased during anti-TNF treatment and this reduction was more marked in anti-TNF responders than in non-responders. These results are in agreement with a number of studies which have investigated the effects of anti-TNF agents on serum cytokines and chemokines and confirm that our patient cohorts are representative (Charles et al., 1999; Duftner et al., 2006; Elliott M.J., 1993; Elliott M.J., 1997; Klimuik PA, 2006; Mastroianni et al., 2005; Ohshima S, 1999; Pedersen et al., 2010a; Taylor et al., 2000). Our results are also in agreement with two of the key mechanisms of action through which anti-TNF leads to arthritis improvement; inhibition of the pro-inflammatory cytokine cascade and a reduction in the chemotactic gradient leading to reduced influx of inflammatory cells into joints (Butler DM, 1995; Charles et al., 1999; Tak et al., 1996; Taylor et al., 2000; Williams et al., 1992).

The most marked and rapid effect of anti-TNF treatment on serum cytokines was the reduction in serum IL6 levels observed in patients with RA, AS and PsA after 1 week on treatment, with further decreases at weeks 4 and 12. Although this decrease was observed in anti-TNF responders and non-responders, the reduction was more marked in the responders to treatment than in the non-responders. These results are in agreement with several other studies investigating the effects of anti-TNF agents on pro-inflammatory cytokines in patients with RA and in patients with spondyloarthritis. A reduction in serum IL6 has been reported to occur within hours of infusion with infliximab, with maintenance of response after the
infusion (Charles et al., 1999; Elliott M.J., 1993; Elliott M.J., 1997; Ohshima S, 1999) and patients who are responders to anti-TNF therapy have been shown to have more marked reduction in IL6 (Chen et al., 2011; Elliott M.J., 1993; Ohshima S, 1999; Pedersen et al., 2010a; Xueyi et al., 2012). The observed reduction in pro-inflammatory cytokines (IL6 and IL8) during anti-TNF therapy is also in agreement with one of the main proposed mechanisms of action through which anti-TNF therapy leads to improvement of inflammatory arthritis; anti-TNF has been shown to inhibit the pro-inflammatory cytokine cascade (Butler DM, 1995; Charles et al., 1999; Ohshima S, 1999; Williams et al., 1992).

Another relatively recently characterised and important role for IL6 is its involvement in the differentiation of Th17 cells. Studies have shown that an inflammatory cytokine milieu with macrophage-derived and dendritic-cell derived IL1β, IL6, IL21 and IL23 allows TGFβ together with IL6 to promote Th17 differentiation and inhibit Treg development (Manel et al., 2008; Volpe et al., 2008). As serum levels of IL6 have been demonstrated to decrease significantly during anti-TNF treatment, the increase in Th17 cells observed with anti-TNF therapy in our study are unlikely therefore to be attributed to increased levels of IL6.

In RA patients, there was a trend towards an increase in serum IFNγ levels with anti-TNF therapy which was evident in both responders and non-responders to treatment. This finding is in agreement with the results from our experiments using IFNγ ELISpot assays and intracellular cytokine staining which demonstrated an increase in circulating IFNγ-producing cells during anti-TNF treatment. Other studies have reported increased IFNγ production in cell culture supernatants from stimulated PBMCs from patients on anti-TNF therapy (Berg et al., 2001; Nissinen et al., 2004).

In the RA cohort, there was a trend towards an increase in serum IL12/23p40 levels with anti-TNF treatment, although this was not statistically significant. In AS and PsA patients there were no significant changes in IL12/23p40 levels with anti-TNF therapy. IL12/23p40 is a subunit shared between the cytokines IL12 and IL23 which have key roles in the differentiation of Th1 and Th17 cells respectively (Langrish et al., 2005; Oppmann et al., 2000). IL12/23p40 is thus of interest in light of our findings in this study of the increased frequencies of Th1 and Th17 cells during anti-TNF treatment. A study using the collagen-induced arthritis mouse model of RA reported that anti-TNF therapy ameliorated arthritis by decreasing numbers of Th1 and Th17 cells in arthritic joints, but also caused an increase in Th1 and Th17 cells in the draining lymph nodes (Notley et al., 2008) By using knockout
mice, the increase in Th1 and Th17 cells was shown to occur through signalling via the TNFp55 receptor, which increased expression of the p40 subunit that is shared between IL12 and IL23. More recently, another study by our group in patients with RA treated with anti-TNF agents showed that the increase in circulating Th17 cells with anti-TNF therapy was accompanied by an increase in IL12/23p40 production in supernatants from PBMCs stimulated with lipopolysaccharide (LPS) and also in the plasma layer of whole blood stimulated with LPS (Alzabin et al., 2012). Taken together these results suggest that a possible mechanism for the increase in Th1 and Th17 cells with anti-TNF therapy may be through the increase in the expression of IL12/23p40. In contrast, a small study in 14 patients with AS failed to detect a change in serum IL12/23p40 levels after 10 weeks of anti-TNF therapy (Wendling et al., 2009). Thus further evaluation of the role of this cytokine subunit and its change during anti-TNF treatment in larger cohorts of patients with different types of inflammatory arthritis is needed.

There was a significant increase in serum TNFα levels during anti-TNF therapy in patients with RA and AS. A rapid and dose-dependent increase in immunoreactive but not biologically active TNFα has been reported to occur by a number of other studies as early as 8-24 hours after an infusion of infliximab and to peak on day 7 after the infusion (Charles et al., 1999; Elliott M.J., 1993). This increase in TNFα has been suggested to occur through the formation of high molecular weight complexes of TNFα bound with the anti-TNF agents thus trapping TNFα in the circulation (Charles et al., 1999; Tracey et al., 2008; Wong et al., 2008). Soluble TNF receptors I and II (TNRI and TNFRII) have been reported to be elevated in serum samples from patients with rheumatoid arthritis and their levels have previously been shown to correlate with disease activity (Elliott et al., 1994). A reduction in the serum levels of TNFRI and TNFRII was observed in the three disease groups in this study, which is in agreement with other studies (Charles et al., 1999; Elliott M.J., 1997; Ohshima S, 1999).

In our study, a number of chemokines, including IL8, IP10, TARC and MIP1β were shown to decrease significantly in RA patients during anti-TNF treatment. IL8 (also called neutrophil chemotactic factor) induces chemotaxis of neutrophils and other granulocytes, causing them to migrate towards sites of infection (Baggiolini and Clark-Lewis, 1992). Interferon gamma inducible protein 10 (IP10) is secreted by a number of cells in response to IFNγ and is
responsible for chemoattraction for monocytes/macrophages and T cells, as well as the promotion of T cell adhesion to endothelial cells (Dufour et al., 2002). TARC is a chemokine which induces chemotaxis in T cells and elicits its effects by interacting with the chemokine receptor CCR4 (Imai et al., 1997). MIP1β is a chemokine which acts as a chemoattractant for natural killer (NK) cells, monocytes and B cells (Bytry RS, 2001). A reduction in the concentration of serum chemokines, including IL8 and MCP1 during treatment with infliximab and also with etanercept has been reported previously in RA patients (Klimiuk et al., 2011; Klimuik PA, 2006). Although in our study both anti-TNF responder and non-responder patients demonstrated a trend towards a reduction in these chemokines during treatment, the reduction was more marked and sustained in anti-TNF responders. The observed reduction in serum chemokine levels is in agreement with one of important mechanisms of action which have been proposed through which anti-TNF therapy leads to amelioration of inflammatory arthritis. Studies have shown that anti-TNF treatment reduces leukocyte migration to inflamed joints through reduced synovial expression of chemokines and vascular adhesion molecules (Paleolog et al., 1998; Tak et al., 1996; Taylor et al., 2000).

Using the CBA assay, the majority of patients in the RA, AS and PsA groups did not have detectable serum IL17 levels. Using the MSD platform to measure serum IL17 levels in the RA cohort did not reveal significant changes in serum levels of this cytokine during anti-TNF therapy. Although some studies have reported increased serum IL17 levels in patients with inflammatory arthritis with anti-TNF treatment (Chen et al., 2011; Xueyi et al., 2012), other studies have not been able to detect serum IL17, or this has been present at very low levels (Jandus et al., 2008; Leipe et al., 2010). The findings in an early in vitro study suggested that IL17 is biologically active at very low concentrations and despite this is still capable of eliciting a biological response (Spriggs, 1997). One of the key effects of IL17 is its ability to synergise with other inflammatory cytokines to mediate downstream effector function, thus augmenting its effects. Therefore, even at very low levels IL17 may be biologically relevant in an inflamed milieu. An important limitation to take into account when measuring serum cytokines is that the changes in serum may not be an accurate representation of the situation in the target tissue, such as the synovial joint. However, although the most important cytokine actions in inflammatory arthritis are likely to be in the joints, the relative inaccessibility of synovial tissue during longitudinal clinical studies makes the direct study of this area difficult. Anti-TNF therapy clearly affects a number of cytokines and chemokines in the
circulation which is in keeping with its postulated mechanisms of action. Thus despite its limitations, studying changes in serum cytokines with anti-TNF therapy has yielded further insights into the mechanisms of action of anti-TNF therapy.

Two different types of multiplex cytokine assays were used to measure serum cytokines in the RA cohort, the cytometric bead array and MesoScale Discovery MultiSpot assay. Although two different panels of serum cytokines/chemokines were analysed using these different systems, some of the cytokines measured were common to both. Some of the measured cytokines (IL10, IL17 and IFNγ) were found to be below the lower limit of detection when measured using CBA, whereas they were detectable using the MSD platform. Only one study to date has compared the measurement of various proinflammatory cytokines in human serum samples by CBA and MSD (Dabitao et al., 2011). This study demonstrated that the MSD platform exhibited better reliability and sensitivity in the quantitation of endogenous serum cytokines compared to CBA, especially of those cytokines with low endogenous levels, such as IL10 and IL12p70. Possible reasons for the differences in sensitivity between the two multiplex assays are not clear at present but may be due to the wider dynamic range of the MSD compared to the CBA, as well as the use of electrochemiluminescence by the MSD assay (Chowdhury et al., 2009). The stimulation mechanism in MSD is electrically uncoupled from the signal detected which is light and this has been postulated to reduce background in comparison to fluorescence, which is used by CBA. In addition, the signal amplification through multiple cycles of each label in the MSD assay has also been proposed as a reason underlying its improved sensitivity (Dabitao et al., 2011).

