Regulatory T cell control of immune responses
during acute *Streptococcus pyogenes* infection

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**Abstract**

*Streptococcus pyogenes*, also known as Group A Streptococci (GAS) causes many diseases, ranging from non-invasive (“Strep throat) to highly invasive (bacterial sepsis). GAS secretes various virulence factors implicated in pathogenesis and immune evasion, the best studied include superantigens. Regulatory T cells (T\(_{\text{regs}}\)) are a subset of CD4\(^+\) T cells, previously described in maintaining the fine balance of immune responses during infectious diseases; however, the precise role during streptococcal infection is unknown. The work in this thesis involves the characterisation of T\(_{\text{regs}}\) in the pathogenesis of GAS infection. Depletion of T\(_{\text{regs}}\) in inducible Foxp3.DTR knockout mice during GAS infection showed significant reduction in systemic bacterial spread and decreased serum levels of pro-inflammatory cytokines (IL-6 and IFN-\(\gamma\)), suggesting T\(_{\text{regs}}\) are detrimental to the host response to this bacterium. Further examination of effector cells indicate a protective role for CD8\(^+\), \(\gamma\delta\)\(^+\) T cells and NK cells in this model of acute sepsis as depletion of either of these subsets exacerbated bacterial burden and altered cytokine production. Utilising a transgenic HLA-DQ8.AB\(^0\) murine model, previously demonstrated to be susceptible to streptococcal superantigens, up-regulation of Foxp3 gene and protein expression in secondary lymphoid tissues during early stages of infection was attributed to the presence of SMEZ superantigen, correlating with decreased bacterial load. SPEA superantigen was found to induce T\(_{\text{H}1}\) cells and enhanced IFN-\(\gamma\) production to lead to the so-called “cytokine storm” and increased bacterial burden. This suggests that GAS through the action of secreted superantigens, modulates the immune response to induce T\(_{\text{regs}}\) and establish a state of tolerance to hinder protective effector cells, such as CD8\(^+\), \(\gamma\delta\)\(^+\) T cells and NK cells and IL-17A production, while promoting pathogenic T\(_{\text{H}1}\) cells to drive potent immune pathology.
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For my parents
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<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AG</td>
<td>Acute Glomerulonephritis</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl Hydrocarbon Receptor</td>
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<td>AMP</td>
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<td>DCs</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DKO</td>
<td>Double Knock Out</td>
</tr>
<tr>
<td>DLN</td>
<td>Draining Lymph Node</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide Triphosphates</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran Sulfate Sodium</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria Toxin</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-Type Hypersensitivity</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>EAU</td>
<td>Experimental Autoimmune Uveoretinitis</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence Minus One</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>GAS</td>
<td>Group A Streptococci</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-Induced TNFR-Related protein</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HSPs</td>
<td>Heat Shock Proteins</td>
</tr>
<tr>
<td>i.m</td>
<td>intra-muscular</td>
</tr>
<tr>
<td>i.p</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>iNKT cell</td>
<td>invariant Natural Killer T cell</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 Receptor-Associated Kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous Immunoglobulin</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo-basepair</td>
</tr>
<tr>
<td>KIRs</td>
<td>Killer-cell Immunoglobulin-like Receptors</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>L-Glut</td>
<td>L-Glutamine</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte-Activation Gene 3</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-Associated Peptide</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine Rich Region</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal Antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Melanoma Differentiation-Associated protein 5</td>
</tr>
<tr>
<td>mDCs</td>
<td>Myeloid DCs</td>
</tr>
<tr>
<td>MFI</td>
<td>Median Fluorescence Intensities</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIC-A/B</td>
<td>MHC class I polypeptide-related sequence A/B</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation primary response gene 88</td>
</tr>
<tr>
<td>NALT</td>
<td>Nasal-Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>NETs</td>
<td>Neutrophil Extracellular Traps</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-Like Receptors</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding Oligomerisation Domain</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen Associated Molecular Patterns</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pDCs</td>
<td>plasmacytoid Dendritic Cells</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination Activating Genes</td>
</tr>
<tr>
<td>REST</td>
<td>Relative Expression Software Tool</td>
</tr>
<tr>
<td>RHD</td>
<td>Rheumatic Heart Disease</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid Inducible Gene</td>
</tr>
<tr>
<td>RLRs</td>
<td>RIG-I-Like Receptors</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RORγt</td>
<td>RAR-related Orphan Receptor gamma t</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SE-A/B/C</td>
<td>Staphylococcal Enterotoxins-A/B/C</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SLO</td>
<td>Streptolysin O</td>
</tr>
<tr>
<td>SLS</td>
<td>Streptolysin S</td>
</tr>
<tr>
<td>SMEZ</td>
<td>Streptococcal Mitogenic Exotoxin Z</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor Of Cytokine Signaling</td>
</tr>
<tr>
<td>SPE-A/B</td>
<td>Streptococcal Pyrogenic Exotoxin-A/B</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>STSS</td>
<td>Severe Toxic Shock Syndrome</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TDO</td>
<td>Tryptophan-2,3-dioxygenase</td>
</tr>
<tr>
<td>T&lt;sub&gt;eff&lt;/sub&gt;</td>
<td>T effector cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T Helper</td>
</tr>
<tr>
<td>THB</td>
<td>Todd Hewitt Broth</td>
</tr>
<tr>
<td>THY</td>
<td>Todd Hewitt Yeast</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 Receptor</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TMB</td>
<td>3, 3′, 5, 5′-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzenesulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF Receptor Associated Factor</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 The immune system

The mammalian host has evolved many different elegant mechanisms to prevent colonisation and damage caused by pathogenic microorganisms. The primary functions of the immune system are to offer immunological recognition: discriminating between danger and non-danger, potent effector functions to eliminate pathogens and also immunological memory to provide lasting protection against re-infection. The immune system can be sub-divided broadly into two groups: innate and adaptive immunity, each with unique but inter-connected qualities.

1.1.1 An overview of the immune response

When the human body first encounters an infectious agent, the primary line of host defence is the skin or mucosal surfaces forming physical barriers. If these barriers are breached, innate immunity is initiated, first to contain and eliminate the infection by various mechanisms such as phagocytosis and induced lysis of pathogens. Metchnikoff in 1905 originally proposed the hypothesis of phagocytic cells as a mechanism of protection against infection (Metchnikoff, 1905), and his theory was later supported by the work of Lurie and Suter (Lurie, 1938; Lurie, 1941; Suter, 1953). Cells composing the innate immune system include: Dendritic Cells (DCs), macrophages, neutrophils, monocytes, Natural Killer (NK) cells, γδ T cells and the complement system, which will be discussed in more detail in Section 1.2.
After entry of the pathogens into the host, the microorganisms will activate the innate immune response. If innate immunity cannot eliminate the invading infectious agent, adaptive immunity is initiated to provide differentiated effector cells leading to enhanced pathogen clearance and creation of immunological memory, ready for quick re-activation upon secondary exposure to the same pathogen.

Adaptive immunity, consisting of T and B lymphocytes is mounted after innate immunity. This slower response is due to antigen-mediated activation of naïve T and B cells followed by clonal expansion, providing differentiated effector cells to aid pathogen eradication (Kamat and Henney, 1976). This is achieved either through cytotoxic T cells (CTL) or T helper (Th) cells, which coordinate the immune response via cytokine production, while B cells are responsible for the production of antibodies to neutralise targets. Adaptive immune cells are key in the formation of immunological memory (Nossal and Mäkelä, 1962; Miller and Sprent, 1971; Vischer et al., 1967). After the primary infection has been eliminated in the resolution phase, secondary encounters with the same pathogen are eradicated more efficiently due to quick re-activation and proliferation of memory cells (Figure 1.1) (Nossal et al., 1965).
1.1.2 An overview of microbial recognition

The four classes of PRRs (TLRs, NLRs, RLRs and CLRs) respond to the presence of microbial ligands or endogenous damaged tissue products to initiate signal transduction pathways. This induces gene expression of pro-inflammatory cytokines and type I interferons and aid pathogen clearance to subsequent activation of adaptive immunity or induce cell apoptosis (Chong and Sriskandan, 2011).

The first step required for activation of the immune response is the recognition and discrimination of self and foreign particles. Innate immunity offers a broad specificity against pathogens using germline-encoded Pattern Recognition Receptors (PRRs) that can recognise common conserved pathogenic features or Pathogen-Associated Molecular Patterns (PAMPs). To date, four major families of PRRs have been characterised since their early identification: Toll-like receptors (Medzhitov et al., 1997), Nod-like receptors (Steimie et al., 1993; Harton et al., 2002), C-type lectin
receptors (Santis et al., 1994) and Retinoic acid-induced gene (RIG)-1-like receptors (Rothenfusser et al., 2005) (Figure 1.2). Signaling through these receptors result in the production of pro-inflammatory cytokines, type I interferons, anti-microbial proteins and chemokines, all of which contribute to the host inflammatory response, as observed during bacterial infection and sepsis (Figure 1.2) (de Jong et al., 2010).

The best studied examples of PRRs are Toll-like Receptors (TLRs), homologs of Toll, which was first discovered in Drosophila in the 1980s (Andersen et al., 1985) and subsequently in mammals by Janeway and Medzhitov (Medzhitov et al., 1997). Found in both invertebrates and vertebrates, TLRs have evolved as pathogen sensors on innate cells but also connect with adaptive immunity via the activation of DCs, which subsequently stimulate adaptive immune cells (Kawai and Akira, 2010). TLRs also bridge the innate and adaptive immune system, expressed by a wide range of different subsets from lymphocytes to monocytes, macrophages, NK cells and DCs (Kadowaki et al., 2001; Muzio et al., 2000; Kabelitz, 2007; Hornung et al., 2002).

TLRs can be broadly classified according to their location within the cell. TLR1, 2, 4, 5, and 6 are found on the cell surface and bind extracellular ligands, typically derived from bacteria (Table 1.1). TLR3, 7, 8 and 9 are localised to endosomes and lysosomes within the cell, recognising nucleic acids from pathogens such as viruses that have been internalised (Table 1.1) (Barton and Kagan, 2009). Interestingly, TLRs can also sense host cell-derived ligands or Damage-Associated Molecular Patterns (DAMPs) as well as pathogen-associated ligands. DAMPs are produced during tissue damage, stress or trauma and tend to be either nuclear or cytoplasmic proteins released into the extracellular space during necrosis (Matzinger, 1994; Seong and Matzinger, 2004). Key examples of DAMPs include heat-shock proteins (HSPs), fibrogen and alarmins such as high mobility group box 1 (HMGB1) (Chang, 2010).
Table 1.1: Broad specificity of recognition by TLRs.

A diverse range of known ligands, covering many different classes of microorganisms are recognised by TLRs (Chong and Sriskandan, 2011).

<table>
<thead>
<tr>
<th>TLR</th>
<th>Ligand</th>
<th>Microbial source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipopeptides</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR2</td>
<td>Peptidoglycans, lipopeptides, atypical LPS</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>zymosan, phospholipomannan</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>GPI anchors</td>
<td>Protozoa</td>
</tr>
<tr>
<td></td>
<td>Heat Shock Proteins (e.g. HSP 70)</td>
<td>Host cells</td>
</tr>
<tr>
<td>TLR3</td>
<td>Poly I:C, dsRNA</td>
<td>Virus</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Mannan, glucuronoxylomannan</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Glycoinositolphospholipids</td>
<td>Protozoa</td>
</tr>
<tr>
<td></td>
<td>RSV fusion protein</td>
<td>Virus</td>
</tr>
<tr>
<td></td>
<td>Heat Shock Proteins (e.g. HSP-60)</td>
<td>Host cells</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacyl lipopeptides</td>
<td>Bacteria</td>
</tr>
<tr>
<td>TLR7</td>
<td>Synthetic imiazoquinoline-like molecules, ssRNA</td>
<td>Virus</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA</td>
<td>Virus</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA</td>
<td>Bacteria, protozoa, virus</td>
</tr>
<tr>
<td>TLR10</td>
<td>Function and ligand unknown</td>
<td>-</td>
</tr>
<tr>
<td>TLR11 (mouse only)</td>
<td>Components of uropathogenic bacteria</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Profilin-like molecules</td>
<td>Protozoa</td>
</tr>
<tr>
<td>TLR12-13 (mouse only)</td>
<td>Function and ligand currently unknown</td>
<td>-</td>
</tr>
</tbody>
</table>

Upon ligand binding to TLRs, a conformational change in the receptor complex is induced, resulting in recruitment of adaptor signaling molecules, and signal transduction cascade within the cell. Two signaling pathways exist for TLRs; Myeloid Differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent pathways, both culminating in gene expression of key inflammatory products. All TLRs except for TLR3 utilise the MyD88-dependent signaling pathway, whereas TLR3 signals solely via the MyD88-independent route. TLR4 is unique as it can transmit signals from the extracellular surface via both pathways (Akira, 2003; Chang, 2010; Jin and Lee, 2008).
In the MyD88-dependent pathway, the cytoplasmic adaptor protein MyD88 binds with TLRs via its Toll/Interleukin-1 Receptor (TIR) domains, resulting in the recruitment of Interleukin-1 Receptor-Associated Kinase-4 (IRAK-4) to associate with the receptor complex via its death-domains (Medzhitov et al., 1998; Wesche et al., 1997). MyD88 facilitates IRAK-4 mediated phosphorylation of IRAK-1, a serine-threonine kinase, which leads to activation and recruitment of BCL10 and subsequent dissociation from the receptor complex (Wesche et al., 1997). BCL10, also known as CLAP is required for the optimal signaling of the transcription factor NF-κB (Srinivasula et al., 1999). Phosphorylated IRAK-1 associates with TRAF6, a polyubiquitin ligase to activate TAK1/TAB complexes and enhances the activity of IkB kinase complex (Figure 1.3) (Cao et al., 1996). AP1 transcription factor is also activated through this process through the MAPK signaling pathway (Medvedev et al., 2000). The consequence of this signaling cascade is the nuclear translocation of NF-κB and thus, expression of pro-inflammatory cytokine genes, such as TNF-α and IL-6 (Figure 1.3) (Liebermann and Baltimore, 1990; Collart et al., 1990). Other adaptor proteins similar to MyD88 such as Mal and TIRAP are also recruited in this pathway but are limited to TLR1, 2 and 4 signaling only (Kenny et al., 2009). The presence of anti-inflammatory cytokines such as IL-10 may suppress the MyD88-dependent pathway by degrading key signaling molecules such as IRAK4 and TRAF6 to prevent induction of pro-inflammatory cytokines (Chang et al., 2009).

The MyD88-independent pathway triggers gene induction of type I interferons, such as IFN-β and occurs via ligand binding to TLR3 and TLR4 (Horng et al., 2001; Yamamoto et al., 2002). The MyD88-independent pathway utilises unique adaptor proteins such as TRIF and TRAM, establishing a signal transduction cascade concluding with the activation of IRF-3 transcription factor to induce IFN-β and IFN-inducible genes via STAT1, TKE ε and TBK1/NF-κB activating kinases (Figure 1.3) (Kawai et al., 2001). TRAM has evolved to be a TLR4-only adaptor protein (Yamamoto et al., 2003; Fitzgerald et al., 2003). It is now emerging that other TLRs, such as TLR2 are capable of inducing type I interferon responses, suggesting that other, unknown signaling pathways may also exist to bring about gene expression changes, depending on the required immune response upon infection (Barbalat et al., 2009).
Figure 1.3: Overall signaling pathways utilised by TLRs.

TLRs respond to various microbial ligands leading to signal transduction via the MyD88 dependent and independent pathways. The MyD88 signaling pathway is depicted on the left hand side of the figure, while the MyD88 independent pathway signaling via TRAM is shown on the right hand side, culminating in transcription factor translocation into the nucleus to induce gene expression. Adapted from (Chong and Sriskandan, 2011).

The net effect of TLR signaling via either MyD88-dependent or independent pathways is induced transcription of pro-inflammatory cytokines such as TNF-α and type I interferons to aid microbial clearance. However, another crucial role of TLR signaling is the activation of DCs and the subsequent initiation and polarisation of adaptive immunity. Combinations of TLR ligands (TLR3, TLR4 and TLR8) can work synergistically to trigger activation of DCs to produce cytokines, such as IL-12 required for differentiation of Th1 cells, (Napolitani et al., 2005; Hou et al., 2008). TLR signaling can result in the up-regulation of MHC class II molecules on DCs to stimulate antigen presentation to cells of the adaptive immune system (Chandran et
al., 2009; Mitchell et al., 2010). TLR2 stimulation may also enhance proliferation of CD8⁺ memory T cells and enhance IFN-γ production (Mercier et al., 2009; Cottalorda et al., 2009) or induce T₉₁7 cell differentiation via the production of polarising cytokine by Langerhans cells (Aliahmadi et al., 2009). T₉₁ cell activity can also be mediated via TLR2 signaling, suggesting that TLR2 may behave as a direct co-stimulatory molecule on T cells, priming them for immediate activation (Imanishi et al., 2007; Komai-Koma et al., 2004).

Nod-like receptors (NLRs) are a family of receptors with the predominant function to detect microbial components within the cytoplasm of the cell (Philpott and Girardin, 2010). These receptors work in parallel with TLRs, such as TLR1, 2 and 7 to recognise infectious agents that have already entered the cell (van Heel et al., 2005). Class II, major histocompatibility complex, transactivator (CIITA) was first identified as a MHC Class II gene transactivator expressed by macrophages, B cells, T cells and DCs (Steimie et al., 1993). However, a splice variant of this protein was later found to share common features with other NLRs, establishing CIITA as the founding member of this class of PRRs (Nickerson et al., 2001).

The human genome encodes 23 NLR family members, all of which exhibit similar structural features, such as an amino-terminal pyrin domain (PYD) or a caspase recruitment domain (CARD), a central nucleotide binding domain (NOD) and a carboxyl-terminal leucine rich repeats (LRRs), a feature shared with TLRs (Inohara et al., 1999). Well studied NLRs include NOD1 and NOD2, which are able to signal through NF-κB in a manner similar to TLRs (Inohara et al., 1999).

NLRs have been highly researched in the past few years within the context of the inflammasome, a multi-protein complex with the main function of facilitating the maturation of some pro-inflammatory cytokines, such as IL-1β (Martinon et al., 2002). NLRs are recruited to the inflammasome, however, the exact composition of this complex can vary, generally containing caspase-1, PYCARD, a NALP and sometimes caspase-5 or caspase-11 (Martinon et al., 2002). NLRs are key components in regulating the activation state of caspase-1, an enzyme, which cleaves the immature pro-form of IL-1β into active IL-1β in response to different
environmental signals. The release of pro-inflammatory cytokines in this manner, first studied during infectious diseases, such as *Shigella flexneri* infection is termed pyroptosis and is distinct from apoptosis (Hilbi *et al*., 1997).