7.4 Conclusion

The work presented in this chapter characterised changes in a panel of serum cytokines, including IL6, IL8, IL10, IL17, IL12p70, IL12/23p40, IFNγ, TNFα, TNFRI and TNFR2 and chemokines, including TARC, MIP1β, MCP4, MCP 1 and IP10 longitudinally during anti-TNF therapy in patients with inflammatory arthritis. Serum IL6 was the cytokine with the most pronounced and significant changes. IL6 levels decreased with anti-TNF treatment in all three disease groups, with the reduction more marked in anti-TNF responders than in non-responders. There was a significant increase in serum TNFα levels in patients with RA and
AS during treatment, but a decrease in serum TNFR I and II levels. In the RA group, a
number of chemokines including IL8, IP10, TARC and MIP1β decreased significantly during
anti-TNF treatment and this decrease was more marked and sustained in patients who were
responders to treatment compared to the non-responders.

In this longitudinal investigation of the effects of anti-TNF therapy on serum cytokine and
chemokines during therapy in patients with RA, AS and PsA, our results are in agreement
with other published studies. This confirms that despite the small number of patients, our
cohorts are representative patient populations for the study of the effects of anti-TNF therapy
on clinical, imaging and immunological parameters. In addition, our results are in agreement
with two of the key mechanisms of action through which anti-TNF therapy has been
proposed to lead to improvement in inflammatory arthritis. First, is the inhibition of the pro-
inflammatory cytokine cascade and second is the reduced cell influx of inflammatory cells
into joints through the downregulation of synovial adhesion molecules and chemokines
(Charles et al., 1999; Ohshima S, 1999; Paleolog et al., 1998; Tak et al., 1996; Taylor et al.,
2000).
Chapter 8. Relationships between clinical and ultrasonographic assessments of disease activity and frequency of circulating Th17 cells during anti-TNF treatment

8.1 Introduction

The work presented in this chapter explores the relationships between clinical, ultrasonographic and immunological changes during anti-TNF therapy in patients with inflammatory arthritis. As the RA cohort comprised the largest number of patients, these relationships were explored mainly within this group. This is the first study to characterise simultaneously clinical, imaging and T cell immunological parameters longitudinally during anti-TNF therapy and to explore the relationships between them in RA patients.

Thus far in this study it has been shown that anti-TNF treatment of this cohort of RA patients over 12 weeks leads to significant improvements in the clinical measures of disease activity; DAS28 score, CRP and ESR levels. Using grey scale and power Doppler ultrasonography (PDUS) to evaluate inflammation in the joints, it was also demonstrated that anti-TNF treatment results in significant reduction in semi-quantitative and quantitative measures of synovial thickening (Trans STi, Long STi, Trans STA and Long STA) and synovial vascularity (Trans VASCi, Long VASCi, Trans PDA and Long PDA), albeit with different kinetics of change. Using the IL17 ELISpot assay, as well as intracellular cytokine staining for CD4+IL17+ cells, a significant increase in the frequency of circulating Th17 cells 12 weeks after anti-TNF initiation was observed in the RA cohort. There was also a significant increase in the frequency of circulating CD4+IFNγ+ (Th1) cells during anti-TNF treatment. The results using IL17 ELISpot and intracellular cytokine staining for CD4+IL17+ cells also suggested that patients who were non-responders to anti-TNF treatment at 12 weeks had a higher frequency of Th17 cells at baseline compared to anti-TNF responders. In view of these findings, the first aim was to explore relationships between clinical, ultrasonographic and T cell immunological changes during anti-TNF therapy to determine if a higher frequency of IL17 or IFNγ-producing cells at baseline is associated with poor treatment response.

One of the key mechanisms of action through which anti-TNF leads to improvement in inflammatory arthritis has been shown to be due to the deactivation of the endothelium via
reduced expression of adhesion molecules and chemokines and diminished angiogenesis, leading to reduced trafficking of inflammatory cells to synovial joints (Paleolog et al., 1998; Tak et al., 1996; Taylor et al., 2000). We showed that anti-TNF treatment induced an increase in the frequency of circulating Th17 cells in both responders and non-responders to treatment. A recent cross-sectional study in RA patients demonstrated that the frequency of Th17 cells in synovial fluid from inflamed knee joints correlated with CRP levels, positive power Doppler signal by ultrasound of the knee joint and increased levels of synovial fluid vascular endothelial growth factor (VEGF) (Gullick et al., 2010). This study also suggested that the presence of power Doppler signal in the joints may therefore be a surrogate marker of the presence of Th17 cells. In view of these findings, the second aim was to explore the relationships between clinical, ultrasonographic and T cell immunological changes during anti-TNF therapy to test the hypothesis whether the changes in the frequency of Th17 or Th1 cells during anti-TNF treatment is related to clinical improvement in RA disease activity and morphological improvement of inflamed joints.

8.2 Results

8.2.1 Relationships between the frequency of circulating Th1 and Th17 cells and clinical measures of disease activity in the RA cohort

There was a significant positive correlation between the numbers of IL17-producing PBMCs at baseline and the change in DAS28 score from baseline to week 1 on treatment (r=0.51, p=0.02) (Figure 8.1A). This suggests that the higher the baseline frequency of IL17-producing cells, the smaller the improvement in DAS28 score 1 week after anti-TNF initiation. Conversely, lower baseline frequencies of circulating IL17-producing cells are associated with a greater reduction in DAS28 scores 1 week after anti-TNF is initiated.

There was also a positive correlation between the frequencies of IL17-producing cells at baseline and the change in numbers of swollen joints (28 joint count) from baseline to week 1 on treatment (r=0.48, p=0.03) (Figure 8.1B). This suggests that a higher baseline frequency of IL17-producing cells is associated with a smaller improvement in the numbers of swollen joints 1 week after anti-TNF initiation. These two results support the hypothesis that higher
Figure 8.1 Higher numbers of circulating IL17-producing cells at the start of anti-TNF therapy are associated with poor treatment response

Positive correlations are shown between the baseline frequency of circulating IL17-producing cells (determined by IL17 ELISpot assay) and the change in DAS28ESR score from baseline to week 1 on treatment (A) and the change in numbers of swollen joints (28 joint count) (B) from baseline to week 1 on treatment in the whole RA cohort. Correlations were performed using Spearman’s rank method. spSFC/10^6, specific spot forming cells per 10^6; DAS28ESR, Disease Activity Score in 28 joints; SJC, swollen joint count.
baseline frequencies of IL17-producing cells may be associated with poor treatment response to anti-TNF therapy.

There were no significant correlations between the baseline frequency of Th1 cells and the change from baseline in DAS28 score, CRP, ESR, swollen joint count or tender joint count.

There were no significant correlations between baseline numbers of IL17-producing cells determined by ELISpot, or percentages of CD4+IL17+ cells determined by FACS and baseline DAS28 score, CRP, ESR, numbers of swollen joints or numbers of tender joints on examination. There were also no significant correlations between baseline numbers of IFN\(\gamma\)-producing cells determined by ELISpot or percentages of CD4+IFN\(\gamma\)+ cells determined by FACS and DAS28 score, CRP, ESR, numbers of swollen or tender joints.

8.2.2 Relationships between the frequency of circulating Th1 or Th17 cells and changes in synovial thickening and vascularity by ultrasound in RA cohort

There was a significant negative correlation between the frequency of IL17-producing cells at baseline (by IL17 ELISpot) and quantitative score of synovial vascularity at baseline (Long PDA) \(r= -0.41, p=0.04\) in the RA patients who had presence of power Doppler signal at baseline \(n=19\). There was also an inverse relationship between the percentage of CD4+IL17+ cells at baseline (by FACS) and Long PDA score at baseline \(r= -0.40, p=0.04\) in the same subset of RA patients. Thus, the presence of higher power Doppler signal within the joints at baseline is associated with lower frequencies of Th17 cells in the peripheral blood, which may suggest that the majority of these cells may be concentrated in the synovial compartment.

No significant correlations were observed between the baseline frequency of IL17-producing cells (determined by IL17 ELISpot) or baseline percentage of CD4+IL17+ cells (determined by FACS) and baseline semi-quantitative or quantitative measures of synovial thickening on ultrasound.
There were no significant correlations between the baseline frequency of IFNγ-producing cells (by IFNγ ELISpot) or baseline percentages of CD4+IFNγ+ cells (by FACS) and baseline semi-quantitative or quantitative measures of synovial thickening or vascularity on ultrasound.

A positive correlation was observed between the frequency of IL17-producing cells at baseline (by IL17 ELISpot) and the change in synovial vascularity (by PDUS) from baseline to week 1 on treatment (r=0.46, p=0.02) (Figure 8.2A) in those RA patients who had power Doppler activity at baseline (n=19). There were also strong positive correlations between the frequency of circulating IL17-producing cells at baseline and the change in synovial thickening (by PDUS) from baseline to week 1 (r=0.72, p=0.0004), from baseline to week 4 (r=0.51, p=0.01) and from baseline to week 12 on treatment (r=0.52, p=0.01) (Figure 8.2B-D) in the whole RA cohort. Similarly, there was a positive correlation between the percentage of peripheral blood CD4+IL17+ cells (by FACS) and the change in synovial vascularity score from baseline to week 1 on treatment (r=0.66, p=0.002) (Figure 8.3A). There were also positive correlations between the percentages of CD4+IL17+ cells at baseline and the change in synovial thickening score from baseline to week 1 (r=0.51, p=0.01) and from baseline to week 4 (r=0.36, p=0.04) (Figure 8.3B and C). The correlation between the percentage of CD4+IL17+ cells at baseline and the change in synovial thickening score from baseline to week 12 did not quite reach statistical significance (r=0.35, p=0.05) (Figure 8.3D). These relationships demonstrate that higher baseline frequencies of Th17 cells are associated with a smaller improvement in synovial vascularity and synovial thickening with anti-TNF treatment. Thus they confirm that higher baseline frequencies of Th17 cells may be associated with poorer anti-TNF treatment response.

There were no significant relationships between the frequency of IFNγ-producing cells at baseline (IFNγ ELISpot) or percentages of CD4+IFNγ+ cells (by FACS) and the change in ultrasound measures of synovial thickening or vascularity during anti-TNF treatment. This suggests that baseline frequencies of Th1 cells are not associated with the determination of anti-TNF treatment response, unlike baseline frequencies of Th17 cells.

The results using the IL17 ELISpot assay and intracellular cytokine staining for CD4+IL17+ cells in this study showed that in RA patients there was a significant increase in the frequency of Th17 cells during anti-TNF treatment and the increase at week 12 was statistically
Figure 8.2  Higher frequencies of circulating IL17-producing cells at baseline are associated with a smaller improvement in ultrasound measures of synovial vascularity and thickening with anti-TNF treatment

Positive correlation between the frequency of circulating IL17-producing cells (determined by IL17 ELISpot assay) at baseline and the change in quantitative score for synovial vascularity (10 MCP Trans PDA score) from baseline to week 1 on treatment (A) in RA patients with presence of power Doppler signal at baseline (n=19). Positive correlations are shown between the frequency of circulating IL17-producing cells (determined by IL17 ELISpot assay) at baseline and the change in quantitative score for synovial thickening (10 MCP Trans STA score) from baseline to week 4 (B) and from baseline to week 12 (D) on treatment in the whole RA cohort. Correlations were performed
using Spearman's rank method. spSFC/10⁶, specific spot forming cells per 10⁶; 10 MCP Trans PDA, composite transverse power Doppler area score for synovial vascularity of ten metacarpophalangeal joints; 10 MCP Trans STA, composite transverse synovial thickness area score of ten metacarpophalangeal joints.
Figure 8.3 Higher percentages of circulating CD4+IL17+ cells at baseline are associated with a smaller improvement in ultrasound parameters for synovial vascularity and thickening with anti-TNF treatment

Positive correlations are shown between the percentages of circulating CD4+IL17+ cells (determined by intracellular cytokine staining using flow cytometry) at baseline and the change in quantitative score for synovial vascularity (10 MCP Trans PDA score) from baseline to week 1 on treatment (A) in RA patients with presence of power Doppler signal at baseline (n=19). Also positive correlations are shown between the percentages of circulating CD4+IL17+ cells at baseline and the change in quantitative score for synovial thickening (10 MCP Trans STA score) from baseline to week 1 (B), from baseline to week 4 (C) and from baseline to week 12 (D) on treatment in the whole RA cohort.
Correlations were performed using Spearman’s rank method. 10 MCP Trans PDA, composite transverse power Doppler area score for synovial vascularity of ten metacarpophalangeal joints; 10 MCP Trans STA, composite transverse synovial thickness area score of ten metacarpophalangeal joints.
There were significant negative correlations between the change in numbers of IL17-producing cells (by ELISpot) from baseline to week 12 on treatment and the change in synovial thickening score (Trans STA) and change in synovial vascularity (Trans PDA and Long PDA) scores from baseline to week 12 on treatment (Table 8.1). There was also an inverse relationship between the change in percentage of CD4+IL17+ cells (by FACS) from baseline to week 12 on treatment and the change in synovial thickening and synovial vascularity scores from baseline to week 12, but these were not statistically significant (Table 8.1). These negative correlations suggest that the increase in the frequency of circulating Th17 cells with anti-TNF treatment is associated with a reduction in synovial swelling and synovial vascularity. Thus, these findings support the hypothesis that anti-TNF treatment may increase the frequency of circulating Th17 cells by promoting a redistribution of cells from inflamed joints, which leads to improvement in synovial swelling and inflammation.

Using the IFNγ ELISpot assay and intracellular cytokine staining, we also showed that anti-TNF treatment induced an increase in the frequency of IFNγ-producing cells from baseline and that the percentages of CD4+IFNγ+ cells increased significantly at 12 weeks compared to baseline (Chapter 5 and Chapter 6). There were significant negative correlations between the change in frequency of CD4+IFNγ+ cells (determined by FACS) from baseline to week 12 on treatment and the change in synovial thickening (Trans STA) and synovial vascularity (Trans PDA and Long PDA) scores from baseline to week 12 on treatment (Table 8.2). These negative correlations suggest that the increase in the frequency of circulating Th1 cells with anti-TNF treatment is associated with a reduction in synovial swelling and synovial vascularity. These findings also suggest that anti-TNF treatment may increase the frequency of circulating Th1 cells by promoting their egress from inflamed joints, which leads to improvement in synovial swelling and inflammation.

### 8.2.3 Correlations between serum cytokines and clinical measures of disease activity in the RA cohort

There was a positive correlation between serum IL6 levels and CRP levels at baseline in the RA cohort (r=0.46, p=0.03) (Figure 8.4). There were no significant correlations between
Table 8.1 Increasing frequency of circulating IL17-positive cells during anti-TNF therapy is associated with improvement in synovial thickening and vascularity on ultrasound

<table>
<thead>
<tr>
<th>Ultrasound parameter</th>
<th>Change in numbers of IL17-producing cells from baseline to 12 weeks on treatment</th>
<th>r</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>Change in 10 MCP Trans STA score</td>
<td></td>
<td>-0.39</td>
<td>0.04</td>
</tr>
<tr>
<td>Change in 10 MCP Long STA score</td>
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<td>0.15</td>
</tr>
<tr>
<td>Change in 10 MCP Trans PDA score</td>
<td></td>
<td>-0.51</td>
<td>0.03</td>
</tr>
<tr>
<td>Change in 10 MCP Long PDA score</td>
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<td>0.007</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Ultrasound parameter</th>
<th>Change in % of CD4+IL17+ cells from baseline to 12 weeks on treatment</th>
<th>r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in 10 MCP Trans STA score</td>
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<td>0.12</td>
</tr>
<tr>
<td>Change in 10 MCP Long STA score</td>
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<td>0.25</td>
</tr>
<tr>
<td>Change in 10 MCP Trans PDA score</td>
<td></td>
<td>-0.38</td>
<td>0.09</td>
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<tr>
<td>Change in 10 MCP Long PDA score</td>
<td></td>
<td>-0.14</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Negative correlations are shown between the change in frequency of IL17-positive cells (determined by IL17 ELISpot assay) from baseline to week 12 on treatment and the change in quantitative ultrasound scores for synovial thickening (Trans STA and Long STA) and vascularity (Trans PDA and Long PDA) from baseline to week 12 and between the change in percentages of CD4+IL17+cells (determined by intracellular cytokine staining using flow cytometry) from baseline to week 12 on treatment and the change in quantitative ultrasound scores for synovial thickening (Trans STA and Long STA) and vascularity (Trans PDA and Long PDA) from baseline to week 12 in RA patients. Correlations were performed using Spearman’s rank method and statistically significant correlations are shown in bold type. 10 MCP Trans PDA, composite transverse power Doppler area score for synovial vascularity of ten metacarpophalangeal joints; 10 MCP Trans STA, composite transverse synovial thickness area score of ten metacarpophalangeal joints.
Table 8.2  Increasing frequency of circulating IFNγ-positive cells during anti-TNF therapy is associated with improvement in synovial thickening and vascularity on ultrasound

<table>
<thead>
<tr>
<th>Ultrasound parameter</th>
<th>Change in % of CD4+IFNγ+ cells from baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>Change in 10 MCP Trans STA score</td>
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</tr>
<tr>
<td>Change in 10 MCP Long STA score</td>
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<td>Change in 10 MCP Trans PDA score</td>
<td>-0.41</td>
</tr>
<tr>
<td>Change in 10 MCP Long PDA score</td>
<td>-0.45</td>
</tr>
</tbody>
</table>

Negative correlations are shown between the change in percentages of CD4+IFNγ+ cells (determined by intracellular cytokine staining using flow cytometry) from baseline to week 12 on treatment and the change in quantitative ultrasound scores for synovial thickening (Trans STA and Long STA) and vascularity (Trans PDA and Long PDA) from baseline to week 12 in RA patients. Correlations were performed using Spearman’s rank method and statistically significant correlations are shown in bold type. 10 MCP Trans PDA, composite transverse power Doppler area score for synovial vascularity of ten metacarpophalangeal joints; 10 MCP Trans STA, composite transverse synovial thickness area score of ten metacarpophalangeal joints.
A positive correlation is shown between baseline serum IL6 levels (determined using MesoScale Discovery Multi-Spot assay) and CRP levels in the RA cohort. Correlations were performed using Spearman's rank method. IL6, interleukin-6; CRP, C-reactive protein.
serum IL6 levels at baseline and the other clinical markers of disease activity; DAS28 score, ESR, swollen joint count or tender joint count.

There were no significant correlations between the baseline levels of the other serum cytokines and chemokines (IL8, TNFα, IL12p70, IL17, IFNγ, TARC, MIP1β, MCP1, MCP4) and clinical measures of disease activity (DAS28 score, CRP, ESR).

8.2.4 Correlations between serum cytokines and ultrasound measures of synovial thickening and vascularity in the RA cohort

There were positive correlations between serum IL6 levels at baseline and baseline semi-quantitative and quantitative ultrasound measures of synovial thickening (Figure 8.5A to D). There were also positive correlations between serum IL6 levels at baseline and baseline semi-quantitative and quantitative ultrasound measures of synovial vascularity (Figure 8.6A to D).

8.2.5 Relationships between clinical, ultrasonographic and T cell immunological parameters in AS and PsA cohorts

There were no significant relationships between the clinical measures of disease activity in the AS cohort (BASDAI, BASFI, BASMI, CRP or ESR) and frequencies of IL17 or IFNγ-producing cells (by ELISpot), or percentages of CD4+IL17+ or CD4+IFNγ+ cells at baseline. There were also no significant correlations between the frequencies of IL17 or IFNγ-producing cells (by ELISpot) or percentages of CD4+IL17+ or CD4+IFNγ+ cells at baseline and GUESS score or power Doppler score of entheses.

There were no significant correlations between changes in the frequencies of circulating Th1 or Th17 cells (determined by ELISpot or FACS) and changes in clinical measures of disease activity or changes in grey scale or power Doppler ultrasound scores of entheses with treatment.