C-type lectin receptors (CLRs) are a family of transmembrane receptors, characterised by the presence of a conserved carbohydrate-binding domain (Figdor *et al*., 2002). Carbohydrate ligands derived from a wide variety of pathogens, such as viruses, bacteria and fungi form the main core of ligands recognised by CLRs. There are four groups of CLRs based on key structural features: type I (mannose receptors), type II, type III (collectins) and type V (NK cell receptors). These receptors upon ligand stimulation induce pro-inflammatory cytokines via a separate signaling pathway from TLRs, but still result in NF-κB translocation into the nucleus (Zhang *et al*., 2009b). This signaling pathway can also modulate TLR-mediated immune responses via NF-κB, enhancing IL-10 gene and protein production via TLR3, 4 and 5 (Gringhuis *et al*., 2007). Synergistic effects of combined signaling via TLR and CLR pathways have also been demonstrated in neutrophils and may possess a regulatory role during *Mycobacterium tuberculosis* BCG infection (Zhang *et al*., 2009b).

CLR s are broadly expressed on most cell types, from macrophages to DCs. Dectin-1 is one of the most commonly studied of the CLR family, recognising β-glucans found in fungi to promote the production of pro-inflammatory cytokines, such as TNF-α and also reactive oxygen species (ROS) via NF-κB signaling (Ariizumi *et al*., 2000; Willment *et al*., 2003; Brown *et al*., 2002; Underhill *et al*., 2005). Another well investigated CLR is DC-SIGN, found on DCs and may be involved in the recognition of sugars on specific viruses, such as influenza and Human Immunodeficiency Virus (HIV) (Carthagena *et al*., 2011; Itano *et al*., 2011). The recognition of these pathogens by macrophages and DCs play a key role in the subsequent activation of the adaptive immunity.

RIG-1-like receptors (RLRs) are the last of the four major branches of PRRs. Characterised as RNA helicases, they are crucial for the recognition of viruses and 3 family members have been identified; RIG-1 (Cheng and Lotan, 1998), melanoma
differentiation-associated gene 5 (MDA-5) (Kang et al., 2002) and LGP2 (Rothenfusser et al., 2005). Localised within the cytoplasm of the host cell, RLRs bind genomic RNA of dsRNA viruses and dsRNA generated as part of the replicative cycle of ssRNA viruses. Upon activation of this class of PRR, anti-viral responses are mounted in the form of type I interferon production via NF-κB signaling (Yoneyama et al., 2005).

RIG-1 has been demonstrated to recognise short dsRNA; up to 1Kb, favouring 5′triphosphate ends enhancing type I interferon induction (Kato et al., 2008). It has also been suggested that in resting cells, RIG-1 may form an inhibitory complex with HSP-90α to regulate its function, suggesting that this RLR may have a role in regulating responses to DAMPs (Matsumiya et al., 2009). MDA-5 recognises longer dsRNA fragments; more than 2Kb in length, such as poly I:C, which is also recognised by TLR3 (Kato et al., 2008). LGP2 was first proposed as a negative regulator of RIG-1 and MDA-5. However, it has now emerged that mice lacking this RLR family member are unable to mount anti-viral responses, suggesting that LGP2 may work synergistically with RIG-1 and MDA-5 (Satoh et al., 2010). There is conflicting data on whether RLRs can have an impact on inflammasome activity and is still under current investigation (Delaloye et al., 2009).

Overall, different receptors have evolved to recognise conserved microbial and host cell derived ligands. Common PAMPs, such as poly I:C are recognised by different receptors (TLR3 and MDA-5) to maximise activation of both innate and adaptive immune cells and induce signal transduction and gene expression of key mediators of inflammation and pathogen clearance mechanisms.

1.2 Innate Immunity

Several cell types contribute to innate immunity due to their rapid activation in response to invading pathogens. These cell types initiate the first stages of immunity to limit spread of infection, actively destroy pathogenic microorganisms and secrete
cytokines and chemokines to recruit and prime adaptive immunity. Each of the key cellular components of innate immunity will be briefly described in the following section.

1.2.1 Macrophages

Macrophages were first identified as important cells involved in the phagocytosis of foreign particles and are derived from monocytes in the tissue upon the differentiation with M-CSF (Cohn and Benson, 1964; Stanley et al., 1976). Macrophages have been functionally sub-divided into two phenotypes; classically (M1) and alternatively activated (M2) macrophages. M1 macrophages are characterised by their induction with pro-inflammatory molecules, such as LPS and IFN-γ, producing an array of different pro-inflammatory cytokines like TNF-α, IL-1β, IL-6 and IL-12 also inducible Nitric oxide Synthase (iNOS) to produce Nitric oxide (NO) (Goldmann et al., 2010; Liu et al., 2011a; Trinchieri, 1997). M2 macrophages, in contrast, produce anti-inflammatory cytokines such as IL-4, IL-10, Transforming Growth Factor-β (TGF-β) and IL-1 decoy receptor and also exhibit arginase activity (Goldmann et al., 2010; Liu et al., 2011a; Bastos et al., 2002).

Apart from cytokine production, macrophages express CD80 and CD86 and MHC class II molecules, allowing antigen presentation to naïve T cells to activate adaptive immunity. Macrophages are also involved in direct killing of microbes by many different mechanisms. For example, this innate cell type can produce ROS and NO or anti-microbial peptides. Macrophages can produce elastase such as MMP12, which is stored in intracellular compartments and mobilised after phagocytosis to kill the microorganisms (Houghton et al., 2009). The role of macrophages during bacterial infection will be discussed further in Section 1.9.3.
1.2.2 Dendritic Cells (DCs)

Dendritic Cells (DCs) are derived from monocytes through differentiation with GM-CSF and IL-4. Very few terminally differentiated DCs are found in circulating blood, usually in the range of 0.5-2% of human Peripheral Blood Mononuclear Cells (PBMCs). Their primary function is antigen processing and presentation to naïve T cells and also cytokine production to create specific microenvironment to allow differentiation and polarisation of $T_{H}$ subsets (Tuettenberg et al., 2011).

DCs can be divided into two groups; CD11c$^+$CD123$^-$ myeloid DCs (mDCs) and CD11c$^+$CD123$^+$ plasmacytoid DCs (pDCs) with differing functions (Ito et al., 1999). Both these classes of cells are capable of producing pro-inflammatory cytokines, such as IFN-$\alpha$ in response to microbial stimuli, although mDCs can also secrete IL-10 and IL-12. TLR expression also differentiates these two subsets (Veckman and Julkunen, 2008). mDCs have a broad range of TLR expression, except for TLR7 and 9, whereas pDCs express predominantly TLR7 and 9 and low levels of TLR1, 6 and 10 (Veckman and Julkunen, 2008). DCs can also express the transcription factor Forkhead box P3 (Foxp3) to gain suppressive activity and can skew the T helper cell differentiation towards $T_{H}2$ and $T_{reg}$ phenotype (Lipscomb et al., 2010).

1.2.3 Neutrophils

Neutrophils, also known as Polymorphonuclear Neutrophils (PMNs) are the most abundant cell type within the human white blood cell population. Essential for the initial defence against extracellular pathogens, neutrophils recognise microbes through the expression of many different PRRs such as NODs and TLRs. PMNs are produced and released from the bone marrow into the blood, offering rapid protection from invading pathogens. The levels of circulating neutrophils have been shown to be regulated by the expression of CXCR4; excessive signaling from this chemokine can lead to neutrophil containment within the bone marrow, whereas CXCR4 deficiency results in uncontrolled release of PMNs into the blood (Eash et al., 2010).
Neutrophils are often guided to the site of infection or inflammation via the release of endogenous pro-inflammatory DAMPs, produced by the process of necrosis and also by cytokines and chemokines such as IL-17A and IL-8 respectively (McDonald et al., 2010). This subset of innate cells have three main mechanisms to attack microorganisms, phagocytosis, release of granules containing anti-microbial peptides and enzymes and ensnaring microbes through the release of a framework of DNA and proteases called Neutrophil Extracellular Traps (NETs). NET formation has been shown to be partly responsible for the neutralisation of Staphylococcus aureus (Pilszczek et al., 2010). Neutrophils have also been shown to be an important source of IL-17 during infection and will be discussed in more detail in Section 1.9.3 (Matsuzaki and Umemura, 2007).

PMNs can also exert regulation on T cell proliferation, effector function and survival during an immune response. This is achieved through the expression of OX40L on neutrophils and OX40 receptor on T cells. During poly-microbial sepsis, the expression of OX40L is significantly up-regulated on circulating neutrophils and macrophages. The importance of this ligand has been demonstrated through the use of OX40L−/− mice, which exhibit increased resistance to sepsis compared to wild-type mice (Karulf et al., 2010). This suggests that this interaction between T cells and neutrophils via OX40 may be detrimental to host survival during severe bacterial infection.

1.2.4 Natural Killer (NK) cells

Natural Killer (NK) cells are non-B, non-T lymphocytes, initially described in terms of their ability to lyse transformed or virally infected cells (Greenberg et al., 1975; Glimcher et al., 1977; Kiessling et al., 1975). While they are now appreciated to perform a far more complex role depending on both cytotoxicity and cytokine release in response to a range of environmental; changes including cellular stress, bacterial and fungal infection, the mechanisms of ligand recognition underpinning activation is often uncharted (Bauer et al., 1999; Vankayalapati et al., 2005; Hidore and Murphy, 1986). NK cells are differentiated by interferons and other macrophage derived
cytokines, such as IL-2, IL-12, IL-18 and IL-15 (Mrózek et al., 1996; Riccardi et al., 1983; Suzuki et al., 1997; Takeda et al., 1998).

Two main subsets of NK cells were initially described in humans based on their expression of key markers; CD56 and CD16 (Nagler et al., 1989). CD56$^{\text{dim}}$CD16$^+$ NK cells represent the major population and are highly cytotoxic through their release of granules (Nagler et al., 1989; Mandelboim et al., 1996; Peritt et al., 1998). CD56$^{\text{bright}}$CD16$^-$ NK cells are responsible for cytokine production, such as IFN-γ, TNF-α, IL-10 and GM-CSF (Cooper et al., 2001). Most NK cells located in the lymph nodes are of the CD56$^{\text{bright}}$CD16$^-$ variety (Fehniger et al., 2003). The basic leucine zipper transcription factor E4bp4, also known as NFIL3 has been shown to be essential for the development of NK cells, indicating this may be an important genetic marker for NK cells (Gascoyne et al., 2009). It has been suggested that anti-inflammatory cytokine; TGF-β may inhibit the development and maturation of NK cells through down-regulation of CD16 expression (Allan et al., 2010).

In mice, four subsets of NK cells have been defined, based on CD11b and CD27 expression, contrasting with the two main classes of NK cells in humans. CD11b$^{\text{dull}}$CD27$^{\text{dull}}$ and CD11b$^{\text{dull}}$CD27$^+$ NK cells represent the most naïve populations; CD11b$^+$CD27$^+$ cells are an intermediate population and CD11b$^+$CD27$^{\text{dull}}$ NK cells being defined as the most differentiated and mature subset (Choissone et al., 2009). CD56$^{\text{bright}}$ human NK cells have been suggested to be similar to CD11$^{\text{dull}}$ mouse NK cell population (Vivier et al., 2008).

NK cell development begins in the bone marrow like other hematopoietic cells, before migration to the periphery. The largest population of murine NK cells is found in the spleen (Grégoire et al., 2007). Murine NK cells express diverse chemokine receptors involved in NK cell trafficking from the bone marrow to the periphery. CCR2, CCR5, CXCR3 and CX3CR1 regulate NK cell recruitment in response to inflammatory signals. CD62L (L-selectin) is also required to allow murine NK cells to enter lymph nodes from the blood, with CCR7 required in human NK cells for the same role (Vivier et al., 2008).
The role of NK cells during infection has been well researched as key mediators of anti-viral effects, such as IFN-γ production, release of cytolytic granules for lysis of infected cells and induction of apoptosis via TRAIL (Scalzo et al., 2007). The expression of IFN-γ is mediated via T-bet transcription factor expression (Werneck et al., 2008). Cytotoxic granules are pre-formed within NK cells, ready for deployment upon activation from cytokine signals from other cells (Reefman et al., 2010). The role of NK cells is not restricted to the field of infectious diseases, but this cell population has also been implicated in autoimmune diseases such as Multiple Sclerosis (MS). For example, NK cells can suppress autoreactive T cell responses in the central nervous system in a murine model of MS (Hao et al., 2010).

The activation of NK cells is dependent on a balance of activation and inhibitory signals through three classes of receptors; Killer-cell Immunoglobulin-like Receptors (KIRs) - the key molecules used by human NK cells and not found in mice, Ly49 lectin-like receptors and NKG2 lectin-like receptors expressed on the cell-surface. KIRs can recognise polymorphic Human Leukocyte Antigen (HLA)-A, B and C allotypes, whereas CD94-NKG2A receptor complex can recognise HLA-E allotypes (Sivori et al., 2010).

NK cell effector function is regulated by interactions between inhibitory receptors and self-MHC class I molecule expression by a process that has been termed ‘licensing’ by Wayne Yokoyama and colleagues (Kim et al., 2005). This will be examined in more detail in Chapter 4. Cells expressing MHC class I molecules are more resistant to NK-mediated killing, this is due to inhibitory signals received via KIRs and NKG2 receptors (Yokoyama and Kim, 2006). NK cell killing of target cells is mediated by an overall activation signal received by receptors such as 2B4 in human NK cells (Betser-Cohen et al., 2010).

A new subset of NK-like cells has been identified in human tonsils, Peyer’s patches and appendix. This unique population of cells expresses CD56 and NKp44 similarly to conventional human NK cells, but also secrete IL-22 in response to IL-23. These so-called “NK-22 cells” are similar to Th17 phenotype due to expression of CCR6, Acyl hydrocarbon receptor (AHR) and RAR-related Orphan Receptor γt (RORγt)
transcription factor (Cella et al., 2010). Further phenotypic analysis has also revealed NK-22 cells as functionally heterogeneous as some can produce IFN-γ, while others secrete IL-17 (Cella et al., 2010). Whether these cells are also present in mice is still the subject of on-going work.

1.2.5 NKT cells.

NKT cells, also known as invariant-(i)NKT cells, are found in the thymus, peripheral lymphoid organs and blood, and share some markers of both NK and T cells. Their ability to release a large, rapid burst of cytokine in response to innate activation marks this cell type an important bridge between adaptive and innate immunity. They express a largely invariant T Cell Receptor (TCR) α chain and one of three different β chains and it is hypothesised they can recognise antigens from glycolipids presented by MHC class I-like β2-microglobulin associated CD1d molecules (Behar et al., 1999; Brossay et al., 1998). CD1d is mainly expressed by DCs, macrophages, particular subsets of B and T cells (Arrenberg et al., 2008). Upon activation, iNKT cells can produce IL-4, IL-10 and IFN-γ, suggesting that they have both regulatory and effector phenotype. The expression of IL-4 and IL-10 may be controlled by the NK specific transcription factor E4bp4 (Motomura et al., 2011), while it has also been suggested that the transcription factor GATA-3 may be required for the maturation of iNKT cells (Kim et al., 2006), highlighting the share features of both NK and T cells.

The regulatory function of NKT cells has been shown in several autoimmune models such as Experimental Autoimmune Uveitis (EAU), where T_{h17} and T_{h1} cell differentiation was diminished in vitro (Oh et al., 2011). Cell contact between NKT and T_{h17} cells was required to inhibit T cell development; whereas the release of IL-4 from NKT was sufficient to suppress T_{h1} differentiation (Oh et al., 2011). NKT cells can also express Foxp3 with regulatory T cell-like phenotype such as CD25, CTLA-4 and GITR expression (Monterio et al., 2010). These cells can also suppress
the progression of disease in Experimental Allergic Encephalomyelitis (EAE) 
(Monterio et al., 2010).

1.2.6 γδ T cells

γδ T cells are a subset of T cells; characterised by their expression of γ and δ chains 
forming the TCR, although this may be a more limited repertoire compared to αβ 
TCR+ T cells due to less available gene segments (Elliott et al., 1988). Their 
development pathway is similar to αβ TCR T cells, beginning in the thymus before 
migration into the periphery (Born et al., 2010). Although less abundant than αβ TCR 
T cells, γδ T cells tend to accumulate in epithelial cell-rich tissue and lymphoid 
tissue. The ligands for γδ TCR activation are still largely unknown (Bonneville et al., 
2010). The function of these T cells has also shown to be fairly plastic, with both 
effector and regulatory roles implicated (Bonneville et al., 2010; Eberl and Moser, 
2009).

Immune surveillance is an important process, protecting the host from any potential 
threats incurred during tissue damage, inflammation and infection. Stress-induced 
MHC class I-related molecules (MIC)-A/B molecules activate γδ T cells in the skin 
or intestines to detect damaged, infected or transformed cells (Groh et al., 1998; 
Sumaria et al., 2011).

γδ T cells can express some receptors found on NK cells, such as NKG2D and 
NKG2A, indicating this cell subset may have overlapping function with NK cells. For 
example, increased expression of MICA/B on infected or transformed cells enhances 
interactions with the activating NKG2D receptor on γδ T cells to lead to target cell 
killing (Nedellec et al., 2010). A particular subset of human γδ T cells characterised 
as Vγ9Vδ2+ also exhibits antigen cross presenting abilities similar to Antigen 
Presenting Cells (APCs) through the expression of adhesion receptors, co-stimulatory 
molecules and MHC class I and II molecules, suggestive that this class of T cells can 
activate other T cells (Meuter et al., 2010).
The effector function of γδ T cells is mediated by cytokine production and release of cytotoxic granules containing perforin, granzymes and TRAIL (Eberl and Moser, 2009). RORγt expression is also required for differentiation of γδ T cells and may also be linked to their ability to secrete IL-17 (O’Brien et al., 2009). Type I interferon signaling can negatively regulate IL-17A and IL-17F production by γδ T cells during bacterial infection (Henry et al., 2010). γδ T cells can also produce the pro-inflammatory cytokine IFN-γ upon activation and CD27 co-stimulation has been suggested to expand this particular population of cells (Ribot et al., 2010). The expression of other markers such as CCR6 and NK1.1 can also distinguish γδ T cells that secrete IL-17 and IFN-γ respectively (Haas et al., 2009).