There were no significant correlations between baseline serum cytokine levels and clinical measures of disease activity or ultrasound changes of entheses in the AS cohort.
Figure 8.5  Correlation between baseline serum IL6 levels and ultrasound measures of synovial thickening in RA cohort

Positive correlations are shown between the baseline serum IL6 levels (determined using MesoScale Discovery Multi-Spot assay) and quantitative ultrasound scores for synovial thickening, Trans STA (A), Long STA (B) and the semi-quantitative ultrasound scores for synovial thickening, Trans STi (C) and Long STi (D). Correlations were performed using Spearman’s rank method. 10 MCP Trans PDA, composite transverse power Doppler area score for synovial vascularity of ten metacarpophalangeal joints; 10 MCP Trans STA, composite transverse synovial thickening area score of ten metacarpophalangeal joints; 10MCP Long STA score, composite transverse synovial thickness area score of ten metacarpophalangeal joints; 10MCP Trans STA score, composite transverse synovial thickening area score of ten metacarpophalangeal joints; 10MCP Long STi score, composite longitudinal synovial thickness area score of ten metacarpophalangeal joints; 10MCP Trans STi score, composite...
transverse synovial thickening index score of ten metacarpophalangeal joints; 10MCP Long STi score, composite longitudinal synovial thickening index score of ten metacarpophalangeal joints.
Figure 8.6  Correlation between baseline serum IL6 levels and ultrasound measures of synovial vascularity in RA cohort

Positive correlations are shown between the baseline serum IL6 levels (determined using MesoScale Discovery Multi-Spot assay) and quantitative ultrasound scores for synovial vascularity, Trans PDA (A), Long PDA (B) and the semi-quantitative ultrasound scores for synovial thickening, Trans VASCi (C) and Long VASCi (D). Correlations were performed using Spearman’s rank method. 10MCP Trans PDA score, composite transverse power Doppler area score of ten metacarpophalangeal joints; 10MCP Long PDA score, composite longitudinal power Doppler area score of ten metacarpophalangeal joints; 10MCP Trans VASCi, composite transverse synovial vascularity index of 10 metacarpophalangeal joints; 10MCP Long VASCi, composite longitudinal synovial vascularity index of 10 metacarpophalangeal joints.
There were no significant correlations between baseline frequencies of Th1 or Th17 cells (by ELISpot or FACS) and clinical measures of disease activity at baseline (swollen joint count, tender joint count, CRP, ESR) in the PsA cohort. There were no significant relationships between the baseline frequencies of Th1 or Th17 cells and the semi-quantitative or quantitative ultrasound scores for synovial thickening or vascularity at baseline in the PsA cohort.

There were no significant correlations between changes in the frequencies of circulating Th1 or Th17 cells and changes in clinical measures of disease activity or changes in ultrasound scores for synovial thickening or vascularity with treatment.

There were no significant correlations between baseline serum cytokine levels and clinical measures of disease activity or ultrasound measures of synovial thickening or vascularity in the PsA cohort.

The lack of significant correlations between T cell immunological parameters and clinical or ultrasonographic measures of disease activity in the AS and PsA cohorts may be due to at least two reasons. The small number of patients in the AS and PsA cohort may have precluded from identifying positive correlations. The study of the association between baseline frequencies of Th17 cells in peripheral blood and AS disease activity assessed by BASDAI score has yielded conflicting results in the literature, with some studies detecting no significant correlation in a group of 20 AS patients (Shen et al., 2009), whereas another study reporting a positive correlation in a group of 222 patients (Xueyi et al., 2012). The second likely contributing reason for the lack of significant associations is due to the limitations of the disease activity measures used in AS and PsA as discussed in Chapter 3.

8.3 Discussion

Anti-TNF treatment in the RA patients led to a significant and sustained improvement in the clinical measures of disease activity, as well as morphological improvement in synovial thickening and vascularity determined by power Doppler ultrasound over 12 weeks. Using ELISpot assays and intracellular cytokine staining, it was shown that there was an increase in the frequency of circulating Th17 and Th1 cells at 12 weeks on treatment compared to baseline in this disease group and this occurred in both responders and non-responders to treatment. There was also an apparent trend for anti-TNF non-responders to have a higher baseline frequency of Th17-producing cells compared to anti-TNF responders. These results
provided a basis to further investigate the relationships between the changes in immunological parameters during anti-TNF treatment and treatment response assessed using clinical measures and power Doppler ultrasonography, in order to evaluate immune correlates of treatment response. This is the first study to characterise simultaneously clinical, imaging and T cell immunological parameters longitudinally during anti-TNF therapy and to explore the relationships between them in a cohort of RA patients. The results of this study provide support for two main hypotheses. Firstly, that high baseline numbers of IL17-producing cells are associated with poor anti-TNF treatment response. Consistent with this, higher baseline numbers of IL17-producing cells correlated positively with a smaller improvement in DAS28 score at 1 week on treatment and a smaller improvement in numbers of swollen joints at 1 week on treatment. There were also significant associations between higher numbers of Th17 cells at baseline and a smaller improvement in synovial vascularity on ultrasound at 1 week, as well as a smaller improvement in synovial thickening at 1, 4 and 12 weeks after anti-TNF initiation.

There were significant negative correlations between the change in frequency of IL17-producing cells and Th1 cells at 12 weeks on treatment relative to baseline and the change in synovial thickening and vascularity on ultrasound, supporting the hypothesis that anti-TNF treatment leads to a redistribution of inflammatory cells (Th17 and Th1 cells) from the joints leading to improvement in synovitis.

Another important and novel finding to emerge from this work is the significant positive correlation between serum IL6 levels and measures of synovial thickening and vascularity by power Doppler ultrasound. IL6 plays a role in promoting angiogenesis of the pannus tissue in RA by inducing vascular endothelial growth factor production. Thus the morphological changes in the joints seen by power Doppler ultrasound are directly linked to the immunopathological changes occurring in these joints (Nishimoto and Kishimoto, 2006).

8.3.1 Relationships between baseline numbers of Th17 cells and RA disease activity

Th17 cells are a highly pro-inflammatory cell type which has been implicated in the pathogenesis of RA (Korn et al., 2009; Miossec et al., 2009). Using IL17 ELISpot and intracellular cytokine staining for CD4+IL17+ cells by FACS, the present study demonstrated that patients with RA showed a trend towards a higher frequency of circulating Th17 cells at baseline compared to healthy controls. However, the baseline clinical disease activity
measures (DAS28 score, CRP, ESR) did not correlate significantly with the frequency of IL17-producing or percentages of CD4+IL17+ cells in our RA cohort prior to anti-TNF treatment. This suggests that despite their involvement in RA disease pathogenesis, Th17 cells may have limited utility as a biomarker to indicate RA disease activity. These findings are in agreement with a cross-sectional study in RA patients which also failed to detect significant relationships between the frequency of peripheral blood Th17 cells and clinical measures of disease activity; DAS28, CRP or ESR (Gullick et al., 2010). Both this study and our study however have been performed in patients with established disease, on a variety of treatment regimens, which may have an influence on peripheral blood Th17 levels. Indeed in a recent investigation of patients with early RA, who were treatment naïve, it was found that the frequencies of peripheral blood Th17 cells determined by FACS, correlated positively with DAS28 score and CRP (Leipe et al., 2010). Additionally, RA disease duration may also affect the frequency of peripheral blood Th17 cells and therefore their relationship with clinical measures of disease activity. We found that RA patients with shorter disease duration (less than 3 years) had a significantly higher frequency of IL17-producing cells at baseline compared to patients with long-standing disease, implicating a role for Th17 cells early in RA disease pathogenesis, which has also been observed in other studies (Coulthard et al., 2012; Raza et al., 2005; van Hamburg et al., 2011). Thus, although Th17 cells are involved in RA disease pathogenesis, the extent of their involvement, the roles they play at different stages of RA and different levels of disease activity and the effect of various treatments on their frequency may affect their correlation with clinical measures of disease activity.

Importantly, significant inverse correlations were observed between the baseline frequency of Th17 cells (determined by IL17 ELISpot and FACS) and ultrasound measures of synovial vascularity in the present study. There were no significant correlations between the baseline frequency of Th1 cells and ultrasound measures of synovial thickening. This suggests that higher power Doppler signal within the joints at baseline prior to anti-TNF therapy is associated with lower frequencies of Th17 cells in the peripheral blood and this could be because the majority of these cells may be located in the synovial compartment. In support of this observation, power Doppler ultrasound-defined active synovitis of affected knee joints has been associated with the presence of local CD4+IL17+ cells in the synovial fluid of patients with RA (Gullick et al., 2010). Moreover, the patients who had a high frequency of CD4+IL17+ cells in the synovial fluid were shown to also have high levels of synovial fluid
vascular endothelial growth factor (VEGF) levels and the knee joint PDUS scores correlated strongly with the presence of VEGF in synovial fluid. In contrast, there were no differences between synovial fluid VEGF levels in patients with high or low Th1 cells. This study further demonstrated a role for local IL17 in stimulating VEGF production and angiogenesis using in vitro experiments by showing that IL17 but not IFNγ increased VEGF production by RA synovial fibroblasts (Gullick et al., 2010). The results of this study are also in agreement with our findings of the lack of a significant correlation between the frequencies of circulating Th1 cells and power Doppler signal. Thus the presence of Th17 cells, rather than Th1 cells in the joints may be associated with the presence of power Doppler signal and active synovitis in RA.

A number of histopathological studies have shown an association of positive PDUS signal with neoangiogenesis (Walther et al., 2001; Walther et al., 2002). A role for IL17 in angiogenesis has also been shown in mouse models of RA where local overexpression of IL17 in the joints induced arthritis with increased vascularity and IL17 was also shown to induce the migration of endothelial cells in vitro and increase angiogenesis in Matrigel plugs (Pickens et al., 2010). Both synovial tissue IL17 mRNA and persistent PDUS signal have been independently associated with increased joint damage progression in RA (Kirkham et al., 2006; Naredo et al., 2008a; Taylor et al., 2004). These findings further support the role of Th17 cells as a highly inflammatory and pathogenic cell type involved in RA pathogenesis by promoting angiogenesis, inducing the production of pro-inflammatory cytokines, promoting osteoclastogenesis and cartilage damage (Annunziato et al., 2009b; Miossec et al., 2009; Pickens et al., 2010; Tesmer et al., 2008). In contrast, IFNγ mRNA levels in synovial tissue have shown strong negative correlations with joint damage progression (Kirkham et al., 2006) and Th1 cells have also been shown to inhibit the development of osteoclasts (Sato et al., 2006).

The presence of synovial thickness determined by grey scale ultrasound reflects synovial swelling, but it cannot accurately distinguish between fibrotic tissue and active synovitis (Qvistgaard et al., 2001). In our cohort, there were no significant correlations between baseline frequencies of Th17 cells and synovial thickness ultrasound parameters. By demonstrating the presence of blood flow in small vessels, power Doppler ultrasonography can make this distinction and delineate the presence of highly vascularised synovium. This, combined with the association between positive PDUS signal specifically and the presence of
Th17 cells in the joints (Gullick et al., 2010) may explain the lack of an association between baseline frequencies of Th17 cells and ultrasound measures of synovial thickening.

A number of studies have proven PDUS to be more sensitive and reproducible than clinical examination in assessing joint inflammation (Backhaus et al., 1999; Szkudlarek et al., 2001; Szkudlarek et al., 2006). This may also explain why there was an association between the baseline frequency of circulating Th17 cells and synovial vascularity, but there is no association with the clinical measures of disease activity.

8.3.2 Higher frequency of IL17-producing cells at baseline is associated with poor anti-TNF treatment response in RA patients

Using IL17 ELISpot and intracellular cytokine staining for CD4+IL17+ cells by FACS, a trend was observed for the anti-TNF non-responders to have a higher frequency of Th17 cells at baseline. To investigate this observation further, relationships between the baseline frequency of Th17 cells and clinical measures of improvement in disease activity, as well as ultrasonographic measures of improvement in synovial thickening and vascularity during anti-TNF treatment were sought. There were significant positive correlations between a higher frequency of IL17-producing cells at baseline and a smaller improvement in DAS28 score and a smaller improvement in the numbers of swollen joints 1 week after anti-TNF initiation. In addition, there were also strong positive correlations between higher frequencies of IL17-producing cells (determined by ELISpot) at baseline and a smaller improvement in synovial vascularity and synovial thickening assessed by PDUS 1 week after anti-TNF initiation. The relationship between IL17-producing cells at baseline and synovial thickening was maintained at 4 and 12 weeks after anti-TNF initiation. Similarly, there were also strong positive correlations between higher percentages of CD4+IL17+ cells (determined by FACS) at baseline and a smaller improvement in synovial vascularity 1 week after anti-TNF initiation and a smaller improvement in synovial thickening 1, 4 and 12 weeks after anti-TNF initiation. Two different and complementary techniques have been used in this study to assess the frequency of Th17 cells at baseline (ELISpot and intracellular cytokine staining) and using both methods, there was a significant relationship between a higher baseline frequency of Th17 cells and poor treatment response assessed by ultrasonographic measures which adds strength to the findings.
In contrast, there were no significant relationships between the baseline frequency of Th1 cells and the improvement in clinical or ultrasonographic measures of disease activity. This suggests that a higher frequency of Th17 cells at baseline may be a marker of poor anti-TNF treatment response. Results from a number of other studies also point to an association between higher baseline levels of IL17 or a higher frequency of Th17 cells and poor anti-TNF treatment response in RA; although in these studies this relationship has been investigated by clinical measures of disease activity only and at a single time point on treatment, rather than longitudinally (Alzabin et al., 2012; Chen et al., 2011). In a study of 18 patients with RA, carried out by our group, a significant negative correlation was found between the percentage of Th17 cells in peripheral blood before the start of anti-TNF therapy and the DAS28 score at 4 weeks after therapy (Alzabin et al., 2012). Higher baseline frequencies of Th17 cells and higher baseline levels of serum IL17 levels have also been reported in patients who were found to be anti-TNF non-responders after 6 months of anti-TNF treatment, compared to responders and logistic regression analysis identified baseline IL17 levels as a significant predictor of therapeutic response (Chen et al., 2011). A cross-sectional study investigating the relationships between Th17 cells in the synovial fluid from affected knee joints and local PDUS signal reported that patients who were found to have high levels of Th17 cells in the synovial fluid were also non-responders to anti-TNF therapy (Gullick et al., 2010).

We found a significant positive correlation between the baseline frequency of Th17 cells and the change in synovial vascularity on ultrasound at 1 week after anti-TNF initiation, but this relationship was not significant at weeks 4 and 12 on treatment. In contrast, there was a significant positive correlation between the baseline frequency of Th17 cells and the change in synovial thickening on ultrasound at 1, 4 and 12 weeks after anti-TNF initiation. These differences may be a reflection of the different kinetics of change of synovial thickening and synovial vascularity parameters observed during anti-TNF treatment in this study. Evaluation of the changes in the quantitative ultrasound scores for synovial thickening within the RA cohort in this study revealed significant improvement after 4 and 12 weeks on treatment, whereas the quantitative scores of synovial vascularity improved significantly after 1 week on treatment, with further improvements after 4 and 12 weeks (Chapter 4). These observations also suggest that changes in synovial vascularity assessed by ultrasound may be a marker of early treatment response.

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8.3.3 Improvement in RA disease activity with anti-TNF treatment is associated with an increase in the frequency of circulating Th17 cells

Anti-TNF treatment induced an increase in the frequency of circulating Th1 and Th17 cells during treatment and the increase of these cell types at 12 weeks was statistically significant compared to baseline. This increase was observed both in anti-TNF responder and non-responder patients and in patients treated with etanercept or adalimumab, suggesting that this is a class effect of anti-TNF agents. One of the key mechanisms of action through which anti-TNF therapy is thought to lead to improvement of arthritis has been shown to be through the reduced trafficking of inflammatory cells to inflamed joints as a result of reduced synovial expression of chemokines and adhesion molecules and a reduction in angiogenesis and VEGF expression (Paleolog et al., 1998; Tak et al., 1996; Taylor et al., 2000). In patients with RA, infliximab has been demonstrated to cause a reduction in the cellularity of inflamed synovial tissue, which parallels the rapid reduction in swollen joints and significant reductions in the number of intimal and sublining macrophages, as well as plasma cells and T cells as early as 48 hours after infliximab infusion (Smeets et al., 2003). Anti-TNF treatment in collagen-induced arthritis, a mouse model of RA, has been shown to reduce numbers of Th1 and Th17 cells in inflamed joints, but to increase their numbers in the draining lymph nodes (Notley et al., 2008). Consistent with this mechanism of action, in the RA cohort studied here, there were significant negative correlations between the change in numbers of Th17 cells from baseline to 12 weeks on treatment and the change in ultrasound scores for synovial thickening and vascularity from baseline to 12 weeks. Thus, as the frequency of Th17 cells increases in the peripheral blood with anti-TNF treatment, there is a corresponding decrease in synovial swelling and vascularity. An inverse relationship between the change in Th1 cells from baseline to 12 weeks on treatment and the change in synovial thickening and vascularity from baseline to 12 weeks was also demonstrated. These results suggest that at baseline it is Th17 cells, but not Th1 cells that are associated with poor anti-TNF treatment response, however both Th17 and Th1 cells increase in peripheral blood during anti-TNF treatment in parallel with improvement in synovial thickening and synovial vascularity. These results provide an immunopathological explanation for the improvement in synovial thickening and vascularity observed during anti-TNF therapy.

This is the first study to link the changes in T cell immunopathology assessed by cellular assays with the morphological changes in inflamed joints assessed by power Doppler
ultrasound during anti-TNF treatment. Only one other study to date has related changes in CD3+ cells determined by immunohistochemical staining on synovial samples during anti-TNF treatment to changes in DAS28 scores and changes in MRI scores in a small cohort of PsA patients. There were significant positive correlations between the reductions in synovial CD3 expression in synovial tissue from an affected knee joint after 12 weeks of etanercept treatment and the reduction in DAS28 score and reduction in MRI synovitis score of the same knee joint (Pontifex et al., 2011).

There were no significant relationships between the increase in the frequencies of circulating Th1 and Th17 cells with anti-TNF treatment and improvement in clinical measures of disease activity with treatment; DAS28 scores, CRP or ESR. Power Doppler ultrasound has repeatedly been shown to be more sensitive and reproducible than clinical evaluation in assessing joint inflammation (Backhaus et al., 1999; Szkudlarek et al., 2001; Szkudlarek et al., 2006). PDUS has been shown to identify ongoing synovial inflammation in a cohort of patients defined as being in clinical remission by their treating physician by clinical examination and 60.4% of these patients had active inflammation as confirmed by increased PDUS signal. This further demonstrates the increased sensitivity of PDUS in the detection of synovial inflammation compared with clinical examination alone and confirms that PDUS provides more objective information to clinical disease activity markers alone (Brown et al., 2006). This may explain the lack of a significant association between the changes in the frequencies of Th1 and Th17 cells during anti-TNF treatment and the change in clinical disease activity scores with treatment.

8.3.4 Relationships between serum IL6 levels and systemic and ultrasonographic measures of RA disease activity

In the present study a significant positive correlation was observed between serum IL6 levels and CRP at baseline in the RA cohort. IL6 is a proinflammatory mediator and has been shown to play a central role in the pathogenesis of RA (Nishimoto and Kishimoto, 2006). It increases the production of acute phase proteins, such as CRP, fibrinogen and serum amyloid A from hepatocytes and a number of other studies have reported a positive correlation
between serum IL6 levels and CRP, linking this cytokine to systemic inflammation (Charles et al., 1999; Houssiau AF, 1988; Madhok R, 1993).

There were no significant correlations between the change in serum IL6 levels with anti-TNF treatment and the change in serum CRP levels in the RA cohort at any of the time points. Another study reported a significant correlation between the reduction in serum IL6 level on day 3 after infliximab infusion and the reduction in CRP at the same time point in a group of RA patients (Charles et al., 1999). This study also reported that the rate of fall of IL6 levels was much more rapid than that for CRP levels. The later time points in our study may explain why there were no significant correlations between the change in serum IL6 levels and the change in CRP with anti-TNF treatment.