This subset of T cells also demonstrates a regulatory role during infection or autoimmunity. γδ T cells can suppress Tregs via high expression of IL-23R, which correlates with cells that are potent producers of IL-17A. IL-23R+ γδ T cells can expand during the murine model of EAE and prevent the induction of Foxp3+ T cells via a IL-23 dependent mechanism (Petermann et al., 2010). γδ T cells can also provide co-stimulation to NK cells to aid tumour cytotoxicity, possibly via CD137L expression (Maniar et al., 2010), indicating this cell type as a potent source of effector and regulatory function.

Overall, the different components of innate immunity offer rapid recognition of the invading pathogen to lead to effective killing and elimination of the ensuing infection. Consequences of innate immunity include neutrophil and phagocyte activation to kill microbes, with cytokine production leading to APC activation, antigen processing and presentation to activate the next branch of host defence; adaptive immunity.

1.3 Adaptive immunity

Adaptive immunity plays a critical role to enhance the clearance of pathogenic microorganisms if innate immunity is unable to effectively clear the infection. This
thesis is mainly concerned with CD4$^+$ T cells, however the role of CD8$^+$ T cells will be further discussed in Chapter 4. In the past few years, the field of CD4$^+$ T cell immunity has diversified due to ongoing attempts to classify T helper cells into distinct subsets on the basis of cytokines and transcription factors required for differentiation, cell-surface phenotype, cytokine profile and effector functions.

1.3.1 $T_{H1}$ and $T_{H2}$ cells

It was originally proposed by Mossmann and Coffman in 1986 that naïve CD4$^+$ T cells could differentiate into cells of different cytokine profile, either T helper ($T_{H1}$)-1 or $T_{H2}$ cells, depending on the strength of the TCR activation signal and the cytokine microenvironment (Mosmann et al., 1986). $T_{H1}$ cells were considered to be involved in the eradication of intracellular pathogens due to their ability to produce their hallmark cytokine, IFN-$\gamma$ (Figure 1.4). $T_{H2}$ cells secrete cytokines such as IL-4, IL-5 and IL-13 in response to allergens and parasitic helminths infection (Figure 1.4). The end result is increased eosinophils, mast cell activation and Immunoglobulin (Ig)-E antibodies responses leading to chronic inflammation (Coffman, 2010).

$T_{H1}$ cells are defined by their expression of T-bet transcription factor (Szabo et al., 2000), whereas $T_{H2}$ cells are characterised in the master regulator; GATA-3 (Zheng and Flavell, 1997) (Figure 1.4). The presence of these respective transcription factors plays a determining role in dictating the cytokine profile associated with each particular T helper cell subset. For example, continuous T-bet expression may silence $il-4$ gene expression, therefore no IL-4 production is observed in $T_{H1}$ cells (Zhuang et al., 2009). GATA-3 can also inhibit $ifn-\gamma$ gene expression in $T_{H2}$ cells (Yu et al., 2009).
Naïve CD4$^+$ T cells differentiate into various T helper subsets in the presence of different cytokines and environmental signals. T helper subsets can be identified by expression of different transcription factors indicated inside the cell and are characterised by the secretion of hallmark cytokines, shown on the right-hand side of the figure. Key signaling pathways that lead to T cell differentiation are also indicated on the right side of the figure.

The cytokine environment is central in providing the necessary signals for naïve CD4$^+$ T cells to differentiate into T helper subsets. For example, IFN-γ and IL-12 produced by DCs and macrophages are required for $T_{H1}$ cell development (Hsieh et al., 1993) (Figure 1.4). In chronic inflammation, IFN-γ production by $T_{H1}$ cells; as mediated by JAK3 and p38-MAPK and STAT1 and STAT4 dependent signaling pathways, may be triggered by the presence of IL-2, 7 and 15 from innate cell sources (Sattler et al., 2009).

The main cytokine required for naïve T cell polarisation to $T_{H2}$ cells is IL-4. IL-4 binding to its cognate receptor, leads to recruitment and phosphorylation of STAT6 to
dimerise and translocate into the nucleus to induce the expression of T\(_h\)2 cell specific genes such as GATA-3 (Kurata et al., 1999). Activation of STAT3 is also required for T\(_h\)2 cell development, as knockout mice lacking STAT3 do not develop T\(_h\)2 cells (Stritesky et al., 2011). This would suggest that STAT3 and STAT6 work synergistically to lead to the differentiation of T\(_h\)2 cells (Figure 1.4).

T\(_h\)2 associated cytokines are not only produced by differentiated CD4\(^+\) T cells. IL-33, a new member of the IL-1 cytokine family, can induce the production of T\(_h\)2 associated cytokines and increase serum immunoglobulins, particularly in mast cells (Chen et al., 2009b). This novel cytokine can act through two receptors; ST2 and IL-1RAcP to lead to classical signaling via NF-κB. It has also been hypothesised that IL-33 may mediate allergic responses by mast cells (Pushparaj et al., 2009). IL-25, also known as IL-17E may also play a role in the development of lineage negative multi-potent progenitor cells to produce T\(_h\)2 related cytokines such as IL-4, IL-5 and IL-13 (Saenz et al., 2010).

The balance of T\(_h\)1 and T\(_h\)2 responses during infection is crucial to efficient clearance of pathogens. This has been demonstrated within the context of Respiratory Syncytial Virus (RSV) infection, whereby IFN-γ receptor knockout mice upon intranasal infection exhibited enhanced T\(_h\)2 responses. The resultant effect was increased eosinophil recruitment to the lung and elevated IL-4, IL-5 and IL-13 cytokine levels (Boelen et al., 2002). This imbalance in CD4\(^+\) T cells lead to worse lung pathology, despite no change in viral load compared to wild-type mice, highlighting the detrimental effect of T\(_h\)2 cells during infection (Boelen et al., 2002). Parasitic infestations and subsequent T\(_h\)2 responses can also augment disease outcome of other bacterial infections. For example, filarial parasites can induce strong T\(_h\)2 responses and down-regulate protective T\(_h\)1-mediated immunity during M. tuberculosis infection (Babu et al., 2009). This finding emphasises T\(_h\)2 responses as detrimental to effective bacterial clearance.

This view of pathogenic effects of T\(_h\)2 cell during infectious diseases may be oversimplistic, as other studies have offered complex contradicting observations. One such study investigating the role of IL-4 pre-treated macrophages on secondary
challenge with *Neisseria meningitides* showed decreased ability to phagocytose this Gram-negative bacterium and was accompanied with increase levels of pro-inflammatory cytokines, such as IL-6 and TNF-α (Varin *et al.*, 2010). These findings are contradicted by other work demonstrating vaccination of soluble FliC protein can enhance T\(_H\)2 responses and lead to augmented T\(_H\)1 immunity and hence better *Salmonella typhimurium* clearance (Bobat *et al.*, 2011). These publications emphasise the fine balance between T\(_H\)1 and T\(_H\)2 mediated immunity during infection.

1.3.2 T\(_H\)17 cells

The T\(_H\)1/T\(_H\)2 paradigm suggested by Mossmann and Coffman was revised when it was found that naïve CD4\(^+\) T cells could differentiate into other subsets with unique cytokine profiles and functions, such as T\(_H\)17 and T\(_R\)egs cells. Earlier studies of autoimmune diseases, such as EAE had postulated a pathogenic role for IFN-γ and T\(_H\)1 cells. However, depletion studies of T\(_H\)1 cells could not fully explain the disease pathology. It was only through the use of IL-12 (p35\(^{-/}\)) and IL-23 (p19\(^{-/}\)) transgenic animals that IL-23 and T\(_H\)17 cells were initially linked to the development of autoimmune diseases like EAE in mice and also both intra- and extracellular pathogen infection (O’ Connor Jr *et al.*, 2010; Weaver, 2009; Cua *et al.*, 2003). This class of T helper cells is marked by the key expression of the pro-inflammatory cytokine IL-17A, although other cytokines such as IL-17F, IL-21, IL-22 and IL-9 have also been linked to the cytokine profile of T\(_H\)17 cells (Nowak *et al.*, 2009; Stephens *et al.*, 2011). This T helper subset was initially acknowledged when human IL-17A was first identified in cDNA libraries of T cells and later confirmed to be secreted as a homodimer by activated memory T cells (Yao *et al.*, 1995; Fossiez *et al.*, 1996). This discovery has since paved the way for a rapid expansion in the study of T\(_H\)17 responses in autoimmunity and infection.

The key genetic marker for T\(_H\)17 cells is the transcription factor ROR\(_\gamma\)t and is essential for IL-17 production (Figure 1.4) (Ivanov *et al.*, 2006; Crome *et al.*, 2009). Other reliable markers for human T\(_H\)17 cells are IL-23R and RORC. However, it has
been suggested that CD161, the human ortholog of murine NK1.1 may also be used a marker for T cells secreting IL-17 (Maggi et al., 2010). Human T_{H17} cells can also express CCR6 and CCR4 (Annunziato et al., 2009), indicating that this subset can express numerous markers.

The cytokines required for T_{H17} cells differentiation from naïve CD4^{+} T cells has been contentiously debated for many years with differences observed between human and murine cells. TGF-β, IL-6 and IL-21 were initially considered to be responsible for the induction of T_{H17} cell development from naïve T cells in mice (Korn et al., 2008; Yang et al., 2008; Volpe et al., 2008). The role of TGF-β during development of T_{H17} cells was debated (O’Garra et al., 2008) as some groups have shown the presence of this cytokine to have a redundant role (Das et al., 2009), although it may be required for IL-9 production (Beriou et al., 2010). IL-1β has also been implicated in T_{H17} cell differentiation in humans (Wilson et al., 2007; Acosta-Rodriguez et al., 2007; Valmori et al., 2010). IL-23, a cytokine composed of p19 and p40 subunits acts during the later stages of T_{H17} cell differentiation to stabilise the phenotype, as oppose to differentiate and the expression of IL-23R is indeed induced by TGF-β (Figure 1.4) (Yang et al., 2008; Tato and O’Shea, 2006; McGeachy et al., 2009). IL-7 is also required for T_{H17} cell survival, supporting the proposed general function of this cytokine to promote the survival of all T cells (Liu et al., 2010b).

Interferon regulatory factor 4 (IRF4) is an important protein in T_{H2} and T_{H17} cell differentiation. Human naïve T cells require IL-1, IL-23 or IL-6 and TGF-β activated signaling for T_{H17} differentiation as it leads to downstream IRF4 and RORγt expression (Chung et al., 2009). The phosphorylation of STAT3 as a consequence of IL-6 mediated signaling is also critical for T_{H17} cell development (Figure 1.4) (Manel et al., 2008). Signaling via AHR may also lead to the production of IL-22 in response to environmental signals (Veldhoen et al., 2008).

Other factors apart from the cytokine milieu may influence T_{H17} cell differentiation. For example, C5aR, a component of the complement cascade may trigger the induction of T_{H17} cells and this has been shown to be the case in an autoimmune model of arthritis (Hashimoto et al., 2010). This suggests the signals received during infection or inflammation may drive the differentiation of this subset of T cells.
IL-17A and IL-17F, the signature cytokines of T\(_{H17}\) cells can form a heterodimer to signal through the same receptor subunits; IL-17RA and IL-17RC (Gaffen, 2009; Onishi and Gaffen, 2010). The receptor for IL-17A consists of a single transmembrane protein and activation leads to induction of other pro-inflammatory cytokines such as IL-6 via NF-κB signaling (Shen and Gaffen, 2008; Moseley et al., 2003). The observed effects of secreted IL-17 include recruitment of neutrophils via the induction of G-CSF and other chemokines such as CXCL8/IL-8 by various cells expressing IL-17R (Moseley et al., 2003).

1.3.3 Regulatory T cells (T\(_{regs}\))

\[
\begin{array}{cccc}
\text{activation} & \text{proliferation} & \text{differentiation} & \text{effector function} \\
\end{array}
\]

Naïve T cells

\(T_{reg}\) → \(T_{reg}\) → \(T_{H17}\) → \(T_{H2}\)

\(T_{reg}\)

\(T_{reg}\)

\(T_{reg}\)

\(T_{reg}\)

\(T_{reg}\)

Figure 1.5: The role of T\(_{regs}\) to modulate immune responses.

T\(_{regs}\) can suppress the immune response in various manners. T\(_{regs}\) can inhibit activation, proliferation, differentiation of naïve T cells and also terminal effector cell function (Sojka et al., 2008).

The immune system must maintain a fine balance between defending the body from pathogens whilst minimising inflammatory damage to host cells. Integral components in coordinating this balance are regulatory T cells (T\(_{regs}\)), a subpopulation of T cells
involved with control of both adaptive and innate responses. T\textsubscript{reg}s have been a highly researched field in the past few years, as their function has been linked to autoimmunity, tumour immunity, control of infection and organ transplantation (Fehérvari and Sakaguchi, 2004; Sakaguchi, 2004). As the name suggests, T\textsubscript{reg}s regulate the function of T effector (T\textsubscript{eff}) cells and other cell types to dampen down the adaptive and innate response (Figure 1.5) (Fehérvari and Sakaguchi, 2004; Sakaguchi, 2004; Sakaguchi \textit{et al.}, 2007).

Two subtypes of T\textsubscript{reg} cells are operationally defined, having different properties: naturally occurring T\textsubscript{reg} (nT\textsubscript{reg}) and inducible T\textsubscript{reg} (iT\textsubscript{reg}) (Figure 1.4). nT\textsubscript{reg} are characterised as CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells, which develop in the thymus and make up 5-10% of all CD4\textsuperscript{+} T cells in humans or mice. T\textsubscript{reg} can also be further defined based on integrin \(\alpha_{E}\beta_{7}\) (CD103) expression; naïve T\textsubscript{reg} are CD103\textsuperscript{−}, while effector and memory T\textsubscript{reg} express this integrin (Zhang \textit{et al.}, 2009a).

It is believed that nT\textsubscript{reg} can recognise a wide array of self-antigens (Belkaid, 2007). Via TCR activation, they suppress the proliferation and cytokine release of CD4\textsuperscript{+}CD25\textsuperscript{−} cells \textit{in vitro} co-cultures in an antigen non-specific manner (bystander suppression) (Belkaid, 2007; Tang and Bluestone, 2008; Zanin-Zhorov \textit{et al.}, 2006). The strength of the TCR signal leading to activation (for example, avidity of antigen) may also play a role in the rapid induction of T\textsubscript{reg} during infection (Shevach \textit{et al.}, 2008). It has been suggested that specialised DCs such as CD103\textsuperscript{+} DCs can promote T\textsubscript{reg} differentiation from naïve T cells, due to their capability to secrete the necessary cytokines for T\textsubscript{reg} induction (Yamazaki \textit{et al.}, 2008; Suffner \textit{et al.}, 2010).

Although T\textsubscript{reg} become anergic upon TCR stimulation, in the periphery IL-2 and IL-7 is essential for their proliferation, whereas TGF-\(\beta\) is required for their differentiatiation and maintenance (Horwitz \textit{et al.}, 2008; Brandenburg \textit{et al.}, 2008; Korn \textit{et al.}, 2008; Shevach \textit{et al.}, 2008; Bayer \textit{et al.}, 2008). TGF-\(\beta\) binding to its cognate receptor activates Smad2 and Smad3, which may be required for \textit{foxp3} induction (Takimoto \textit{et al.}, 2010). CD28 co-stimulation with B7 ligands on DCs can lead to signaling to induce thymocytes to express Foxp3 (Guo \textit{et al.}, 2008; Scottà \textit{et al.}, 2008). Upon TCR activation and CD28 co-stimulation, transcription factors are induced to bind to
their respective promoter sequences and it has been suggested that the NF-κB signaling pathway may be vital in specific foxp3 induction (Soligo et al., 2011). This is further supported as c-Rel, a component of NF-κB as a requirement for the generation of nT<sub>regs</sub> and homeostatic proliferation of peripheral T<sub>regs</sub> (Isomura et al., 2009; Ruan et al., 2009; Deenick et al., 2010; Vang et al., 2010).

iT<sub>regs</sub> are conventional CD4<sup>+</sup>CD25<sup>−</sup> cells that upon exposure to self or foreign antigens develop in the periphery in the presence of IL-10 and IL-27 to become Tr1 cells, or in the presence of TGF-β and Runx1 and Runx3 signaling to induce foxp3 expression to form T<sub>H3</sub> cells (Klunker et al., 2009). Foxp3 expression is a loose requirement in these cell types (Hansen et al., 2007; Dardalhon et al., 2008; Nicolson et al., 2006; Vieira et al., 2004) and their suppressive potency is considerably lower than that of nT<sub>regs</sub> (Huter et al., 2008). IL-35 may be involved in the conversion of naïve T cells into iT<sub>regs</sub> (Collison et al., 2010) and indeed this cytokine is also produced by Foxp3<sup>+</sup> T<sub>regs</sub> as well (Collison et al., 2007). It has been suggested that iT<sub>regs</sub> are unstable as upon further re-stimulation in the absence of TGF-β, these cells lose Foxp3 expression.

T<sub>regs</sub> can suppress the proliferation and cytokine production of different innate and adaptive immune cells (Figure 1.5). CD4<sup>+</sup>CD25<sup>hi</sup>Foxp3<sup>+</sup> T cells can suppress IFN-γ production from γδ T cells during <i>M. tuberculosis</i> infection (Mahan et al., 2009) and may be important in maintaining intestinal homeostasis by suppressing activated γδ T cells (Park et al., 2010). The interaction between T<sub>regs</sub> and neutrophils is unclear. Neutrophil infiltration to the site of inflammation may be limited by the presence of T<sub>regs</sub> (Richards et al., 2010), although it has also been shown that human CD4<sup>+</sup>CD25<sup>hi</sup> cells can produce IL-8 to actively recruit neutrophils (Himmel et al., 2011). T<sub>regs</sub> induced during acute Fried retrovirus infection can dampen down CD8<sup>+</sup> T cell responses (Dietze et al., 2011). B cells and auto-reactive antibody production are implicated in the autoimmune disease Systemic Lupus Erythematosus (SLE) and activated T<sub>regs</sub> may suppress these B cells via cell-contact dependent mechanisms to induce apoptosis to limit pathology (Iikuni et al., 2009).
Suppressive features are not just limited to CD4\(^+\) T\(_{reg}\), but CD8\(^+\) T\(_{reg}\) cells in humans particularly have also identified to share the phenotype. Foxp3 expression can also be induced in naïve CD8\(^+\) T cells (Tuovinen et al., 2008; Cosmi et al., 2003). In general, CD8\(^+\)Foxp3\(^+\) T cells share the same features as their CD4\(^+\) counterparts but are less suppressive in comparison but still require TGF-\(\beta\) and TCR engagement for differentiation (Mahic et al., 2008; Mayer et al., 2011).

1.3.4 Foxp3 and T\(_{reg}\) development

Foxp3 is a transcription factor that represses a variety of downstream effectors of the T cell activation pathway like il-2 transcription, while inducing expression of such genes as cd25 and ctla-4 (Li and Greene, 2008; Sadlon et al., 2010). The importance of Foxp3 is shown by the existence of scurfy mice, which have a frameshift mutation in foxp3 and so lack the forkhead domain of this transcription factor. These mice exhibit widespread autoimmunity due to dysfunctional T\(_{reg}\) cells (Brunkow et al., 2001). The autoimmune defect can be rescued by the adoptive transfer of wild-type Foxp3\(^+\) cells (Huter et al., 2008). Patients with IPEX syndrome also mirror this phenotype with the production of nT\(_{regs}\) expressing mutated Foxp3 leading to severe impaired suppressive activity (Roncarolo and Gregori, 2008).

The status of Foxp3 as the defining marker of T\(_{reg}\) is contentious, as it has been suggested it is not the main determinant factor in T\(_{reg}\) cells development, rather it helps stabilise the phenotype (Hansen et al., 2007; Pacholczyk et al., 2007). This proposal has been contradicted by McFaddon and colleagues (McFadden et al., 2007) and Fontenot and colleagues (Fontenot et al., 2003), who suggest Foxp3 to be the master switch in T\(_{reg}\) differentiation in terms of nT\(_{regs}\) versus iT\(_{regs}\) development. The forced over-expression of Foxp3 in CD4\(^+\)CD25\(^-\) T cells can down-regulate the secretion of T\(_{h1}\) and T\(_{h2}\) associated cytokines, supporting the theory that Foxp3 expression represses other effector functions (Aarts-Riemens et al., 2008).

Apart from Foxp3, many different proteins and signaling pathways have been implicated in T\(_{reg}\) development. The DNA-binding inhibitor Id3 may be involved in
T\textsubscript{reg} development as TGF-β signaling pathways can lead to the production of this protein and can prevent GATA-3 from binding to the foxp3 promoter (Maruyama \textit{et al.}, 2011) Dec1 is a helix-loop-helix transcription factor and act to maintain the T\textsubscript{reg} phenotype by up-regulation of CD25 expression and prolong cell survival (Miyazaki \textit{et al.}, 2010). The transient inhibition of the mammalian target of rapamycin (mTOR)-signaling enhances T\textsubscript{reg} cell proliferation, implicating this pathway as an important regulatory switch in T\textsubscript{reg} development (Delgoffe \textit{et al.}, 2009; Procaccini \textit{et al.}, 2011).

1.3.5 \textit{T}\textsubscript{reg} associated markers

Early microarray data comparing gene expression patterns between CD4\textsuperscript{+}CD25\textsuperscript{+} and CD4\textsuperscript{+}CD25\textsuperscript{-} T cells have suggested 77 out of 11,000 murine genes were significantly differentially expressed upon TCR stimulation (McHugh \textit{et al.}, 2002). From these genes, Foxp3 is the most reliable murine activation marker for T\textsubscript{reg} cells, although Lymphocyte Activation Gene-3 (LAG-3), Cytotoxic Lymphocyte Antigen-4 (CTLA-4) and Glucocorticoid-Induced Tumor necrosis factor Receptor (GITR) are also used due to high constitutive expression along with CD25 in T\textsubscript{reg}s. Foxp3 expression in humans is not restricted purely to T\textsubscript{reg}s (Hori, 2008; Ziegler, 2006; Ziegler, 2007) as human CD4\textsuperscript{+} T cells can up-regulate Foxp3 following TCR stimulation unlike murine cells (Morgan \textit{et al.}, 2005; Walker \textit{et al.}, 2003). This high expression of Foxp3 in activated human CD4\textsuperscript{+} cells only appears to be a transient effect (Cosmi \textit{et al.}, 2003; Morgan \textit{et al.}, 2005).

CTLA-4 is an important receptor expressed by activated T cells with most research connected with T\textsubscript{reg}s, due to their high expression profile. Foxp3 can induce the expression of \textit{ctla-4} via an interaction with the transcription factor NF-AT. It has been suggested that this receptor is key in the suppressive activity of T\textsubscript{reg}s as demonstrated by CTLA-4\textsuperscript{-/-} mice exhibiting extreme autoimmune defects (Zheng \textit{et al.}, 2008b; Tang \textit{et al.}, 2008; Wing \textit{et al.}, 2008; Friedline \textit{et al.}, 2009).

The importance of CTLA-4 during immune responses against pathogens has been highlighted by studies focused on bacterial infection with \textit{Listeria monocytogenes}. 

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This intracellular Gram-positive bacterium is a predominant cause of acute gastrointestinal tract infection, with antigen specific CD4\(^+\) and CD8\(^+\) T responses induced. However, upon CTLA-4 blockade, these T cells responses are heightened and increased bacterial clearance is observed (Rowe et al., 2009), indicating CTLA-4 may not be beneficial for an efficient immune response against \textit{L. monocytogenes}.

GITR is another proposed T\textsubscript{reg} associated marker, however, it is also expressed by other subsets of T cells such as CD8\(^+\) CTL cells. GITR is a co-stimulatory factor for T cells to induce T cell proliferation and IL-2 production (van Olffen et al., 2009). The role of GITR on CTLs has been demonstrated by the administration of agonistic anti-GITR antibody to enhance expansion of these cells during viral infection (Snell et al., 2010). GITR signaling leads to enhanced survival of CD8\(^+\) effector cells by increased anti-apoptotic Bcl-X\(_L\) expression suppressing cell death pathways.

LAG-3, also known as CD223 is found on activated NK and T cells and is closely related to the CD4 co-receptor. It can bind to MHC class II molecules with a higher affinity than CD4 and large amounts of this receptor have been found in intracellular compartments (Liang et al., 2008; Woo et al., 2010). The expression of this receptor is regulated by two transmembrane metalloproteinase called ADAM10 and ADAM17, which can cleave LAG-3 from the surface of other cells such as DCs to produce soluble LAG-3 (Workman et al., 2009).

While Foxp3 is regarded a faithful marker for murine T\textsubscript{regs}, human T\textsubscript{regs} have been found to express other key markers. Latency-associated peptide (LAP) associates with dimeric mature TGF-\(\beta\) to prevent downstream signaling within the cell. Many different cells express the receptor for latent TGF-\(\beta\) or GARP, such as megakaryocytes and activated human Foxp3\(^+\) T\textsubscript{regs} (Stockis et al., 2009; Tran et al., 2009). It has been suggested that GARP, an orphan TLR composed of LRRs, may represent another marker for T\textsubscript{regs} in humans as it allows for discrimination between suppressor and non-suppressor cells within the CD25\(^+\) population (Wang et al., 2009; Battaglia and Roncarolo, 2009). Another marker for T\textsubscript{regs} in mice is CD49b (integrin \(\alpha_2\)), although this is also primarily been used as a marker for NK and NKT cells (Han et al., 2009b). In humans, CD127 (IL-7R\(\alpha\)) can also be used as a marker for T\textsubscript{regs}.
indicating the possible heterogeneous nature (Finney et al., 2010; Simonetta et al., 2010).

Since the discovery of Foxp3, many other transcription factors have now been implicated in T_{reg} development. For example, Helios, a family member of the Ikaros transcription factor family has been correlated with Foxp3 induction and is expressed in 70% of Foxp3^{+} T_{reg} cells in mice (Thornton et al., 2010). Ets-1 is the founding member of a family of winged helix-turn-helix transcription factors and is involved in many biological processes such as haematopoiesis, angiogenesis and tumour progression. Interestingly, when this transcription factor is knocked out in CD4^{+} T cells, Foxp3 expression is decreased correlating with diminished suppressive activity, suggesting that Ets-1 may play a role in foxp3 induction (Mouly et al., 2010). The discovery of a role for these transcription factors in T_{reg}s development, suggest a possible fine-tuning approach to regulate T_{reg} differentiation and suppressive activity.

1.3.6 \( T_{reg} \) suppressive function

Figure 1.6: Proposed suppressive mechanisms exerted by \( T_{reg} \) on target cells.

Four mechanisms by which \( T_{reg}s \) exert their suppressive activities upon \( T_{eff} \) cells have been proposed; [A] production of immunosuppressive cytokines, [B] direct induction of apoptosis of target cells, [C] disruption of metabolic processes in target cells and [D] targeting DCs directly to prevent further adaptive immune activation (Vignali, 2008).
The precise mechanism by which T\textsubscript{reg} act upon T effector (T\textsubscript{eff}) cells is still not fully understood. The four proposed main mechanisms for T\textsubscript{reg}-mediated suppression are the release of immunosuppressive cytokines, induced apoptosis of target cells; disruption of metabolic processes in target cells or targeting DCs directly (Figure 1.6) (Vignali, 2008).

T\textsubscript{reg} can produce many immunomodulatory cytokines upon activation. The key cytokines are IL-10 and TGF-β (Figure 1.6A). IL-10 can inhibit MHC class II and co-stimulatory CD80 and CD86 molecule expression on monocytes and macrophages (Couper et al., 2008). This anti-inflammatory cytokine can also limit the production of IFN-γ, IL-2, IL-4 and IL-5 production from CD4\textsuperscript{+} T cells (Couper et al., 2008). TGF-β is another immunosuppressive cytokine can inhibit the secretion of immunoglobulins (Corthay, 2009). A newly discovered suppressive cytokine, IL-35 is also found to be secreted by T\textsubscript{reg}. The role of this cytokine has been disputed as human T\textsubscript{reg} have been not found constitutively express this cytokine like their murine counterparts (Bardel et al., 2008).

The second mechanism of suppression is induced apoptosis of adaptive immune cells like follicular DCs, B cells and CD4\textsuperscript{+} T cells (Figure 1.6B) (Elpek et al., 2007; Hotchkiss and Karl, 2003). This is achieved through high expression of granzymes A and B and perforin on T\textsubscript{reg} (Vignali et al., 2008; Shevach, 2009). Galectin-1 is also secreted by T\textsubscript{reg} to bind glycoproteins such as CD45, CD43 and CD7 to induce apoptosis and inhibition of pro-inflammatory cytokines (Shevach, 2009).

The third mode of suppression mediated by T\textsubscript{reg} is metabolic disruption of the target cell via direct cell-to-cell contact between nT\textsubscript{reg} and T\textsubscript{eff} cells, possibly involving CTLA-4, membrane bound TGF-β and GITR interactions (Figure 1.6C) (McFadden et al., 2007; Savage et al., 2008). These interactions repress il-2 transcription in the target cell leading to apoptosis (Pandiyan et al., 2007; Scheffold et al., 2007). Apoptosis is also triggered through the production and release of cAMP, a potent secondary messenger that can inhibit T cell growth, differentiation and proliferation via gap junctions between T\textsubscript{reg} and T\textsubscript{eff} cells (Bopp et al., 2009).
T\textsubscript{regs} can suppress the proliferation of target cells by blocking IL-2 as a means of cytokine deprivation to eliminate T\textsubscript{eff} cells by apoptosis, independent of APCs (Thornton and Shevach, 1998; Scheffold \textit{et al}., 2007; Pandiyan \textit{et al}., 2007). Fas-FasL mediated pathways can also achieve induced cell death of target effector cells. High expression of an ectoenzyme, CD39 is also found on T\textsubscript{regs}, hydrolysing extracellular ATP and ADP into AMP. It has been suggested the presence of ATP can promote T\textsubscript{H}17 differentiation and human CD39\textsuperscript{+}Foxp3\textsuperscript{+} T\textsubscript{regs} can suppress T\textsubscript{H}17 cells in EAE murine models of autoimmunity (Moncrieffe \textit{et al}., 2010). Suppressive T\textsubscript{regs} can also inhibit the effects of secreted TNF-\textalpha by releasing soluble TNFRII into the extracellular space to bind this cytokine and reverse the inflammatory response (van Mierlo \textit{et al}., 2008).

The last suppressive mechanism by which T\textsubscript{regs} are suppressive is targeting DCs directly to prevent T cell activation (Figure 1.6D) (Belkaid and Rouse, 2005). T\textsubscript{regs} can interact with DCs via LAG-3 and MHC class II molecules to block DC maturation and co-stimulatory signals to T cells (Liang \textit{et al}., 2008). Tryptophane is an essential amino acid, required for the synthesis of essential cellular factors such as NAD\textsuperscript{+} and serotonin. This amino acid is degraded by two enzymes; tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), members of the kynurenine pathway (Curti \textit{et al}., 2009). TDO is primarily expressed in the liver, whereas IDO is expressed by many different tissues and induced by inflammatory stimuli (Curti \textit{et al}., 2009). IDO mediated suppression exerted by T\textsubscript{regs} is achieved by inhibiting DC production of pro-inflammatory cytokines such as IL-6 and TNF-\textalpha before cell death is induced (Hill \textit{et al}., 2007; Hotchkiss and Nicholson, 2006; Sakaguchi \textit{et al}., 2009).

Overall, various potent modes of suppression have been proposed for T\textsubscript{regs}. It would appear that perhaps \textit{in vivo}, a combination of these different mechanisms may be utilised by T\textsubscript{regs} in order for this cell type to exert maximum effect on the immune response.
1.3.7 Other T helper subsets

The repertoire of T helper subsets is continuously expanding over time. A new subset of CD4$^+$ T helper cells, T$_{H}9$ cells have been suggested to produce IL-9 and are a distinct population from T$_{H}2$ cells. The cytokines required to differentiate naïve CD4$^+$ T cells in to T$_{H}9$ cells has been contested with the suggestion that TGF-$\beta$ and IL-4 may drive the development of this particular T cell subset (Veldhoen et al., 2008). Whereas, TGF-$\beta$ and IL-1$\beta$ has also been shown to differentiate naïve CD4$^+$ T cells to produce IL-9, independent of IL-4 (Uyttenhove et al., 2010).

IL-9 is been heavily implicated in allergic diseases, such as allergic asthma (Soroosh and Doherty, 2009). However, this cytokine may also have a role in autoimmunity as demonstrated by the co-transfer of T$_{H}9$ and T$_{H}17$ cells to induce EAE disease phenotype (Jäger et al., 2009). The role of T$_{H}9$ cells during infectious disease is still largely unknown and warrants further investigation. Although the cytokine profile of T$_{H}9$ cells may have been studied, the master regulator is still unknown and IRF4 signaling has been suggested to be required as part of the induction process (Staudt et al., 2010).

IL-22, a new member of the IL-10 related cytokine family is generally considered a signature T$_{H}1$ cytokine. However, IL-22 is also linked due to its association with ROR$\gamma$t with the differentiation and expression of IL-17 in T$_{H}17$ cells. In humans, a new subset of memory T helper cells defined by their expression of CCR6$^+$CCR4$^+$CCR10$^+$ have been found to secrete IL-22 and IL-13 but not IFN-$\gamma$ or IL-17 and have been tentatively named T$_{H}22$ cells (Trifari et al., 2009). However, these cells may not represent a new T helper subset due to the presence of RORC, the human ortholog of ROR$\gamma$t. AHR expression may also regulate the expression of IL-22 in these cells, leading to the suggestion that these are exactly T$_{H}17$ cells (Trifari et al., 2009). To date, these cells have also not been identified in mice and are still under investigation.

With the expansion of cytokines known to be produced by T cells, the categorisation of CD4$^+$ T cells into discrete defined T helper subsets may prove to be a challenge in
the future. This may be due to changes in cytokine profiles according to the microenvironment and this will be discussed in the next section.

1.3.8 T cell subset stability and plasticity

Since the dissolution of the two T helper subset paradigm proposed by Mossmann and Coffman, the stability of T helper subsets has been much discussed in the past few years. The expression of specific transcription factors and cytokines were once regarded to repress the differentiation of other T helper subsets and maintain the stability of the final cell phenotype. This has been enforced through the use of certain signaling pathways, however, as these signaling pathways appear to be interlinked, this leaves the possibility of plasticity open for discussion. It is now emerging that T helper cell subsets are not rigid phenotypes resulting in final differentiation state but may be more be flexible than originally considered.

It has been suggested an equilibrium between Foxp3 and RORγt exist in CD4⁺ T cells, indicative of plasticity between T_{regs} and T_{H17} cells. TGF-β can induce the expression of Foxp3 in naïve CD4⁺ T cells leading to T_{reg} development, whereas IL-6 has an inhibitory effect. However, TGF-β together with IL-6 and IL-21 in mice can induce RORγt activation and hence leading to T_{H17} differentiation (Bettelli et al., 2008; Korn et al., 2008; Manel et al., 2008). It is interesting to note that transgenic mice over-expressing IL-6 to have increased nT_{reg} levels with normal suppressive function, although iT_{regs} development was impaired, suggesting that IL-6 has a more profound effect on the conversion of naïve CD4⁺ T cells into iT_{regs} (Fujmoto et al., 2011). IL-6 can induce Suppressor Of Cytokine Signaling (SOCS)-3 to inhibit T_{H17} cell development, although TGF-β can reverse this suppression to promote T_{H17} cell differentiation (Qin et al., 2009).

Curiously, human Foxp3⁺ T cells can still express IL-17 if they express CCR6 while retaining their suppressive quality, supporting the hypothesis of plasticity between T_{regs} and T_{H17} cells or a shared dual phenotype (Beriou et al., 2009). The presence of
IL-6 and IL-4 together can down-regulate the expression of Foxp3 in nT\textsubscript{regs}, resulting in a loss of suppressive function (Kastner \textit{et al.}, 2010). This suggests the cytokine environment is important for maintaining T cell phenotypes or inducing plasticity.

The inverse relationship between IL-6 and TGF-\beta can be further manipulated by Retinoic Acid (RA), a vitamin A metabolite, which drives T\textsubscript{reg} generation by enhancing TGF-\beta signaling via Smad3 and NF-AT and preventing T\textsubscript{H}17 development in mice and humans (Bettelli \textit{et al.}, 2008; Lochner \textit{et al.}, 2008; Huehn \textit{et al.}, 2009; Wang \textit{et al.}, 2009a; Nolting \textit{et al.}, 2009). RA is produced by CD103\textsuperscript{+} DCs to induce iT\textsubscript{regs} in the gut to inhibit T\textsubscript{H}17 cells (Xiao \textit{et al.}, 2008). RA not only drives T\textsubscript{reg} development but can also promote the proliferation of activated human T cells via the production of IL-2 (Ertesvag \textit{et al.}, 2009).