There were also significant positive correlations between baseline serum IL6 levels and the semi-quantitative and quantitative markers of synovial thickening and vascularity on ultrasound. Only one other study has linked serum IL6 levels to the degree of synovial vascularity assessed by power Doppler ultrasound and this study was carried out in 19 patients with juvenile chronic arthritis (Shahin et al., 2002). Correlations between power Doppler ultrasound measures of synovial vascularity and clinical measures of disease activity have been extensively reported (Filippucci et al., 2006; Ribbens et al., 2003; Salaffi et al., 2008), however no other studies to our knowledge have investigated relationships between serum cytokines and power Doppler activity in RA patients. These relationships are consistent with the roles of IL6 in RA immunopathogenesis. IL6 has been found to induce production of VEGF synergistically with TNFα, to activate and recruit T cells by regulating chemokine secretion and to induce the expression of adhesion molecules, thus having a central role in inflammation (Nishimoto and Kishimoto, 2006).

8.4 Conclusion

This is the first study to investigate the relationships between the changes in the frequencies of circulating Th1 and Th17 cells and the changes in clinical and ultrasonographic measures of disease activity during anti-TNF treatment in RA patients. Higher frequencies of Th17 cells at baseline, but not Th1 cells, were demonstrated to be associated with poorer anti-TNF
treatment response in the RA cohort. Higher baseline frequencies of Th17 cells correlated positively with smaller improvements in DAS28 score, swollen joint counts and synovial thickness and vascularity scores on ultrasound with anti-TNF treatment. These findings, together with our observation that anti-TNF non-responders in the RA group have a trend towards higher baseline frequencies of Th17 cells suggest that Th17 cells may be a marker of anti-TNF non-response. Significant negative correlations were also observed between the change in frequency of Th1 and Th17 cells from baseline to 12 weeks on anti-TNF treatment and the change in synovial thickness and vascularity over the same period of time. This suggests that anti-TNF treatment induces a redistribution of inflammatory Th1 and Th17 cells from inflamed joints, which leads to improvement of arthritis and of the synovial swelling and inflammation detected by ultrasound. These results provide an immunopathological explanation for the improvement in synovial thickening and vascularity observed during anti-TNF therapy. This observation is consistent with the mechanism of action of anti-TNF agents in inducing an efflux of inflammatory cells from inflamed joints through a reduction in the expression of chemokine and adhesion molecules in the synovium, as well as reduced angiogenesis (Paleolog et al., 1998; Tak et al., 1996; Taylor et al., 2004).

These findings require confirmation in larger study cohorts of RA patients and also investigation of whether similar relationships exist in larger cohorts of patients with AS and PsA. In addition, studying whether patients with higher baseline frequencies of Th17 cells may have more IL17-driven disease and whether such patients would derive greater benefit from treatment with biologic agents targeting IL17 is warranted. If these findings are confirmed, the characterisation of Th17 cells as a marker of anti-TNF non-response raises the possibility of being able to individualise treatment of inflammatory arthritis in the future.
Chapter 9. General Discussion

The objective of the work presented in this thesis was to conduct a prospective, longitudinal investigation of patients with inflammatory arthritis during 12 weeks of anti-TNF treatment using clinical, imaging and T cell immunological assessments to gain an understanding of the immune correlates of treatment response or failure. Specifically, the main aims were firstly to prospectively follow a cohort of patients with RA, AS or PsA at pre-determined protocol visits to evaluate changes in clinical disease activity measures as well as morphological changes in the target tissue by power Doppler ultrasound (PDUS) during the first 12 weeks of anti-TNF treatment, thus robustly characterising treatment response. Secondly, the next aim was to determine the effect of anti-TNF treatment on the frequency and phenotype of circulating Th1 and Th17 cells in this patient cohort using two different but complementary cell-based assays; ELISpot and intra-cellular cytokine staining. Thirdly, the aim was to investigate relationships between the clinical, ultrasonographic and T cell immunological changes during anti-TNF treatment in the RA cohort in order to determine the immunopathological correlates underlying treatment response or failure.

Anti-TNF treatment has revolutionised the management of inflammatory arthritis, it leads to improvement in arthritis signs and symptoms in RA, AS and PsA, as well as slowing radiographic progression in RA and PsA (Singh JA, 2009). However, despite its success in the majority of patients with inflammatory arthritis, about 20-30% of patients do not respond to anti-TNF treatment or experience significant side effects, such as infections (Hyrich et al., 2006). Assessing inflammatory arthritis disease activity accurately is vital in order to be able to adjust treatment regimens with the aim to ‘treat to target’ and achieve low disease activity or induce remission and in the long term prevent joint damage (Smolen et al., 2010). A number of disease–specific composite clinical disease activity scores have been developed, but each has its limitations in that each score, such as DAS28, PsARC and the Bath Indices commonly used in RA, PsA and AS respectively, comprise components which can be subjective, insensitive to change, only assess a limited number of the joints involved, or limited aspects of the disease process (Anderson et al., 2011; Mease, 2011b; van Tubergen and Landewe, 2009). Power Doppler ultrasound has become an invaluable tool in rheumatology as multiple studies have demonstrated its increased sensitivity in the detection of synovial and entheseal inflammation compared with clinical examination alone (Backhaus...
et al., 1999; D'Agostino et al., 2003; Szkudlarek et al., 2001; Szkudlarek et al., 2006). Moreover, PDUS has been shown to also be more sensitive than clinical outcome measures in evaluating treatment response (de Miguel et al., 2009; Naredo et al., 2008a; Ribbens et al., 2003; Salaffi et al., 2008; Taylor et al., 2004).

Th17 cells are a highly pro-inflammatory T helper cell subset, which have been shown to contribute to arthritis pathogenesis (Korn et al., 2009; Miossec et al., 2009). The signature cytokine of Th17 cells, IL17, has pleiotropic effects on effector cells of the immune system and induces the production of other pro-inflammatory cytokines, such as TNFα, IL1β, IL6 by macrophages and is strongly synergistic in its actions with these cytokines (Korn et al., 2009). IL17 also contributes to cartilage and bone damage by promoting the release of matrix metalloproteinases, and increases osteoclast differentiation leading to bony erosions and mediates angiogenesis in inflamed joints (Anunziato et al., 2009b; Miossec et al., 2009; Pickens et al., 2010; Tesmer et al., 2008). In collagen-induced arthritis, a mouse model of RA, anti-TNF treatment was shown to increase the numbers of Th1 and Th17 cells in the draining lymph nodes, while reducing numbers of these cells in inflamed paws and leading to arthritis improvement (Notley et al., 2008). This effect of anti-TNF treatment on circulating Th17 cells has also been reported in patients with inflammatory arthritis, including RA (Aerts et al., 2010; Alzabin et al., 2012) and AS (Xueyi et al., 2012). In addition, a number of early studies investigating the effects of anti-TNF treatment on the frequency of circulating Th1 cells have also reported an increase in this cell type in patients with RA (Maurice et al., 1999; Nissinen et al., 2004) and AS (Baeten et al., 2001; Zou et al., 2003). However, each of these studies has investigated the changes in the frequency of Th17 or Th1 cells during anti-TNF therapy in one type of inflammatory arthritis only and the majority of the studies have investigated the changes in cytokine-producing cells at a single time point on anti-TNF treatment compared to baseline, rather than longitudinally. Furthermore, in these studies, disease activity has also been assessed using only composite clinical disease activity scores and only two studies have related the immunological changes observed with anti-TNF to treatment response (Chen et al., 2011; Xueyi et al., 2012).

This is the first study to characterise longitudinally the changes in clinical and ultrasonographic measures of disease activity alongside changes in the frequency of circulating Th1 and Th17 cells simultaneously in 3 different types of inflammatory arthritis and to investigate the relationships between them.
As part of the prospective clinical study, 25 patients with RA, 15 patients with AS and 8 patients with PsA were recruited and followed at predetermined protocol visits. This allowed the determination of the effect of anti-TNF treatment on validated clinical disease activity measures in patients with RA, AS and PsA and the results are detailed in Chapter 3. Using established and validated disease-specific definitions of treatment response, we identified anti-TNF responders and non-responders within each disease group. The anti-TNF non-response rate was around 30% as would be expected (Villeneuve and Haraoui, 2010), thus confirming that our patient populations are representative of others. In addition, the changes in the composite clinical disease activity measures observed during anti-TNF therapy in the two larger cohorts; RA and AS patients were in line with the reported changes in these parameters from a number of the clinical trials of anti-TNF therapies (Table 3.11 and Table 3.13) (Brandt et al., 2000; Braun et al., 2002; Elliott M.J., 1993; Elliott et al., 1994; Maini et al., 1998).

The present study is also the first longitudinal ultrasound investigation using both semi-quantitative and quantitative measures of synovial thickening and vascularity to characterise changes in the MCP joints of patients with RA and PsA during anti-TNF treatment including shorter term (1 week and 4 weeks) and medium term (12 months) endpoints. In addition, we used both grey scale and power Doppler ultrasound to characterise changes in lower limb enthesal pathology of AS patients longitudinally during anti-TNF treatment (Chapter 4). Thus, using PDUS, we were able to assess quantitatively the evolution of morphological changes in the target tissue at multiple time points during the first 12 weeks on anti-TNF treatment. The use of PDUS provided an objective and sensitive measure of inflammation in the joints and entheses in RA, PsA and AS patients which was shown to be highly reproducible (Table 4.1, Table 4.2 and Table 4.3). PDUS endpoints complemented the information obtained from changes in clinical outcome measures during anti-TNF treatment and allowed further characterisation of anti-TNF responders and non-responders as described in Chapter 4. In the RA cohort, there were strong positive correlations between the semi-quantitative and quantitative ultrasound measures of synovial thickening and vascularity and numbers of swollen joints on examination. There were also strong positive correlations between the semi-quantitative and quantitative ultrasound scores for synovial vascularity and DAS28 scores, thus confirming the validity of PDUS as a biomarker of synovitis (Table 4.4). These findings are in agreement with other studies (Backhaus et al., 1999; Ellegaard et al.,
2009; Larché et al., 2010; Szkudlarek et al., 2001; Szkudlarek et al., 2006; Walther et al., 2001; Walther et al., 2002). The ultrasound scores for synovial thickness and vascularity improved during anti-TNF treatment in the RA cohort, but exhibited different kinetics of change with treatment, with the synovial vascularity measures showing earlier and more marked improvement compared with the measures of synovial thickening (Table 4.5). Power Doppler signal has been shown to reflect the vascularisation of the pannus in RA and to correlate with histological changes of synovitis and synovial membrane microvascular density (Walther et al., 2001; Walther et al., 2002). One of the mechanisms of action of anti-TNF agents is through the reduction of neovascularisation and angiogenesis in the synovial tissue by reducing VEGF expression (Paleolog et al., 1998). Thus anti-TNF appears to act rapidly to reduce synovial vascularity and therefore inflammation, which is reflected by the improvement in ultrasound measures of vascularity. The reduction in synovial thickness assessed by grey scale ultrasonography is a slower process as it is likely to represent a decrease in the swelling and inflammation of the synovium, which is a combination of reduction in the infiltration of inflammatory cells in the joints, reduced expression of inflammatory cytokines and chemokines, as well as a reduction in synovial tissue vascularity (Tak et al., 1996; Taylor et al., 2000; Wong et al., 2008).

There was a clear difference between anti-TNF responders and non-responders in the change in ultrasound measures of synovial thickening and vascularity during anti-TNF treatment (Figures 4.2-4.9). Thus, these measures were able to objectively differentiate between anti-TNF responders and non-responders. Responders demonstrated a significant improvement in synovial thickening and vascularity at 1, 4 and 12 weeks on treatment, whereas there were no significant changes in these parameters in the non-responder group. The ultrasound measures of synovial vascularity were better able to discriminate between the responder and non-responder groups compared to ultrasound measures of synovial thickening, which has also been shown by others (Larché et al., 2010; Seymour et al., 2012; Seymour, 2012; Taylor et al., 2004).

In the present study, we used two different and complementary functional cell-based assays; ELISpot and intracellular-cytokine staining in order to evaluate changes in the frequency of circulating Th17 and Th1 cells during anti-TNF treatment (Chapters 5 and Chapter 6). By using the IL17 ELISpot assay we were able for the first time to determine changes in the frequency of circulating IL17-producing cells in patients with inflammatory arthritis during
anti-TNF therapy. The advantages of the ELISpot assay is that it provides an objective analysis of cellular function, it is reproducible (Table 5.3 and Table 5.4) and particularly suited to studying low frequency responses, such as IL17-producing cells because of its high sensitivity (Lehmann and Zhang, 2012).

At baseline, patients with RA, AS and PsA showed a trend towards having a higher frequency of Th17 cells compared to healthy controls (Figure 5.3A and Figure 6.1). This is supported by a number of other studies which have demonstrated increased frequencies of Th17 cells in the peripheral blood of patients with AS, RA or PsA by flow cytometry, or increased IL17 production by ELISA of cell culture supernatants from these disease groups compared to healthy controls (Chen et al., 2011; Gullick et al., 2010; Jandus et al., 2008; Leipe et al., 2010; Shen et al., 2009; Xueyi et al., 2012). We also investigated the relationships between the frequency of Th17 cells and clinical and ultrasonographic measures of disease activity in the RA cohort at baseline (Chapter 8). While there were no significant correlations between the baseline clinical disease activity measures (DAS28 score, CRP, ESR) and the frequency of circulating Th17 cells at baseline in our RA cohort, there were significant inverse correlations between the baseline frequency of Th17 cells (determined by IL17 ELISpot and FACS) and ultrasound measures of synovial vascularity. This shows that higher power Doppler signal within the joints at baseline is associated with lower frequencies of Th17 cells in the peripheral blood, suggesting that the majority of these cells may be located in the synovial compartment. Consistent with this notion, a cross sectional study in RA patients highlighted PDUS-defined synovitis as a possible surrogate marker for Th17 cells (Gullick et al., 2010). PDUS-defined synovitis of affected knee joints in RA was associated with the presence of CD4+IL17+ cells in the synovial fluid and the patients with a higher frequency of synovial CD4+IL17+ cells also had higher synovial VEGF levels. These findings are also consistent with the role of IL17 in inducing angiogenesis in an animal model of RA (Pickens et al., 2010).

Using ELISpot and intra-cellular cytokine staining, we demonstrated an increase in the frequency of Th17 cells and Th1 cells during anti-TNF treatment in patients with RA, AS and PsA (Chapter 5 and Chapter 6). These results were obtained using two different techniques and were consistent, thus strengthening our findings. The observed increase in the frequency of Th1 cells during anti-TNF treatment is in agreement with a number of studies (Maurice et al., 1999; Nissinen et al., 2004; Zou et al., 2003). The increase in IL17-producing cells with anti-TNF treatment has also been reported by others (Aerts et al., 2010; Alzabin et al., 2012).
The increase in circulating Th1 and Th17 cells observed during anti-TNF therapy occurred in the three different disease groups and the time course of the increase was similar in patients treated with etanercept and those treated with adalimumab. Etanercept and adalimumab differ in structure and pharmacological properties. Adalimumab is a humanised monoclonal antibody, whereas etanercept is a TNF receptor fusion protein linked to the Fc portion of human IgG and although both compounds can neutralise soluble TNF, they differ in other pharmacological properties, including the ability to bind transmembrane TNF, form complexes and to induce antibody-dependent cellular cytotoxicity (Taylor, 2010). Despite these differences treatment with both agents induced an increase in Th1 and Th17 cells, which suggests that this is a class effect of anti-TNF agents rather than being agent-specific.

We further investigated the relationships between the changes in the frequency of circulating Th1 and Th17 cells and the changes in the clinical and ultrasonographic measures of disease activity during anti-TNF treatment in the RA cohort (Chapter 8). There were significant negative correlations between the change in numbers of Th17 cells (determined by IL17 ELISpot and FACS) from baseline to 12 weeks on treatment and the change in ultrasound scores for synovial thickening and vascularity from baseline to 12 weeks (Table 8.1). Thus, as the frequency of Th17 cells increases in the peripheral blood with anti-TNF treatment, there is a corresponding decrease in synovial swelling and vascularity. An inverse relationship between the change in Th1 cells (determined by IFNγ ELISpot and FACS) from baseline to 12 weeks on treatment and the change in synovial thickening and vascularity from baseline to 12 weeks was also demonstrated (Table 8.2). Our results suggest that increases in both Th17 and Th1 cells in the peripheral blood during anti-TNF treatment are associated with improvement in synovial thickening and synovial vascularity. These findings provide an immunopathological explanation for the improvement in synovial thickening and vascularity observed during anti-TNF therapy in patients with RA. This is the first study to link changes in T cell immunopathology assessed by cellular assays with the morphological changes in inflamed joints assessed by power Doppler ultrasound during anti-TNF treatment. These correlations are consistent with the mechanism of action of anti-TNF agents. One of the key mechanisms of action through which anti-TNF has been shown to lead to arthritis improvement is through the reduction in trafficking of inflammatory cells to joints through the reduced synovial expression of chemokines and adhesion molecules and also reduced angiogenesis and synovial VEGF expression (Paleolog et al., 1998; Tak et al., 1996; Taylor et
al., 2000). In patients with RA, infliximab causes a reduction in the cellularity of inflamed synovial tissue, with significant reductions in the number of intimal and sublining macrophages as well as plasma cells and T cells which parallels the rapid reduction in swollen joints as early as 48 hours after infliximab infusion (Smeets et al., 2003). Consistent with this mechanism, analysis of the changes in serum chemokine levels including TARC, MIP1β and IP10 in the RA cohort revealed significant decreases during anti-TNF treatment in the whole cohort and these changes were also more marked and sustained in the treatment responders (Table 7.4) (Chapter 7). Thus the negative correlation between the increase in peripheral blood Th1 and Th17 cells with anti-TNF treatment and the decrease in synovial thickness and vascularity on ultrasound suggests that anti-TNF treatment induces an efflux of inflammatory cells from the joints leading to improvement in swelling and inflammation.

Another possible mechanism through which anti-TNF may cause an increase in circulating Th1 and Th17 cells is through the increase in the p40 subunit shared between IL12 and IL23, the key cytokines involved in the differentiation of Th1 and Th17 cells respectively. A study by our group using the collagen-induced arthritis (CIA) mouse model of RA reported that anti-TNF therapy ameliorated arthritis by decreasing numbers of Th1 and Th17 cells in arthritic joints, but also caused an increase in Th1 and Th17 cells in the draining lymph nodes (Notley et al., 2008). By using knockout mice, the increase in Th1 and Th17 cells was shown to occur through signalling via the TNFp55 receptor, which increased expression of the p40 subunit shared between IL12 and IL23. A similar mechanism was found to occur in a mouse model of reactive arthritis, where Yersinia-induced reactive arthritis in mice lacking TNFR p55 was associated with more severe disease than in wild type animals. Increased levels of IL17, IFNγ, IL23 and IL12p70 were found in the arthritic joints of these mice and antibody blockade of IL17 and IFNγ was shown to reduce arthritis severity. The increase in Th1 and Th17 responses in the TNFRp55/- mice was shown to be mediated by an increase in IL12/23p40 (Eliçabe et al., 2010). The results from these two studies in animal models of arthritis suggest that in addition to its proinflammatory role, TNFα may also have a role in limiting Th1 and Th17 responses in the context of autoimmune arthritis, which may represent a regulatory negative feedback mechanism serving to limit the duration of T cell driven inflammation. Indeed, in vitro studies have also shown that TNFα can selectively inhibit p40 expression in human and mouse myeloid cells in an IL10-independent manner and this effect was also shown to not be due to TNFα-induced apoptosis (Ma et al., 2000; Zakharova and Ziegler, 2005). Moreover, TNFα-induced p40 inhibition was shown to be cytokine-specific
as TNFα did not inhibit other proinflammatory cytokines, IL12p35, IL1β or IL6 (Ma et al., 2000).