AHR has been implicated in regulating T\textsubscript{reg} and T\textsubscript{H}17 cell development by inhibiting T\textsubscript{H}17 differentiation by STAT1 activation (Kimura \textit{et al.}, 2008). These findings are further supported when a synthetic chemical, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is injected into mice, to bind AHR and induce immune suppression (Marshall \textit{et al.}, 2008). IL-27, another cytokine produced by innate cells can suppress T\textsubscript{H}17 responses and together with the action of TGF-\beta can induce the differentiation of IL-10 producing T cells with Tr1-like properties, influencing the balance between T\textsubscript{regs} and T\textsubscript{H}17 cells (Bettelli \textit{et al.}, 2008).

Despite the reported expression of ROR\textgamma\textsubscript{t} in T\textsubscript{H}17 cells, it has been observed that less than half of these cells produce IL-17, its hallmark cytokine (Lochner \textit{et al.}, 2008). The ratio of T\textsubscript{regs} and T\textsubscript{H}17 cells during murine models of infection and inflammation tend to be constant (Lochner \textit{et al.}, 2008) and it has been proposed that a possible source of TGF-\beta for T\textsubscript{H}17 differentiation may be the T\textsubscript{regs} themselves (Zheng \textit{et al.}, 2008). Only 25pg/ml TGF-\beta is required for T\textsubscript{H}17 conversion, whereas 2ng/ml is needed for T\textsubscript{reg} differentiation (O’Garra \textit{et al.}, 2008) indicating the immune system has evolved a subtle mechanism for fine-tuning the desired immune response, with a slight skewing towards T\textsubscript{H}17 differentiation.

It is not only T\textsubscript{regs} and T\textsubscript{H}17 cells that exhibit plasticity. GATA-3, the transcription factor for T\textsubscript{H}2 cells along with Foxp3 both have a competitive role in iT\textsubscript{reg}
development (Manel et al., 2008). GATA-3 can inhibit foxp3 induction by binding to its promoter and hence drive TH2 cell development over iTreg conversion. The reverse can also be said of Foxp3 binding to GATA-3 to block expression to promote Treg development (Dardalhon et al., 2008). Growth factor independent-1 (Gfi-1) is a transcriptional repressor and is transiently induced upon TCR stimulation. Gfi-1 can select GATA-3hi cells to allow for the differentiation of TH2 cells, while suppressing TH17 and Treg cell development (Zhu et al., 2009b).

Epigenetic studies have revealed cis-regulatory elements within the ifn-γ, il-17a and il-17f loci, suggesting that these genes can also be remodeled in T cells and expression patterns are not fixed, confirming the theory of plasticity within T cells phenotype (Mukasa et al., 2010). T-bet and IFN-γ can directly inhibit TH17 cell development via Runx1 activity (Lazarevic et al., 2010; Villarino et al., 2010) and also Treg differentiation (Caretto et al., 2009). Runx3, another member of the Runx family can interact with T-bet to suppress il-4 expression and promote ifn-γ expression (Kohu et al., 2009; Yagi et al., 2010). Sphingosine 1-phosphate (S1P) is a lysophospholipid and signaling via its G protein-coupled receptor can block iTreg development, while promoting TH1 differentiation (Ohkura and Sakaguchi, 2009; Liu et al., 2010a). These results further emphasise the plastic nature of T cells and the interactions of transcription factors in T helper cell differentiation.

The plasticity of T cells in humans and mice in vivo is now been demonstrated. CD4+ T cells extracted from human tumours have exhibit a TH17 phenotype, although can be differentiated into either IFN-γ producing cells or Foxp3+ cells in vitro upon TCR stimulation and expansion (Ye et al., 2011). The opposite can also be said to be true, as human memory Foxp3+ cells have been found to produce IL-17 ex vivo (Ayyoub et al., 2009). Foxp3+ cells infiltrating the central nervous system in the murine model of EAE express of IL-17 and IFN-γ (Espoito et al., 2010) and Foxp3+ cells can up-regulate T-bet during inflammation via STAT1 signaling in this model (Koch et al., 2009). TH1 cells in EAE can also co-produce IL-17 and IFN-γ or “ex-TH17” cells can shut down IL-17 production to become IFN-γ+ T cells (Kurschus et al., 2010; Hirota et al., 2011). Foxp3+ cells can also up-regulate GATA-3 to develop TH2 phenotype (Wang et al., 2010b). It would appear that Foxp3+ cells may represent the most
Research is now emerging that Foxp3⁺ Tregs can also co-express other T helper cell associated transcription factors. For example, it has been demonstrated that some Foxp3⁺ cells can express T-bet and CXCR3 in the presence of IFN-γ during type 1 inflammation (Koch et al., 2009). These Foxp3⁺T-bet⁺ cells do not produce IFN-γ but still retain their suppressive activity (Koch et al., 2009). GATA3 expression has also recently been shown to control foxp3 gene expression and consequently downstream effector function (Wang et al., 2011). This suggests that T-bet and GATA3 expression may be required for Foxp3⁺ cells to respond to different environmental signals, such as inflammation.

Overall, adaptive immunity has evolved many differentiated CD4⁺ T helper subsets, each equipped with unique functions, which are required to combat infection.

1.4 *Streptococcus pyogenes*

Bacteria can be broadly divided into two large groups according to a simple histological test known as Gram staining. Hans Christian Joachim Gram achieved this in 1884 through the discovery of different dyes to differentiate bacteria according to the physical properties of their outer cell wall structure (Gram, 1884).

For Gram staining, bacterial samples are heat-fixed before staining with the primary stain, Crystal Violet (purple) before fixation with a mordant agent Gram’s iodine. The sample is then decolourised by acetone and counter-stained with safranin (pink) (Moyes et al., 2009). After this protocol, Gram-positive bacteria, such as Streptococci, Staphylococci and Clostridium species remain purple due to the presence of Crystal Violet-iodide complexes trapped within the thick cell wall composed of Peptidoglycan. Gram-negative bacteria, however do not have a cell wall...
and hence lose their purple colour upon decolourisation and are counter-stained pink. Key examples of Gram-negative bacterium include *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae* and *Bordetella pertussis*. Gram staining remains an important microbiological test and offers an easy means to classify bacterial species.

In 1933, Rebecca Lancefield developed a method to differentiate Streptococci strains according to expression of cellular carbohydrates into five distinct groups; A, B, C, D and E (Lancefield, 1933). To date, this classification has now been extended to reflect the emergence of new groups (G-H and K-V) across many different host species (Facklam *et al.*, 2002).

*Streptococcus pyogenes* is a Gram-positive bacterium and an example of a Group A Streptococci (GAS). It is defined by the Lancefield classification scheme by the surface expression of Group A carbohydrate, composed of N-acetylglucosamine linked to a rhamnose polymer backbone and also further characterised according to surface M protein serotype based on work carried out by Lancefield in 1928 (Lancefield, 1928).

To date, more than 100 M proteins encoded by *emm* genes have been identified with more serotypes added in the past decade to the Lancefield classification scheme (Facklam *et al.*, 2002). In the past, identification of *emm* types was carried out by the Lancefield capillary precipitin technique or the slide agglutination procedure with the use of polyclonal anti-sera binding directly to M proteins. Nowadays, the standard method of *emm* typing involves Polymerase Chain Reaction (PCR)-based techniques amplifying the 5’ variable region of the *emm* gene, which defines the M serotype (Beall *et al.*, 1996; Vitali *et al.*, 2002).

*S. pyogenes* (Figure 1.7) is typically cultured on blood agar and exhibits β-haemolytic activity, resulting in zones of red blood cell lysis around the bacterial colonies, an observation made by J. H. Brown in 1919 (Brown, 1919). This feature was later attributed to secreted virulence factors called Streptolysins, which will be later discussed in Section 1.6.2.
S. pyogenes is stereotypically classified as an extracellular pathogen as it replicates and mediates its pathogenesis from outside the cell via secreted virulence factors. This is in contrast to intracellular pathogens; such as Salmonella and Listeria species, which replicate inside host cells. S. pyogenes is a human obligate pathogen and naturally colonises the throat or skin to result in asymptomatic carriage or mild non-invasive infections. However, severe invasive disease can be induced by this bacterium as well.

Other examples of streptococcal species include Group B Streptococci (GBS) strains, such as Streptococcus agalactiae, the major cause of sepsis and meningitis in neonates, young infants and pregnant women (Lancefield and Hare, 1935). S. agalactiae has a complex etiology associated with the transmission of pathogenesis to neonates. This Gram-positive bacterium can be found naturally in mucosal surfaces, such as the vagina and can be pass on to newborns during birth. Similar to GAS, GBS exhibits β-haemolytic activity and also found naturally colonised asymptotically in healthy adults.
1.5 GAS infection

1.5.1 GAS associated pathology

*S. pyogenes* is the causative agent of numerous human diseases, ranging from mild non-invasive (impetigo and pharyngitis or “strep throat”) to severely invasive disease (bacterial sepsis and necrotising fasciitis) associated with high mortality of approximately 20-50% of affected patients (Cunningham, 2000).

Epidemiologists have long studied bacterial sepsis due to the large burden it carries on the national health care service, estimated to be approximately $17 billion annually in the United States of America (Martin et al., 2003). From 1979 to 2000, the epidemiology of sepsis was studied in America, indicating that Gram-positive bacterial species were the most common cause of sepsis from the late 1980s onwards and was the overall 10\textsuperscript{th} leading cause of death in the US (Martin et al., 2003).

*S. pyogenes* has become an prevalent human pathogen due to the resurgence of invasive M1 and M3 serotype strains since the 1980s across many different developed countries, such as Norway (Martin and Høiby, 1990) and Australia (Carapetis et al., 1995). Some of these studies have correlated certain virulence factors, such as M proteins in contributing to the rise in particular strains to cause invasive human disease (Schwartz et al., 1990). Although a more recent study has shown that GAS strains with increased virulence are not the main contributing factor to the resurgence of invasive disease but rather the expression of such virulence factors enables the pathogen to spread throughout the population more effectively (Rogers et al., 2007).

The increase number of invasive *S. pyogenes* infection was not just limited to America, but also true in Britain. An upsurge of M1 and M3 serotype *S. pyogenes* associated invasive infections was recorded between 1980-1990 (Colman et al., 1993). An increase in the number of patients with invasive disease caused by *S. pyogenes* and *Streptococcus pneumoniae* has been more recently recorded above
seasonally expected levels in England (Zakikhany et al., 2011). This was observed throughout all age groups and concurrent with H1N1 viral infection, suggesting the rise in seasonal flu may contribute to the increased number of cases of invasive diseases caused by *S. pyogenes* (Zakikhany et al., 2011).

1.5.2 Non-invasive GAS infection

Most healthy adults are asymptotic carriers of *S. pyogenes*, naturally colonised on the skin and particularly in the throat with a high percentage of asymptotic carriers testing culture-positive after throat swabs. In some cases, *S. pyogenes* can cause mild infection of the skin or throat, presenting itself as impetigo, tonsillitis, pharyngitis or more commonly known as “Strep throat”. These types of infection can vary depending on the season. For example, pharyngitis tends to occur during autumn and winter, while impetigo appears more frequently during the summer (Cunningham, 2000).

A small percentage of young children present with recurrent tonsillitis, with the hypothesis formed that intracellular *S. pyogenes* can persist within tonsil tissue (Osterlund and Engstrand, 1997). Certain *emm* serotypes are associated with *S. pyogenes* induced throat infection, for example, *emm*1, 89, 2 and 12 (Blandino et al., 2010). Mouse models of pharyngitis have demonstrated that both type I and interferon (IFN)-γ responses are important in controlling dissemination of *S. pyogenes* from nasal-associated lymphoid tissue (NALT), the murine equivalent of human tonsils to the draining lymph nodes (Hyland et al., 2009). This demonstrates that immune responses are very efficient at limiting non-invasive GAS diseases, such as pharyngitis.

Scarlet fever is another example of a *S. pyogenes* associated infection in the throat. It is characterised by many symptoms such as skin rash (Figure 1.8), “strawberry tongue” and sore throat. Adults are less susceptible to scarlet fever infection, with young children being the largest affected age group. Many outbreaks correlating with different *emm* type isolates (*e.g.* *emm*75 and *emm*2) have been reported in the 21st
century, indicating this is still a prevalent disease (Dong, 2009). This infection is usually associated with the presence of secreted virulence factors called superantigens, which will be discussed later in Section 1.6.4.

![Image](image1.png)

**Figure 1.8: Characteristic skin rash induced by scarlet fever infection.**

Scarlet fever is caused by secreted exotoxins such as superantigens from *S. pyogenes*, resulting in presented symptoms of characteristic skin rash as shown above, sore throat, fever, bright red tongue with “strawberry” texture.

Certain *S. pyogenes* serotypes (*e.g.* M49 and M55) can cause skin infection, such as impetigo or pyoderma and tend to be distinct from isolates that cause pharyngitis as discussed previously (Bisno and Stevens, 1996). These infections are limited to the face and extremities of the body and are highly contagious. *S. aureus* co-infection is commonly found during some clinical episodes of impetigo (Akiyama et al., 1999). The production of Exofoliative Toxin (ET) from *S. aureus* may be responsible for the blisters in bullous impetigo (Kato et al., 2011). However, this infection can be easily treated with antibiotics.

### 1.5.3 Invasive GAS infection

Severe invasive diseases have also been linked with *S. pyogenes* deep tissue infection, resulting in necrotising fasciitis, Streptococcal Toxic Shock Syndrome (STSS) and bacterial sepsis (Johansson et al., 2010), which is a leading cause of mortality in intensive care units in America (Martin et al., 2003). Bacterial sepsis primarily affects
older individuals; with more than 60% of septic patients aged 65 years or older and 80% of deaths from this disease occur in aged patients. The term sepsis describes a systemic response to infection and clinical signs of severe sepsis include organ dysfunction and sepsis-induced hypoperfusion (Bone et al., 1992).

M1 and M3 serotypes of *S. pyogenes* have gained notoriety in the press, commonly referred to as “flesh-eating” bacteria as the pathogen responsible for necrotising fasciitis (Figure 1.9). Progression of disease is mediated by the secretion of virulence factors, such as exotoxins, which lead to the destruction of the fascia, adipose tissue and muscle. Most cases of necrotising fasciitis caused by *S. pyogenes* show no point of entry by this bacterium and a previous episode of blunt trauma may contribute to the onset of disease (Nuwayhid et al., 2007).

**Figure 1.9: Necrotising fasciitis.**

Necrotising fasciitis or “flesh eating disease” usually occurs at a site of trauma, leading to the destruction of skin and deeper tissue commonly associated with *S. pyogenes* infection. Images courtesy of Prof. S. Sriskandan.

Bacterial sepsis is a complex disease with various factors influencing the onset of pathology (Sriskandan and Altmann, 2008; Shimaoka and Park, 2008; Rittirsch et al., 2008). The initial pro-inflammatory response is key in the development of sepsis as reviewed by Chong and Sriskandan (Chong and Sriskandan, 2011). Numerous pathways starting from early recognition of the pathogen lead to the downstream production of pro-inflammatory cytokines and chemokines such as IL-6, TNF-α and IL-8 (Damas et al., 1992; Miyoshi et al., 2010), NO and the activation of complement can contribute to the recruitment of immune cells, vasodilation and coagulation (Levi, 2010), propagating the symptoms of bacterial sepsis (Figure 1.10).
Figure 1.10: Cascade of pro-inflammatory mechanisms leading to sepsis.

Microbial products and endogenous damage tissue antigens are recognised by PRRs. This induces gene expression of various products to initiate the pro-inflammatory events and leads to the propagation of the symptoms of sepsis presented by patients (red boxes) (Chong and Sriskandan, 2011).

The symptoms of severe sepsis have been attributed to the ill defined “cytokine storm”, whereby an excessive release of pro-inflammatory cytokines, such as TNF-α and IL-1β lead to large systemic inflammatory response observed in patients (Hotchkiss and Nicholson, 2006; Russell, 2006; Remick, 2007). However, this may be a simplistic view, as the administration of anti-cytokine antibodies either in the clinical setting or murine models does not offer protection from toxic shock (Reinhart and Karzai, 2001). Despite, high levels of TNF-α associated with severe sepsis, this cytokine may be beneficial. A clinical trial of a TNF-receptor: Fc fusion protein has been shown to bind and neutralise excessive TNF-α during sepsis and increased mortality (Fisher Jr et al., 1996).

The initial hyper-inflammatory response is understood to aid the host in clearing the bacterial infection. However, regulatory mechanisms designed to control the immune response become dysfunctional, leading to immune suppression (Venet et al., 2008b;
Hotchkiss and Karl, 2003; Shimaoka and Park, 2008). At this stage, septic patients present increased IL-10 cytokine levels and tend to have a worse prognosis (Gogos et al., 2000). T cell anergy, a state of T cell unresponsiveness to antigen and enhanced Th2 responses mediated by p38 MAPK signaling are also observed during the later stages of STSS (Song et al., 2000). Sepsis also eventually leads to overall decreased ability to present antigens to T cells due to down-regulated expression of MHC class II molecules such as HLA-DR on monocytes (Le Tulzo et al., 2004; Fumeaux and Pugin, 2002). Large-scale apoptosis of immune cells also exacerbates the later stages of sepsis mediated immune suppression and will be discussed in more detail in Section 1.9.1.

1.5.4 GAS infection associated sequelas

Several autoimmune diseases are triggered as a consequence of S. pyogenes infection, emphasising the impact of different pathology induced by this Gram-positive bacterium. Many of these disorders are less prevalent in the West due to better health care regimens but remain a major health problem in developing countries, particularly affecting the young. Key examples of autoimmune GAS associated sequelas include acute rheumatic fever and acute glomerulonephritis.

Acute Rheumatic Fever (ARF) is a delayed autoimmune sequela following GAS pharyngitis infection (Ellis et al., 2010). The disease exhibits many diverse symptoms, ranging from inflammation of the joints, heart, central nervous system and the skin (Guillherme and Kalil, 2010). Arthritis is the earliest feature of this autoimmune disease and ARF still remains the largest cause of acquired heart disease (rheumatic heart disease) in children, occurring within weeks post S. pyogenes infection (Stollerman, 1997).

The progression of Rheumatic Heart Disease (RHD) is proposed to be mediated through the actions of auto-reactive antibodies and T cells, cross-reactive with both streptococcal components and host tissue such as cardiac myosin peptides (Kaplan, 1963). This has been further confirmed through studies with T cell clones recognising
both streptococcal M5 peptides and myosin in a process known as molecular mimicry (Faè et al., 2006).

The induction and progression of Acute Glomerulonephritis (AG), another example of an autoimmune GAS associated sequela is very similar to ARF. However, unlike ARF, AG is usually induced after streptococcal skin infection as well as after pharyngitis (Rodriguez-Iturbe and Musser, 2008). AG is an inflammatory condition of the glomerular capillaries in the kidneys, resulting in acute and chronic renal failure in children and young adults. It has been well recognised as a consequence of scarlet fever infection as described in 1812 (Wells, 1812). Clinical features of this disease include discoloured urine due to hematuria and edema of the face and extremities of the body (Couser, 1999).

Progression of AG may be related to the deposition of immune complexes in the kidney such as C3 complement component and IgG (Couser, 1999). Many different streptococcal antigens have been suggested to bind in the glomerulus leading to pathogenesis. One such host antigen called Nephritis-associated Plasmin receptor (NAPlr), which is homologous to streptococcal GAPDH has been found deposited in the kidney, leading to disease (Oda et al., 2010). Other studies have focused on the role of antibodies against Streptococcal Pyrogenic Exotoxin B (SPEB), leading to the activation of complement and ensuing inflammation in the kidney (Cu et al., 1998).

In conclusion, due to the many different associated consequences of GAS infection, *S. pyogenes* remains a prevalent human pathogen both in industrialised and developing countries.

1.5.5 Treatment of *S. pyogenes* infection

Given the range of different diseases caused by *S. pyogenes*, treatment is also varied to reflect this diversity of presented symptoms. *S. pyogenes* is sensitive to antibiotics
such as penicillin, which still remains the number one choice of treatment for non-invasive infection such as impetigo and pharyngitis (Wessels, 2011).

For invasive disease and bacterial sepsis, the administration of antibiotics, usually penicillin in combination with clindamycin given intravenously may slow the progression of disease. Intravenous fluids are also administrated to maintain blood pressure. Other therapies and interventions may be required for severe cases of invasive disease and necrotising fasciitis. For example, surgical debridement may be required to remove the infected necrotic tissue to limit further spread of infection and aid the recovery of the unaffected healthy tissue underneath (García-Casares et al., 2010).

The lack of protective antibodies against virulence factors formed during streptococcal infection is usually linked to worse prognosis during invasive disease. Intravenous polyspecific Immunoglobulin (IVIG) is a potential therapy against bacterial sepsis and necrotising fasciitis. This treatment offers high poly-specificity generated by antibodies pooled from several thousand donors against streptococcal virulence factors like superantigens and M proteins (Johansson et al., 2010). The effectiveness of IVIG treatment has been demonstrated in experimental animal models, whereby the effects of secreted superantigens were neutralised and enhanced bacterial killing and reduced systemic inflammation observed (Sriskandan et al., 2006). The use of this therapeutic has also now been extended to the clinic successfully (García-Casares et al., 2010).

The requirement for vaccination has been highlighted due to the resurgence of invasive GAS infection and the enormous burden it bears on the health care service in the past few decades. However, the choice of streptococcal target to induce antibody production has been faced with many difficulties to overcome the sheer diversity in M serotypes and also without inducing auto-reactive antibodies as highlighted in the development of ARF and AG autoimmune sequels. Other GAS targets for vaccine suggested include fibronectin-biniding protein, streptococcal pyrogenic exotoxins and cell wall carbohydrates (Pandey et al., 2009).
1.6 Streptococcal virulence factors

*S. pyogenes* produces different virulence factors to mediate disease pathology and prolong survival within the host. In this section, I will briefly describe the most commonly studied streptococcal virulence factors.

### 1.6.1 CovRS regulatory system

![Diagram: Gene expression induced by the CovRS two-component regulatory system.](image)

Environmental signals induce the phosphorylation of CovS (HPK), which in turns phosphorylates CovR (RR protein) to initiate expression of many different genes involved in the production of capsule, secreted proteins and regulation and adaption to the environment.

Two-component regulatory systems are found ubiquitously and utilised by most prokaryotes, archea and a few eukaryotic organisms to sense and respond to changes within the environment. Two-component systems consist of a phosphotransfer scheme, involving two specific complexes; a membrane bound Histidine Protein Kinase (HPK) and a Response Regulator (RR) protein, usually found in the
cytoplasm of the cell. Upon extracellular signal transduction, the histidine kinase domain of the HPK, transfer a phosphate group to the RR protein (Stock et al., 2000).

The expression of key virulence factors in *S. pyogenes* is mediated by a two-component regulatory system called CovRS or CsrRS, with CovS representing the HPK and CovR, the RR protein (Graham et al., 2002). When this regulatory system is inactive, it leads to over-expression of the hyaluronic acid capsule resulting in a mucoid phenotype (Graham et al., 2002). CovR, exerting its regulatory role during late exponential or stationary phase, is a repressor of approximately 10-15% of the genome with many different genes linked to stress responses and virulence as highlighted by microarray studies (Figure 1.1) (Graham et al., 2002).

Mutations in CovRS in M1 serotype *S. pyogenes* isolates can switch from a non-invasive phenotype to hyper-virulence, characterised by increased resistance to neutrophil mediated killing and enhanced survival within the host. These mutants can arise spontaneously during invasive infection. Enhanced virulence may also be due to increased expression of SdaI DNase to degrade the DNA framework of NETs (Walker et al., 2007). It has also been found that M1 protein and hyraluronic acid capsule expression are indispensable and enhance survival of these hyper-virulent CovRS mutants (Cole et al., 2010).

Subtleties in the regulation exerted by the CovRS system have been demonstrated through the use of single *covR* and *covS* knockout GAS mutants. For example, CovR retains some regulatory control in the absence of CovS but CovS cannot function without CovR (Treviño et al., 2009). *covS* knockout mutants were found to be severely out-competed by wild-type isolates in an ex vivo model of upper respiratory tract infection (Treviño et al., 2009). This indicates *covS* mutants may be positively selected for during invasive disease but not during upper respiratory non-invasive infections such as pharyngitis.
1.6.2 Exotoxins

*S. pyogenes* can secrete different exotoxins to mediate its detrimental effects on the host. For example, GAS can produce a carboxylic esterase encoded by the *sse* gene, under the control of CovRS regulatory system. This esterase contributes to virulence, as mice immunised with a SSE knockout isolate are more resistant to lethal subcutaneous GAS infection and exhibit diminished invasion of skin tissue (Zhu et al., 2009a). If the *sse* gene is deleted, decreased severity of tissue destruction and limited dissemination of GAS from the skin is also observed (Zhu et al., 2009a). This highlights the importance of secreted exotoxins to enhance the survival of GAS within the host.

Sepsis is characterised by devastating cell death during the later stages of disease. This is presented as apoptosis and necrosis of immune cells, such as macrophages. *S. pyogenes* can induce cell death via the production of pore-forming toxins, such as Streptolysin O (SLO) and Streptolysin S (SLS) (Gilbert, 2010).

SLO is an oxygen-dependent secreted protein that binds cholesterol in eukaryotic membranes to form large holes and hence induces apoptosis. SLS is also another oxygen-dependent protein secreted by *S. pyogenes* with potent haemolysin activity to induce necrosis (Goldmann et al., 2009). This exotoxin is also responsible for the zones of red blood cell lysis when *S. pyogenes* is cultured on blood agar. SLS also inhibits neutrophil recruitment upon infection, enhancing the survival of the bacterium within the host by affecting the production of chemotactic gradients required to attract neutrophils to the site of infection (Lin et al., 2009).

Another key exotoxin produced by *S. pyogenes* is Streptococcal Pyrogenic Exotoxin B (SPEB). SPEB is a cysteine protease with broad specificity, which can modulate host proteins like matrix proteins to mediate bacterial entry and virulence. It is produced as a precursor zymogen before autocatalytic processing and *speb* is also under the control of CovRS regulation. SPEB can cleave anti-bacterial chemokines such as IP10, and CXCL10 (Egesten et al., 2009). Interestingly, this exotoxin can also degrade streptococcal targets such as superantigens (Nooh et al., 2006). It has
been suggested that this virulence factor has also been an attractive target for streptococcal vaccine design in the past but process has been hindered as SPEB may be implicated in the induction of AG autoimmunity (Ulrich, 2008).

IL-8 is an important chemokine, responsible for creating a chemotactic gradient to aid the migration of neutrophils to the site of infection and subsequent activation by induced CXCR1 or CXCR2 expression. *S. pyogenes* can produce a cell wall bound subtilisin-like serine protease, called SpyCEP to specifically cleave IL-8 and hence prolonging GAS survival (Edwards *et al.*, 2005). SpyCEP is encoded by *cepA* gene under the control of CovRS regulation and can also be released as a soluble enzyme (Turner *et al.*, 2009). Virulence of particular streptococcal isolates can be attributed to the high expression of SpyCEP (Zinkernagel *et al.*, 2008). This protease can also cleave other chemokines such as Granulocyte Chemotactic protein-2 (GCP-2) and Growth-Related Onco-gene-α (GRO-α) to induce the same effect as IL-8 deactivation (Sumby *et al.*, 2008).

1.6.3 M proteins

M proteins are virulence factors expressed on the bacterial cell wall and membrane, which act as surface antigens. They form fibrillar α-helical coiled-coil dimers, protruding from the bacterial surface. The C-terminal, located near the cell surface is conserved amongst M proteins, whereas the N-terminal is highly immunogenic (Robinson *et al.*, 1993). As previously mentioned in Section 1.4, over 100 different M protein serotypes have been identified reflective of the sheer diversity in the C terminal of this group of virulence factor.

This example of surface proteins can interact with fibrinogen, which in turn can bind via β-integrins expressed on neutrophils. This triggers the release of heparin-binding protein (HBP), which is a mediator of vasodilation, one of the clinical symptoms of sepsis (Macheboeuf *et al.*, 2011). This effect has also been witnessed on human
peripheral blood monocytes in a TLR2 dependent manner (Påhlman et al., 2006), perpetuating the inflammatory response observed during septic shock.

M proteins can also interact with other cells of adaptive immunity. M proteins secreted by *S. pyogenes* can interact with CD46 on CD4⁺ T cells to induce IL-10 production and bystander suppression of T effector cells via granzyme B expression (Price et al., 2005). *S. pyogenes* may even directly bind to soluble CD46 to enhance survival (Lökvist et al., 2008). It has also been shown soluble M1 protein, devoid of its membrane anchor can induce T cell proliferation and cytokine production, such as IFN-γ, acting in a manner very similar to superantigens (Påhlman et al., 2006).

M proteins have been a suggested target for streptococcal vaccine design for many years as neutralising antibodies can be produced against these surface expressed proteins. It has been known that epitopes from M5 protein (M5308-319 and M517-31) can be presented by macrophages via MHC class II molecules and presented to CD4⁺ T cells (Degnan et al., 1997; Delvig and Robinson, 1998; von Delwig et al., 2002). A B cell epitope designated J8 has also been identified from the conserved region of M proteins (Pandey et al., 2009). Peptide synthesised from J8 was found to stimulate T and B cells to offer protection from GAS infection in mice (Pandey et al., 2009). However, research in this field of vaccine design has yet to yield any successful results in the clinic due to the sheer diversity in M protein serotypes (Smeesters et al., 2008) and cross reactivity with human tissue exhibited by such neutralising antibodies.

Other approaches have been developed in the hunt for non-M protein based vaccine targets. Genomic surface protein libraries were searched for potential candidates for vaccine design. One key finding was Spy 1536 proteins. If this gene is deleted in *S. pyogenes*, it leads to decreased bacterial binding to human plasma and extracellular matrix protein and it would appear that M protein expression is also dependent on the presence of this gene (Fritzer et al., 2010). Spy 1536 offers real potential as a vaccine target as protection against GAS infection has been shown upon deletion of protein in murine models of sepsis (Fritzer et al., 2010).
### Superantigens

Table 1.2: Functional properties of superantigens secreted by *S. pyogenes*.

The 11 different superantigens secreted by *S. pyogenes*, along with molecular weights, specificity with human TCR Vβ chains and potency of binding is shown as the concentration of superantigen required to stimulate half the number of PBMCs *in vitro* (P_{50}) (Proft and Fraser, 2003).

<table>
<thead>
<tr>
<th>Superantigen</th>
<th>Molecular weight (KDa)</th>
<th>Human TCR Vβ specificity</th>
<th>P_{50} (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPEA</td>
<td>26.0</td>
<td>2.1, 12.2, 14.1, 15.1</td>
<td>-</td>
</tr>
<tr>
<td>SPEC</td>
<td>24.4</td>
<td>2.1, 3.2, 12.5, 15.1</td>
<td>0.1</td>
</tr>
<tr>
<td>SPEG</td>
<td>24.6</td>
<td>2.1, 4.1, 6.9, 9.1, 12.3</td>
<td>2</td>
</tr>
<tr>
<td>SPEH</td>
<td>23.6</td>
<td>2.1, 7.3, 9.1, 23.1</td>
<td>50</td>
</tr>
<tr>
<td>SPEI</td>
<td>26.0</td>
<td>6.9, 9.1, 18.1, 22</td>
<td>0.1</td>
</tr>
<tr>
<td>SPEJ</td>
<td>24.6</td>
<td>2.1</td>
<td>0.1</td>
</tr>
<tr>
<td>SPEL/K</td>
<td>27.4</td>
<td>1.1, 5.1, 23.1</td>
<td>1</td>
</tr>
<tr>
<td>SPEM</td>
<td>26.2</td>
<td>1.1, 5.1, 23.1</td>
<td>10</td>
</tr>
<tr>
<td>SSA</td>
<td>26.9</td>
<td>1.1, 3, 15</td>
<td>-</td>
</tr>
<tr>
<td>SMEZ-1</td>
<td>24.3</td>
<td>2.1, 4.1, 7.3, 8.1</td>
<td>0.08</td>
</tr>
<tr>
<td>SMEZ-2</td>
<td>24.1</td>
<td>4.1, 8.1</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*S. pyogenes* is an example of a bacterium that expresses a wide range of superantigens. So far, 11 superantigens have been identified from GAS (Table 1.2) (Thulin *et al.*, 2006). Unlike other streptococcal virulence factors such as SPEB, superantigens are encoded on temperate bacteriophages instead of chromosomal DNA (Ferretti *et al.*, 2001; Proft *et al.*, 2001; Proft and Fraser, 2003).

The production of superantigens appears to be conserved within the Streptococci genus. For example, *Streptococcus equi*, a Group D Streptococci, associated with the infectious disease of strangles in horses, produces various superantigens such as SeeH, SeeI, SeeL and SeeM with similar immunogenic features to those secreted by GAS (Paillot *et al.*, 2010). *S. aureus* is another common Gram-positive human pathogen that utilises superantigens, such as Staphylococcal Exotoxins (SE)-A, SEB and SEC to exert pathogenesis (Proft and Fraser, 2003).
The prevalence of invasive GAS isolates may be attributed to *emm* type and also by the expression of different superantigens. A study in Australia examined the genotype of 107 clinical GAS isolates and expression of the 11 different known superantigens. It was revealed that certain *emm* types were found to contain particular combinations of superantigens genes (Commons *et al.*, 2008). For example, *emm1* isolates were found to contain *spea, speg, spej* and *smez* but do not possess *spec, ssa* and *speh* (Commons *et al.*, 2008). This may indicate that superantigens play a role in the prevalence of different *emm* types during infection. However, this can be argued from the other point of view, suggesting that the prevalent of certain superantigens are the main determinants in invasive disease (Lintges *et al.*, 2010).

While the presence of superantigens is not inextricably linked to the ability of GAS to cause sepsis, there is evidence linking specific clinical episodes to the presence of potent superantigens. A high proportion of all pathogenic GAS isolates encode one or more superantigens such as Streptococcal Pyrogenic Exotoxin A (SPEA). *spea* is present in 40-90% of *S. pyogenes* strains associated with invasive disease but only in 15-20% of non-invasive strains (Sriskandan *et al.*, 1999). *In vivo* experiments of mice given recombinant SPEA protein have been shown to develop pyrexia and hypotension (Lee and Schlievert, 1989).

Superantigens are unlike conventional antigens as they bind to MHC Class II molecules in a region away from the peptide-binding groove and to the TCR variable-β chain of the T cell, and do not require prior antigen processing (Dellabona *et al.*, 1990). Many different models have been proposed for how superantigens cross-link with MHC molecules and the TCR. Early crystal structures with SEC superantigen, suggested the protein displaces the TCR and MHC class II molecule like a wedge so there was no direct contact between TCR and antigenic peptide (Fields *et al.*, 1996). Other models have suggested that some superantigens (*e.g.* SEH) have zinc-binding motifs to offer high affinity interactions with TCR and MHC class II molecule in a bridge-like structure (Figure 1.12) (Petersson *et al.*, 2001).

Streptococcal superantigens exhibit preferential binding to MHC class II molecules, with highest binding affinity to HLA-DQ, then -DR and weakest with –DP due to constriction in binding conformations (Sriskandan and Altmann, 2008; Rajagopalan...
et al., 2007). This is highlighted in structural studies of SPEA, whereby a crucial disulphide bonds are required to induce superantigenic effects and strong affinity binding with HLA-DQ molecules (Kline and Collins, 1996).

Figure 1.12: Schematic of superantigen binding to MHC class II molecule and TCR.

Binding of superantigens to MHC class II molecule and TCR leads to T cell activation, proliferation and cytokine release (Llewelyn, 2005).

Three groups of superantigens can be defined according to their specificity to TCR structures as reviewed by Bueno and colleagues (Bueno et al., 2007). The first group contains superantigens such as SEB will recognise any TCR β chain via their CDR2 loops (Hayball et al., 1994). The second group (e.g. SPEA) requires more specific interactions and recognises TCR Vβ chain via CDR1 and CDR2 loops. The last group of superantigens (e.g. SPEC) is highly specific utilising all three CDR loops of the TCR (Kline and Collins, 1996; Sundberg et al., 2002). Hence each superantigen binds to favoured Vβ families, resulting in TCR signaling and specific T cell expansion (Figure 1.12) (Llewelyn, 2005; Llewelyn et al., 2004; Llewelyn and Cohen, 2002). For example, Streptococcal Mitogenic Exotoxin-Z (SMEZ), the most potent superantigen, favours Vβ8+ T cells in mice (Unnikrishnan et al., 2002).