TNFα-mediated inhibition of IL12/23p40 may also occur in human disease. A study by our group in RA patients treated with anti-TNF agents showed that the increase in circulating Th17 cells at 8-12 weeks on anti-TNF therapy was accompanied by an increase in IL12/23p40 production in supernatants from PBMCs stimulated with lipopolysaccharide (LPS) and also in the plasma layer of whole blood stimulated with LPS at 8-12 weeks on treatment (Alzabin et al., 2012). Serum levels of IL12/23p40 in the RA cohort in the present study also showed a trend towards an increase with anti-TNF treatment, although this was not statistically significant (Figure 7.6) (Chapter 7). In AS and PsA patients, there were no significant changes in serum levels of IL12/23p40 during treatment. This is consistent with the results from a small study in 14 patients with AS which failed to detect a change in serum IL12/23p40 levels after 10 weeks of anti-TNF therapy (Wendling et al., 2009).

Taken together, these findings suggest that anti-TNF agents may act through several mechanisms to lead to an increase in circulating Th1 and Th17 cells during treatment and these may potentially differ in the different types of inflammatory arthritis. Further evaluation of the role of this cytokine subunit and its change during anti-TNF treatment in larger cohorts of patients with different types of inflammatory arthritis is needed.

Interestingly, in AS and PsA patients, but not in RA patients there was also a significant increase in the frequency of CD8+IFNγ+ cells during anti-TNF treatment (Figure 6.14E and Figure 6.17C), which has also been reported by others (Baeten et al., 2001; Maurice et al., 1999; Zou et al., 2003). These differences between RA and the spondyloarthropathies in the increase in CD8+IFNγ+ cells during anti-TNF therapy may be due to the differing pathogenesis of these conditions and the greater contribution of innate immune responses and autoinflammation to spondyloarthritis pathogenesis compared to RA (Ambarus C, 2012; Lories and Baeten, 2009).

No significant changes were observed in the phenotype of circulating Th17 cells with anti-TNF treatment with respect to the expression of the memory marker, CD45RO and homing receptor, CCR6 (Chapter 6). However, at baseline patients with inflammatory arthritis were demonstrated to have a significantly higher expression of CD45RO and CCR6 on the surface
of Th17 cells compared to healthy controls (Figure 6.3 and Figure 6.4), suggesting that the Th17 cells in these disease states are highly differentiated. The increased expression of the chemokine receptor CCR6 may facilitate the homing of these cells to inflamed tissues (Hirota et al., 2007; Matsui et al., 2001). The increased expression of CD45RO and CCR6 on Th17 cells in patients with inflammatory arthritis compared to healthy controls has also been reported by others (Homey et al., 2000; Jandus et al., 2008; Leipe et al., 2010; Matsui et al., 2001).

Another interesting finding to emerge from this study was that anti-TNF non-responders in the RA cohort showed a trend towards a higher baseline frequency of Th17 and Th1 cells compared to responders and this trend was observed using results from both ELISpot (Figure 5.10) and intra-cellular cytokine staining (Figure 6.12) (Chapter 5 and Chapter 6). Two other studies have suggested that a higher baseline frequency of Th17 cells (Alzabin et al., 2012) or higher serum IL17 levels (Chen et al., 2011) in RA patients may be associated with poor anti-TNF treatment response, which was assessed by change in DAS28 score from baseline. We investigated this hypothesis further by exploring relationships between clinical, ultrasonographic and T cell immunological changes during anti-TNF therapy to determine if a higher frequency of IL17 or IFN-γ-producing cells at baseline was associated with poor treatment response (Chapter 8). Indeed, higher baseline numbers of IL17-producing cells correlated positively with a smaller improvement in DAS28 score at 1 week on treatment and a smaller improvement in numbers of swollen joints at 1 week on treatment (Figure 8.1). Furthermore, we also found significant correlations between higher numbers of Th17 cells at baseline and a smaller improvement in synovial vascularity on ultrasound at 1 week, as well as a smaller improvement in synovial thickening at 1, 4 and 12 weeks after anti-TNF initiation (Figure 8.2). In contrast, there were no significant correlations between the baseline frequency of Th1 cells and improvement in clinical or ultrasonographic measures of disease activity. This suggests that a higher frequency of Th17 cells, but not Th1 cells at baseline is associated with poor anti-TNF treatment response. We have used two different and complementary techniques (ELISpot and intracellular cytokine staining) to assess the frequency of Th17 cells prior to anti-TNF initiation and using both methods, we found a significant relationship between a higher baseline frequency of Th17 cells and poor treatment response assessed by ultrasonographic measures. If these associations are also confirmed in larger patient cohorts and in patients with other types of inflammatory arthritis aside from
RA, the characterisation of Th17 cells as a marker of anti-TNF non-response raises the possibility of being able to tailor biologic therapy of inflammatory arthritis according to individual patient immunological profile. Thus although it was the change in both Th17 and Th1 cells from baseline that was associated with improvement in clinical and ultrasonographic measures of disease activity, it was a higher baseline frequency of Th17 cells only that was associated with poorer treatment response. Further investigations into whether those patients with higher baseline frequencies of circulating Th17 cells may have more IL17-driven disease and whether these patients may derive greater benefit more from treatment with anti-IL17 biologics or a combination of anti-IL17 and anti-TNF agents is warranted.

In the present study, we also characterised longitudinally changes in a panel of serum cytokines during anti-TNF treatment in all three disease groups using multiplex assays and ELISA (Chapter 7). Serum IL6 was the cytokine that showed the most pronounced and significant changes, demonstrating significant reduction with anti-TNF treatment in RA and AS cohorts (Figures 7.2 and 7.11) and a trend towards a decrease in the PsA cohort (Table 7.7). In the RA cohort, the reduction in serum IL6 levels was more marked and sustained in anti-TNF responders compared to non-responders. In this patient cohort, there was a significant positive correlation between serum IL6 levels and CRP at baseline. IL6 is a proinflammatory mediator and has been shown to play a central role in the pathogenesis of RA (Nishimoto and Kishimoto, 2006). It increases the production of acute phase proteins, such as CRP, fibrinogen and serum amyloid A from hepatocytes and a number of other studies have reported a positive correlation between serum IL6 levels and CRP (Charles et al., 1999; Houssiau AF, 1988; Madhok R, 1993). There were also significant positive correlations between baseline serum IL6 levels and the semi-quantitative and quantitative markers of synovial thickening and vascularity on ultrasound in the RA cohort, consistent with the known role of IL6 in inducing production of VEGF synergistically with TNFα (Nishimoto and Kishimoto, 2006). Only one other study in patients with juvenile chronic arthritis has linked serum IL6 levels to the degree of synovial vascularity as assessed by power Doppler ultrasound (Shahin et al., 2002).

There was also a significant increase in serum TNFα levels in patients with RA and AS during treatment, but a decrease in serum TNFRI and II levels. The results of the changes in serum cytokines during anti-TNF therapy are in agreement with other published studies.
confirming that our cohorts are representative patient populations for the study of the effects of anti-TNF therapy.

Our findings in the present study raise a number of specific questions which warrant further investigation. Studies investigating whether the changes in Th1 and Th17 cells observed during anti-TNF therapy are a transient phenomenon or whether they are long-lasting are needed. A study in RA patients found that responders to anti-TNF therapy at 6 months had a lower frequency of Th17 cells than non-responders compared to baseline (Chen et al., 2011). Thus although in our study at 12 weeks both responders and non-responders to anti-TNF demonstrated an increase in Th1 and Th17 cells, it may be that at later time points the regulation of the increased frequency of Th1 and Th17 cells may differ between those patients who are responding and those who are not responding to anti-TNF. Furthermore, elucidation whether this increased frequency of Th1 or Th17 cells has pathogenic potential is needed to determine whether the increase in these cell types may underlie the development of certain side effects or the relapse of symptoms once anti-TNF is stopped. Further studies investigating the changes in the frequency and expression of Th1 or Th17 cells and their cytokines at the site of disease activity in parallel to changes in the peripheral blood and the relationships between these changes with treatment response would provide direct insight into the changes occurring in the target tissue during treatment.

9.1 Concluding remarks

In this longitudinal prospective study, a cohort of 25 patients with RA, 15 patients with AS and 8 patients with PsA were followed at predetermined protocol visits during the first 12 weeks of anti-TNF treatment. Improvement in composite disease activity scores from baseline were used to define treatment responders and non-responders. Semi-quantitative and quantitative ultrasound measures demonstrated reduction in synovial thickening and vascularity during anti-TNF treatment but showed different kinetics of change, with synovial vascularity measures showing a more rapid and marked reduction than synovial thickening measures. Using IL17 and IFN\(\gamma\) ELISpot assays and intra-cellular cytokine staining of PBMCs, anti-TNF treatment was shown to induce an increase in the frequency of circulating Th1 and Th17 cells from baseline in all three disease groups. Using multiplex assays and ELISA, anti-TNF treatment was shown to lead to a significant decrease in serum
inflammatory cytokines, IL6 and IL8, as well as decreasing serum chemokines, including TARC, IP10 and MIP1β. Investigations of the relationships between the clinical, ultrasonographic and T cell immunological changes during anti-TNF therapy revealed negative correlations between the changes in the frequency of Th1 and Th17 cells from baseline to 12 weeks on treatment and the changes in ultrasound measures of synovial thickening and vascularity from baseline to 12 weeks on treatment. Thus the increase in peripheral blood Th17 and Th1 cells during anti-TNF treatment was associated with a reduction in synovial thickening and vascularity. These results provide an immunopathological explanation for the improvement in synovial thickening and vascularity observed during anti-TNF therapy.

Higher baseline frequencies of Th17 cells correlated positively with smaller improvements in DAS28 score, swollen joint counts and synovial thickness and vascularity scores determined by PDUS during anti-TNF treatment. Higher baseline frequencies of Th17 cells, but not Th1 cells were shown to be associated with a poorer treatment response, characterised by both clinical and ultrasound parameters, suggesting Th17 cells may be a marker of poor response to TNF blockade. The findings in the present study, if confirmed in large patient cohorts, indicate the characterisation of a marker of anti-TNF non-response and raise the possibility of being able to individualise treatment of inflammatory arthritis in the future.
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