Superantigen binding with T cells has been found to activate up to 20-30% of the host T cell population (Lappin and Ferguson, 2009), whereas conventional antigens only
activates approximately 1 in $10^4$ to 1 in $10^6$ T cells (Li et al., 1999). The resultant effect is T cell proliferation and cytokine production, such as IL-1, IL-6, IFN-γ and TNF-α (Figure 1.12) (Cunningham, 2000) as demonstrated when streptococcal supernatants are used to stimulate human PBMCs (Unnikrishnan et al., 2002). High levels of TNF-α are measured from murine spleens within 1 hour of exposure to purified superantigens (Sriskandan and Altmann, 2008).

HLA-transgenic mice have been exploited in the study of superantigen driven immune responses. HLA-DR3 mice immunised with purified SEB via an intra-nasal route developed inflammation of the airways and lung (Rajagopalan et al., 2006). The immune response was further studied and typical effects of superantigen mediated T cell modulation were observed. For example, specific Vβ8+ expansion of CD4+ and CD8+ T cells were seen along with increased IFN-γ production (Rajagopalan et al., 2006). A similar set of results was also attained after infection with SPEA in HLA-DQ8 transgenic mice (Welcher et al., 2002).

SMEZ induces a cytokine response approximately ten times higher than with SPEA (Yang et al., 2005). However, unlike other superantigens, SMEZ is the most polymorphic as demonstrated by the identification of 22 different smez alleles from 21 different M serotype GAS isolates over a 15-year period in New Zealand (Proft et al., 2000). The creation of many different allelic variants of smez may be caused by the process of gene mosaism, similar to homology gene recombination between two copies of the gene (Proft et al., 2000). Due to the highly polymorphic nature of SMEZ, very few neutralising antibodies have been detected in normal adult sera (Proft et al., 2000). This suggests that along with SMEZ representing the most potent superantigen to date, it is also the most prevalent and the most variable.

Other superantigens such as SPEI and SPEJ have been less well researched. These two virulence factors are the second most potent superantigens after SMEZ (Proft et al., 2001). SPEI induced Vβ18+ T cell expansion, whereas SPEJ favoured Vβ2 and to a lesser degree Vβ8+ T cells (Proft et al., 2001). In vivo experiments have demonstrated that rabbits given 500μg of recombinant SPEJ all succumb to sepsis, indicative the potency of this superantigen (McCormick et al., 2001).
Superantigens have not only been exclusively found to interact with T cells but also B cells as well. *Moraxella (Branhamella) catarrhalis* is a Gram-negative bacterium associated with respiratory diseases, particularly in children. It is capable of secreting superantigens, such as *Moraxella* IgD-binding protein (MID), which targets human IgD. This virulence factor can cross-link with B Cell receptors (BCRs) to induce proliferation and cytokine production of B cells (Jendholm et al., 2009), reminiscent of streptococcal superantigens binding to TCR of T cells. However, MID also requires signaling via TLR9 to exert its effects (Jendholm et al., 2009). Superantigens secreted by viruses such as Lymphocytic Choriomeningitis Virus (LCMV) can also expand Vβ specific T cells (Punkosdy et al., 2011), emphasising the conservation of this class of virulence factor amongst various pathogenic microorganisms.

### 1.6.5 Capsule

![Figure 1.13: Mucoid appearance of *S. pyogenes.*](image)

When heavily encapsulated *S. pyogenes* isolates are grown overnight on blood agar, they exhibit a mucoid phenotype, appearing like oil droplets on the agar (A), compared to smaller, more opaque wild-type isolates (B) (Stollereman and Dale, 2008).

*S. pyogenes* is a typical Gram-positive bacterium as it expresses an outer capsule composed of hyaluronic acid (Kendall et al., 1937). This is not uncommon, as many
Pathogenic bacteria such as *S. aureus* also express large polysaccharide capsules to avoid phagocytosis (Wilkinson *et al.*, 1979). Protection from the innate immune system is achieved by blocking opsonins, such as C3 from coating the microorganisms and direct attachment to macrophages (Foley and Wood Jr, 1959; Dale *et al.*, 1996; Whitnack *et al.*, 1981). Host immunity can produce natural antimicrobial peptides, such as the Hyaluronic Acid-Binding Peptide (HABP), PEP35 to trigger an immune response. This peptide has been shown to promote neutrophil recruitment during *S. aureus* infection to aid bacterial clearance (Lee *et al.*, 2010a).

Hyper-virulence of covS M1 serotype GAS mutants is accompanied by increased expression of capsule, leading to decreased adherence to epithelial cells and increased resistance to phagocytosis (Lyons and Ward, 1935; Hollands *et al.*, 2010). Encapsulated GAS isolates are also able to manipulated host cells by binding to the hyaluronic acid binding protein, CD44 to allow entry into epithelium (Schrager *et al.*, 1998). The adaptive immune system can be impeded by the concealment of key surface antigens by capsule proteins shielding the pathogen from elimination. These features of capsule offer great protection from the host immune system.

Overall, *S. pyogenes* has adapted to use many different pathogenic virulence factors to evade the immune system and hence prolonging survival within the host.

1.7 Immunogenetics of sepsis susceptibility

Individuals infected with the same strain of *S. pyogenes* can confer different outcomes in disease progression. This suggests that there may be genetic factors, unrelated to pathogens involved in the susceptibility of sepsis and severity of disease. Specific HLA class II haplotypes have been extensively studied due to the preferential binding of streptococcal superantigens to TCR and MHC class II molecules, which may influence the immunological response.
From analysis of human PBMCs from healthy donors with known HLA genotypes stimulated with 2 clinical streptococcal isolates, it was found that HLA-DR15 and DQ6 alleles were associated with protection against STSS. Whereas, HLA-DR7 and DR5 alleles were indicators of high risk of developing invasive disease (Nooh et al., 2011). More extensive analysis of HLA haplotypes and different clinical manifestations of S. pyogenes invasive disease have also indicated that patients with the DRB1*1501/DQB1*0602 haplotype were less likely to develop severe sepsis (Kotb et al., 2002). The DRB1*14/DQB1*0503 and DRB1*11/DQB1*0301 haplotypes in this study were shown to be a high risk factor for severe sepsis and necrotising fasciitis (Kotb et al., 2002). This indicates that a genetic component does exist as a risk factor for developing severe invasive streptococcal disease.

The allelic variation of MHC class II molecules in humans may influence different affinity to bacterial antigens and hence the varying susceptibility to invasive streptococcal disease. This is also reflected in mice. C3H/HeN and CBA/J mice are highly susceptible to GAS infection and sepsis, whereas DBA/2, C57BL/10 and BALB/c mice are resistant to disease and survive infection (Medina et al., 2001; Goldmann et al., 2005b). This has been linked to an association with H2 genotype. Transgenic BALB/c mice carrying the H2K region of C3H/HeN mice are equally susceptible as wild-type C3H/HeN strain to GAS infection with increased levels of IFN-γ, IL-12 and IL-6 in the blood (Goldmann et al., 2005b). This indicates that MHC class II or H2 genotype in either humans or mice have a major influence on the susceptibility and severity of streptococcal disease.

Genetic risk factors are also associated with cytokine genes as well. As TNF-α, IL-6 and IL-1β are considered hallmark cytokines during the initial pro-inflammatory stage of sepsis, genomic analysis of these genes has produced some interesting results regarding genetic susceptibility to S. pyogenes infection. Many Single Nucleotide Polymorphisms (SNPs) have been identified in the promoter region of tnf-α. One such SNP at position -308bp exhibited strong association with patients who developed severe septic shock (Mira et al., 1999). The same is true for il-6 and il-1β genes and also three SNPs in il-10 have been isolated, leading to decreased production and increased severity of sepsis (Sutherland et al., 2005; Lowe et al.,
However, correlating mutations in key cytokine genes with disease severity, susceptibility and prognosis across multiple genetic backgrounds has given inconsistent results. This suggests that larger sample sizes may be required or the genetic correlation is very weak (Papathanassoglou et al., 2006; Arcaroli et al., 2005).

Point mutations in PRRs have also been investigated. This may be important as a decreased ability to detect and respond to microbial ligands may have a huge impact on the downstream responses to skew the onset of disease. Two specific SNPs (D229G and T399I) in humans tlr4 have been identified, with some correlation to increased risk to septic shock due to decreased cytokine production (Kumpf et al., 2010). Interestingly, the D229G SNP in tlr4 has also been studied within the context of autoimmune diseases, such as MS with little impact on clinical disease found (Kroner et al., 2005). Another example of a polymorphism in a PRR gene is R753Q SNPs in human tlr2, initially identified within a cohort of white healthy donors and may results in a sequestered response to bacterial infection (Schröder et al., 2003; Lorenz et al., 2000).

Overall, it would appear that certain MHC class II haplotypes are associated with susceptibility to GAS infection, presumably due to preferentially binding of superantigens to MHC class II molecules and TCR. However, mutations in key PRRs, such as tlr2 and tlr4 may also play a key initial role in determining the severity of disease.

1.8 Mouse models of sepsis and inflammatory diseases

Different animals models of sepsis and other inflammatory diseases have been developed in the past few decades to help researchers study the immune responses mounted during systemic infection. Each model has advantages and disadvantages associated and will be discussed in the following section.
1.8.1 Cecal Ligation and Puncture (CLP) model

The Cecal Ligation and Puncture (CLP) model of sepsis is the most widely used model for experimental sepsis. It has been claimed to be the most realistic model for induction of poly-microbial sepsis.

The cecum is an endogenous source of microorganisms and rupture of this tissue results in the release of this bacterial microenvironment into the peritoneal cavity, causing bacterial peritonitis and eventually sepsis and toxic shock when bacteria gain entry into the bloodstream (Rittirsch et al., 2009). The advantages of using the CLP model to study bacterial sepsis are that this technique can be adapted to induce sepsis with varying degrees of severity and may reflect a more realistic model of human disease but technically, it is hard to achieve consistency between experiments (Rittirsch et al., 2009).

While most studies have focused on particular cell types of the innate and adaptive immune response, the complement system may play a significant role during sepsis. C5a has been proposed to have an inflammatory role in CLP-treated mice. This component of the complement cascade interacts with both DCs and γδ T cells to lead to the production of IL-17, which has been shown to be pathogenic in a CLP model of sepsis (Xu et al., 2010). C5a has also been found to alter the intracellular signaling in neutrophils in vitro and also during the onset of sepsis in vivo (Xu et al., 2010). Mice lacking either C1q and therefore lacking classical complement signaling activity or fd knockout mice, which lack alternative pathway have been shown to have increased mortality and bacterial burden in CLP models of sepsis (Dahlke et al., 2011). This highlights the importance of early innate immunity, such as complement in the activation of neutrophils and downstream effective bacterial clearance.

HSP-70 has been implicated in the pathology of sepsis, particularly in aged patients. This chaperon protein has also studied in murine models of sepsis. For example, young HSP-70+/− mice do not display any difference in survival outcome compared to age-matched WT controls post CLP surgery (McConnell et al., 2011). There was however, a difference in older mice, where the presence of HSP-70 prevented
mortality by reducing sepsis-induced apoptosis and systemic inflammation (McConnell et al., 2011), indicating that DAMPs, such as HSPs are key in activating protective immune response.

Many different disease models have emphasised the importance of cytokine production during infection. IL-33 can also influence the migration of neutrophils to the site of infection to aid bacterial clearance in CLP model (Alves-Filho et al., 2010). Decreased serum levels of IL-6 and TNF-α, potent inducers of systemic inflammation were also observed upon IL-33 treatment (Alves-Filho et al., 2010). This suggests that IL-33 is protective and may represent a therapeutic agent against the onset of sepsis. Another possible therapeutic agent is recombinant IL-7, shown to induce proliferation of CD4+ and CD8+ T cells and suppress apoptosis mediated by CLP-induced sepsis (Unsinger et al., 2010). A similar role was also found for IL-15, which can protect CD4+, CD8+ T cells, NK cells and DCs from apoptosis during sepsis, improving overall survival during systemic disease (Inoue et al., 2010). However, whether this cytokine can be used as a therapeutic for treatment of sepsis remains unknown.

The use of the CLP model has highlighted an interesting caveat in the study of Tregs during sepsis. CLP-treated mice exhibit an increase in the number of Tregs with suppressive activity. However, these mice are unable to contain the development of an implanted tumour due to enhanced immune suppression after the symptoms of sepsis has subsided (Cavassani et al., 2010). This is also mirrored in humans, where patients who have survived sepsis show evidence of a sustained immunosuppressive state. Mice subjected to poly-microbial sepsis are more susceptible to secondary bacterial infection, such as Pseudomonas aeruginosa, caused by decrease neutrophil recruitment as a consequence of immune suppression (Delano et al., 2010).

Models of Acute poly-microbial Peritonitis (AP) are similar to CLP models of sepsis, as a surgical connection in the form of 18-gauge silicon tube is inserted in the cecum and out into the peritoneum, allowing microbiota to enter this cavity from the intestines. AP-operated mice exhibit elevated serum levels of IL-6 and IL-10 compared to sham controls, accompanied with an influx of neutrophils (Barrera et al., 2011). This mirrors immunological changes observed in CLP models of sepsis. These
cytokine changes also correlate with increased bacterial burden in AP-treated mice, suggesting there is a dysfunction in the clearance of the resultant infection and also reflect a decreased proliferation of T cells (Barrera et al., 2011). This indicates appropriate adaptive immunity is not sufficiently mounted to offer protection from disease.

1.8.2 Animal models of colitis

Different murine models have also been developed to study other inflammatory diseases, such as colitis. Colitis usually refers to as the inflammation of the colon and it can be classified based on various disease phenotypes, such as autoimmune Crohn’s disease to infectious colitis, caused by the presence of Clostridium difficile and Shigella dysenteriae. The presence of gut microbiota is important in the induction of colitis, as mice housed in pathogen-free environments do not develop this disease (Feng et al., 2010). The study of colitis has offered interesting results regarding how the immune system can induce an inflammatory response and may be extrapolated to the study of sepsis.

The most commonly used model of colitis involves the transfer of auto-reactive CD4\(^+\) T cells into RAG-deficient mice, resulting in inflammation of the large intestine. This highlights the potency of T cells in the induction of inflammation. This is emphasised by the requirement of IL-7 by memory colitogenic T cells to proliferate during the development of colitis in RAG-1\(^{-/-}\) mice (Tomita et al., 2009). Co-stimulatory signals between CD27 expressed on T cells and CD70 on DCs may affect the development of colitis. This has been proposed in an animal model of colitis, whereby signaling via the CD27-CD70 co-stimulatory pathway was shown to be detrimental (Manocha et al., 2009; Cox et al., 2011). The blockade of CD70 or CD27 prevented the induction of colitis upon the transfer of autoreactive CD4\(^+\)CD45RB\(^{hi}\) T cells into RAG-deficient mice (Manocha et al., 2009).

If T cells are important to drive inflammation, it is not inconceivable that IL-2 is also required to promote the proliferation of these antigenic T cells. This has been
demonstrated when CD4+CD45RBhi T cells from a IL-2−/− mouse cannot induce colitis when adoptively transferred into RAG-knockout mice (Kameyama et al., 2010). γδ+ TCR T cells may also be responsible for the onset of colitis through the production of IL-17 (Nanno et al., 2008; Do et al., 2011). A similar pathogenic role for T cells has been implicated in Inflammatory Bowel Disease (IBD). Neutralisation of IL-23 ameliorates murine models of IBD, due to decreased T cell proliferation and Th17 development in the intestines (Ahern et al., 2010), supporting the theory that T cells mediated immunity is not always advantageous to the host.

As with sepsis, a possible detrimental role of TNF has been highlighted through the studies of colitis, where CD4+ T cells over-expressing the TNF receptor, TNFR2 caused exacerbation of the disease (Dayer Schneider et al., 2009). Mice administrated with TNF have even been shown to develop toxic shock as a result. This effect may be mediated via TNF stimulating the production of type I interferons from macrophages and can be reversed by knocking out IFN-β in mice to protect them from induced shock (Huys et al., 2009). IL-17 from Th17 cells may also exacerbate TNF-induced shock (Takahashi et al., 2008). While TNF may be pathogenic during transfer models of colitis, the protective role of IL-10 from Tregs has been demonstrated to suppress the onset of colitis (Murai et al., 2009), emphasising the importance of immune regulation during inflammation.

The administration of certain chemicals to animals can also artificially induce colitis. The intra-rectal administration of trinitrobenzene sulphonic acid (TNBS) in susceptible strains of mice has been shown to induce Th1 cells to cause colitis, while treatment with anti-CD70 antibody can inhibit disease (Manocha et al., 2009). Decreased foxp3 expression has also been found in a chemical induction model of transient colitis (Reardon et al., 2008). Activation of the AHR pathway through the administration of TCDD inhibited dextran sulphate sodium (DSS)-induced colitis, suggestive that this signaling pathway, generally associated with Th17 cell development may be protective in murine models of colitis (Takamura et al., 2010), which may explain the decrease in Tregs observed by Reardon and colleagues.
Overall, the use of animal models of sepsis and other associated inflammatory
diseases has provided us with many key findings of how the immune system responds
during infection and inflammation. They have also provided important tools to new
therapeutics to better treat patients with sepsis.

1.9 Immune responses during GAS bacterial infection and sepsis

As the high mortality and morbidity associated with sepsis represent a huge
economical burden on the health care service, much research has been conducted to
better understand how the host immune system responds to systemic bacterial
infection. I will present a brief summary of our current understanding of how
apoptosis, autophagy, innate and adaptive immune response contribute to the onset
and development of sepsis.

1.9.1 Apoptosis during GAS infection and sepsis

Large-scale apoptosis, particularly of lymphocytes is observed during the
immunosuppressive phase of severe sepsis. This is attributed to apoptosis via both
Fas and mitochondrial-mediated pathways. However, blocking either pathway only
partially protects mice from CLP-induced sepsis (Chang et al., 2007), indicating that
other pathways may operate to induce apoptosis and still remains a source of
research. Successful capture of pathogens, such as S. pyogenes by phagocytosis can
induce gene expression changes to up-regulate apoptotic pathways. For example,
neutrophils that have engulfed S. pyogenes increase gene expression of components
connected to TNF-signaling pathways (TNF-α, TRAIL) and also BAX and BCL2A1,
important apoptotic mediators (Kobayashi et al., 2003).
A consequence of increased apoptosis of cells during sepsis is a defect in Delayed-Type Hypersensitivity (DTH) responses as mediated by CD4+ T cells. CLP-operated mice have delayed DTH responses but return to basal levels by day 10 after surgery (Unsinger et al., 2010). These apoptotic cells can induce a state of tolerance, mediated by TRAIL-expressing CD8+ T cells to inhibit CD4+ T cells and hence the inhibition of delayed DTH responses (Unsinger et al., 2010). Human Tregs have also been shown to induce apoptosis via Fas-FasL mediated interactions of monocytes after stimulation with bacterial endotoxins (Venet et al., 2009). Septic patients can down-regulate their expression of HLA-DR on monocytes (Fumeaux and Pugin, 2002). This results in an increase in Treg population and subsequent suppression of the immune response leading to anergy (Venet et al., 2009).

### 1.9.2 Autophagy of GAS

*S. pyogenes* is generally considered an extracellular pathogen, but can invade non-phagocytic cells by endocytosis to persist within the host. This has been found to be the case for neutrophils (Staali et al., 2003), macrophages (Thulin et al., 2006) and lung epithelial cells (LaPenta et al., 1994). This Gram-positive bacterium can also be within minutes engulfed rapidly by human neutrophils, (Kobayashi et al., 2003). Although, GAS has been shown to be more resistant to phagocytosis compared to other bacteria, such as *S. aureus* and *L. monocytogenes* (Kobayashi et al., 2003).

*S. pyogenes* can escape transportation to the lysosome via the secretion of SLO (previously discussed in Section 1.6.2), which has been demonstrated to inhibit GAS internalisation by keratinocytes and intracellular trafficking to lysosomes mediated by clathrin-dependent mechanisms (Håkansson et al., 2005; Logsdon et al., 2011). The host cell can still degrade internalised *S. pyogenes* once free in the cytoplasm. This is achieved through autophagy, the major pathway leading to the degradation of cytoplasmic components within eukaryotic cells. Autophagy is very efficient at eliminating GAS as shown *in vitro*. Internalised *S. pyogenes* within HeLa cells are held within intracellular membrane bound structures called GAS-containing Autophagosome-Like Vacuoles (GCAV) (Yamaguchi et al., 2010). Once the
bacterium is captured within GCAVs, it can fuse with lysosomes, leading to degradation (Nakagawa et al., 2010). This remains a critical mechanism of bacterial clearance and will be discussed in more detail in Chapter 4.

1.9.3 Innate immunity to S. pyogenes infection and sepsis

Different aspects of immunity within the context of GAS infection or sepsis have previously been examined in murine and human studies. I will give a brief overview of our current understanding of the varying contribution from DCs, macrophages, neutrophils, NK, NKT and γδ T cells in the onset and development of sepsis.

Only a few clinical GAS isolates are able to demonstrate DC maturation in vitro with human monocyte-derived DCs. Although, a ratio of 1:1 of live bacteria to DCs may mimic early infection (Cortes and Wessels, 2009). S. pyogenes has been suggested to induce maturation of DCs in vivo as characterised by the up-regulation of surface expression of MHC and co-stimulatory molecules. A M1 serotype S. pyogenes isolate was used to stimulate mDCs and pDC at a higher ratio of 5:1 live bacteria to DCs and showed increased expression of CD80 and CD40 on pDCs and also the production of cytokines associated with the disease phenotype from mDCs (IL-10, IL-12 and TNF-α) (Veckman and Julkunen, 2008).

Upon S. pyogenes activation, DCs were shown to be capable of stimulating naïve T cells to proliferate and differentiate into a Th1 phenotype (Veckman and Julkunen, 2008). pDCs can undergo in vivo maturation upon SEA superantigen stimulation, although this was shown to require the presence of T cells and IFN-γ (Muralimohan and Vella, 2006). This emphasises how innate immune cells can respond to microbial infection to prime adaptive immunity.

A protective role for DCs in sepsis has been postulated. If this cell type is depleted in CD11c-DT transgenic mice, significant increase in bacterial load was found in the draining lymph node and liver after sub-cutaneous infection with S. pyogenes (Loof et
This result is also mirrored by experiments demonstrating the lack of DCs contribute to increased mortality of mice in poly-microbial models of sepsis, TLR2 and TLR4 may also be involved in the apoptosis of this valuable cell type (Pène et al., 2009). This highlights the importance of DCs in preventing the dissemination of bacteria into the lymph nodes and other systemic organs.

Transcriptome analyses of murine macrophages infected with a M23 serotype S. pyogenes isolate revealed up-regulation of many key genes such as cytokines and chemokines (Goldmann et al., 2007). M1 and M2 macrophage associated markers, such as iNOS and arginase were also up-regulated upon infection, implicating this cell type is implicated in the elimination of S. pyogenes (Goldmann et al., 2010). Monocytos may also play a role during the onset of sepsis. For example, serum extracted from septic patients exhibited enhanced levels of IDO activity, correlating to increased severity of disease (Tattevin et al., 2010).

While DCs and macrophages have been shown to be protective during some models of S. pyogenes infection, the role of neutrophils is still unclear with contradicting evidence found. Neutrophils may have a protective role during animal models of poly-microbial sepsis, as their failure to migrate to the site of infection correlates to worse disease prognosis (Freitas et al., 2009). In this model of CLP, IL-17 and subsequent signaling via IL-17 receptor was shown to recruit neutrophils and be beneficial to the host (Freitas et al., 2009). However, blockade of IL-17 and hence reduced neutrophil recruitment in CLP models of sepsis has also been shown to be protective, contradicting other groups’ data (Flierl et al., 2008).

Another potential protective role of neutrophils has also further been highlighted through studies of TNF-α receptors; TNFR1 and TNFR2. If these receptors are knocked out in mice, it was deemed protective in a poly-microbial model of sepsis. Enhanced neutrophil recruitment was observed to aid bacterial clearance in the peritoneal cavity and overall enhanced survival (Seche et al., 2009). It was also shown in this model, TNF reduces the expression of CXCR2 on neutrophils to affect their ability to migrate to the site of infection and also induced apoptosis of infected
target cells (Secher et al., 2009). This supports other observations of the detrimental effects of TNF as revealed in animal models of colitis.

NKT cells during sepsis have also previously been examined in CLP models of polymicrobial sepsis, showing a decrease of overall frequency after surgery (Hu et al., 2009). However, this also correlated with an observed increase in the frequency of cytokine producing iNKT cells (Hu et al., 2009), suggesting that the remaining cells may have gained potent effector function. Despite these observed changes, NKT cells may be pathogenic, as inhibition of antigen presentation or activation by CD1d molecules blocks the release of pro-inflammatory cytokines and decreased mortality (Hu et al., 2009). NK cells may also be pathogenic during poly-microbial sepsis, as mice depleted of this cell type are less susceptible to mortality (Sherwood et al., 2004).

NKT cells may be sensitive towards superantigens stimulation due to their expression of TCR. For example, Vβ7+ and Vβ8+ NKT cells can proliferate upon stimulation with SEB and produce TNF-α in a similar fashion as T cells (Ragin et al., 2006). However, as NKT cells represent such a small percentage of the lymphocyte population, their contribution towards the pathogenesis of sepsis is minimal compared to T cells. NK cells may also indirectly respond to superantigens as well, although this is less well understood. Superantigens such as SEB and SPEA have been shown to activate Kupffer cells in the liver to produce pro-inflammatory cytokines like IL-12 to activate NK and NKT cells (Dobashi et al., 1999).

γδ T cells, another key cell type of the innate immunity branch have been implicated as pathogenic in adoptive cell transfer models of colitis. This particular cell population via the production of IL-17 may contribute to the differentiation of colitogenic Th17 cells, exacerbating the disease phenotype (Do et al., 2011). Mice subjected to CLP-surgery, followed by lung injury were found to have increased number of γδ T cells in the lung, despite low levels of cytokine production (Hirsh et al., 2004a). The authors suggested that γδ T cells might be beneficial to recovery post CLP surgery. This observation has been strengthened by experiments showing that IL-7 treated γδ T cells produced IL-17 to recruit neutrophils, aiding bacterial
clearance in a CLP model (Kasten et al., 2010). However, in septic patients, decreased circulating γδ T cells have been noted, contradicting data from murine models (Venet et al., 2008a). Overall, from these finding, it would appear that γδ T cells are both pathogenic and protective in sepsis and their role requires further clarification.

In conclusion, various cell components of the innate branch of immunity are implicated in the development of sepsis, either from human studies or animal models of disease. Data is still lacking for some of these cell types, particularly in the context of S. pyogenes infection and more research is required.

1.9.4 Adaptive immunity to S. pyogenes infection and sepsis

The presence and activity of T cells have been implicated in models of S. pyogenes infection and also sepsis. There is evidence to suggest that particular T helper cell subsets may be pathogenic or protective during bacterial infection, which I will discuss in the following section.

IL-17A has been proposed to be a protective cytokine in mouse models of colitis, with T\(_{H17}\) cells representing the primary source. Colitis is believed to be a T\(_{H1}\) cell mediated disease and the effects of IL-17A can inhibit this specific cell population to suppress gut inflammation (Awasthi and Kuchroo, 2009). IL-23 secreted by APCs during inflammation such as IBD, can contribute to the disease phenotype by activating T\(_{H1}\) cells (O' Connor Jr et al., 2010), while IL-17 has been shown to modulate T cell driven colitis. Although the effects of this cytokine did not prevent the onset of the disease (O' Connor Jr et al., 2010), suggesting only a minor protective role.

CD4\(^+\) T cells isolated from CLP-operated mice were found to proliferate poorly and produce less IFN-γ than sham treated mice (Scumpia et al., 2007). Upon treatment with anti-GITR agonistic antibody, this phenotype was reversed and increased T cell-
dependent antibody class switching was observed along with increased production of T_{H1} and T_{H2} cytokines (Scumpia et al., 2007). This suggests that GITR induced signaling may be beneficial to restore T cell function during sepsis. However, the role overall of CD4^{+} T cells may be pathogenic in a mouse model of AP due to limited bacterial clearance (Busse et al., 2007).

Co-stimulation is an important signal received by T cells from APCs to induce activation and differentiation. B7-H3 expression is induced by human monocytes, macrophages and DCs in the presence of inflammatory cytokines. This has been studied in septic patients, whereby increased levels of soluble B7-H3 in plasma were found and correlated with increased survival from sepsis (Zhang et al., 2010). A possible mode of action of B7 molecules in this setting is the augmentation of proliferation of antigen-specific T cells and enhance the induction of CTLs (Zhang et al., 2010).

The induction of T helper subsets during S. pyogenes infection has not been well characterised to date. Most studies have concentrated on the effect of superantigens on T cell immunity. However, upon intra-nasal infection with S. pyogenes in mice, T_{H1} and T_{H17} cells producing IFN-γ or IL-17A respectively have been identified (Costalonga et al., 2008; Wang et al., 2010a). A T_{H2} phenotype as mediated by p38 kinase signaling pathways has been implicated during the later stages of CLP-induced sepsis, as characterised by an induced state of immune suppression (Song et al., 2000). This suggests that effector T cells may contribute to amelioration of infection.

The role of T_{regs} has also been investigated within the context of colitis. The presence of the cytokine IL-21 during an adoptive T cell transfer model of colitis decreased TGF-β mediated foxp3 induction, and together with IL-6 and TGF-β favoured T_{H17} induction (Fantini et al., 2007). This emphasises the importance of the cytokine milieu during inflammation in establishing T helper cell differentiation. It has also been shown that CD4^{+}CD45RB^{lo} and CD8^{+}CD122^{+} T_{regs} act synergistically to prevent the development of disease in a similar model of colitis, suggesting that in this experimental setting, T_{regs} may be protective (Endharti et al., 2011).
The role of $T_{\text{regs}}$ during sepsis however is controversial. During the later stages of systemic infection, there is an increase in the number of circulating $T_{\text{regs}}$, as supported by elevated intracellular expression of Foxp3, TGF-$\beta$ and IL-10 in septic patients (Kessel et al., 2009). Increased $T_{\text{reg}}$ number also correlated with heightened suppression of $T_{\text{H1}}$ responses in septic patients (Monneret et al., 2003). This observation of increased frequency of $CD4^{+}CD25^{+}$ T cells has also been mirrored in CLP-operated mice (Wisnoski et al., 2007). Whether this correlates with worse prognosis is still unclear. Adoptive transfer of $in vitro$ stimulated $CD4^{+}CD25^{+}$ T cells into CLP-operated mice showed decreased mortality and bacterial burden in a dose-dependent manner and would suggest that $T_{\text{regs}}$ may be beneficial in this model of poly-microbial sepsis (Heuer et al., 2005).

$iT_{\text{regs}}$ can be induced $in vivo$ in an experimental model of colitis, indicating that Foxp3 expression is required for $iT_{\text{regs}}$ maintenance and suppressive function (Haribhai et al., 2009). During the later stages of immune suppression in sepsis, immature DCs can engulf apoptotic DCs to become tolerogenic and promote the differentiation of naïve T cells into $T_{\text{regs}}$ (Kushwah et al., 2010), further supporting previous evidence of increased levels of $T_{\text{regs}}$ in septic patients.

Pre-administration of superantigens, such as SEB can prime the immune response, resulting in effective eradication of pathogens. For example, mice pre-treated with SEB or SPEA before lethal infection with $L. monocytogenes$ are protected from infection (Okamoto et al., 2001). This effect is mediated via enhanced cytotoxic activity of $CD8^{+}$ T effector cells (Okamoto et al., 2001). Immunisation of superantigens into mice can induce deletion of superantigen-reactive $CD4^{+}$ T cells by anergy, with n$T_{\text{regs}}$ and Tr1 cells remaining to dampen pathogenic immune responses (Eroukhmanoff et al., 2009). In an adoptive T cell transfer model of colitis, SCID mice given $CD4^{+}CD45RB^{hi}$ cells together with SEB were shown to have impaired development of $CD4^{+}$ T cells expressing Foxp3. This suggests superantigens may have an impact on $T_{\text{reg}}$ differentiation (Heriazon et al., 2009). Superantigens are not alone in their ability to induce cytokine responses in T cells. Cytolysins, such as alpha toxin produced by $S. aureus$ can induce potent IL-17 responses from $CD4^{+}$ T cells (Niebuhr et al., 2011). This emphasises how bacterial products can influence T cell mediated immunity.
While the role of T\(_{\text{regs}}\) during infection has been studied both in animal models and in man, phenotypic differences may arise between murine and human T\(_{\text{regs}}\). For example, as previously discussed in Section 1.3.5, Foxp3 is not a reliable marker for T\(_{\text{regs}}\) in humans (Morgan et al., 2005). It should also be noted that humans can express two isoforms of Foxp3, one lacking exon 2 with diminished il-2 repression and suppressive activity compared to WT Foxp3 (Allan et al., 2005). Not all antibodies can distinguish the two isoforms of human Foxp3, which may offer one explanation why findings in animal models of infection may differ from actual human disease.

In summary, there is evidence for particular T helper subsets and associated cytokines in the control or progression of poly-microbial models sepsis. These findings are mirrored in septic patients. Whether this is also true during acute \(S.\) \textit{pyogenes} infection in murine and human studies is still largely unknown.

1.9.5 \textit{The role of TLRs during sepsis}

It is essential for cells of the immune system to discriminate self from infectious agents to initiate the chain of events to contain and eliminate the potential threat. This is largely achieved through PRRs expressed on many different immune cells. A crucial role for TLRs has been implicated during \(S.\) \textit{pyogenes} infection and sepsis as increased TLR2 and TLR4 expression are generally observed in septic patients (Tsujimoto et al., 2008; Knapp, 2010).

TLR signaling culminates in the translocation of NF-\(\kappa\)B into the nucleus to initiate expression of key genes associated with the inflammatory response. Without subunits forming this transcription factor, such as Rel family members, increased mortality was observed in sub-lethal poly-microbial models of sepsis, indicating that signaling via NF-\(\kappa\)B is important to control the inflammatory response (Coutine et al., 2011). The importance of NF-\(\kappa\)B signaling has also been highlighted in lymphocytes, as it
can also control apoptosis. During sepsis, lymphocyte apoptosis correlates with decreased NF-κB activity. This suggests key anti-apoptotic genes regulated by NF-κB are not expressed and may be a factor leading to increased T cell anergy observed during the later stages of sepsis (Groesdonk et al., 2007).

TLR2 is not exclusively expressed on innate and adaptive immune cells. Non-hematopoietic cells, such as endothelial cells also express TLR2 and may respond to Lipoteichoic Acid (LTA) to promote leukocyte migration (Gillrie et al., 2010). Endothelial cells may provide protective signals to the immune system. If NF-κB signaling is knocked out in endothelial cells, partial reversal of endotoxic driven hypotension is observed (Ding et al., 2009), emphasising the required role of NF-κB signaling by non-hematopoietic cells in the control of inflammatory responses (Pasparakis, 2009).

There is an interesting link between TLR and MHC class II signaling pathways. It would appear that MHC class II knockout animals are protected from the stimulatory effects of LPS and bacterial infection due to impaired cytokine production, particularly by macrophages and DCs (Liu et al., 2011b). This suggests that MHC class II molecules promote TLR induced pro-inflammatory cytokines, possibly via the activation of Btk kinase (Liu et al., 2011b).

The immune system has evolved an elegant system to prevent excessive triggering of TLR-mediated signaling. This is achieved through the expression of soluble extracellular TLR receptors that are capable of binding and sequestering microbial ligands. This reduces inflammation and implicates TLR signaling may not always be beneficial to the host during infection and sepsis (Raby et al., 2009). This concept is also highlighted in lethal infection with L. monocytogenes, leading to TLR2-mediated neutrophil apoptosis (Navarini et al., 2009). Contradictory, TLR signaling during CLP models of sepsis may be beneficial as MyD88−/− mice exhibit increased mortality compared to operated wild-type mice, due to decreased apoptosis of T and B cells (Peck-Palmer et al., 2008). This suggests that in some cases, TLR signaling may function to dampen the systemic response to infection.
Various ligands derived from S. aureus have been postulated to bind to TLR2. These include peptidoglycans (PGN), LTA and CpG unmethylated DNA. Mice lacking TLR2 have increased susceptibility to the effects of LTA, correlating with an impairment to produce TNF-α (Gillrie et al., 2010). However, during in vivo infection with S. aureus infection, TLR2 may negatively regulate the production of pro-inflammatory cytokines and promote bacterial survival within macrophages by induced phagocytosis (Gillrie et al., 2010; Watanabe et al., 2007; Shiratsuchi et al., 2010). In a mouse model of otitis media, S. pneumoniae infection of TLR2−/− mice resulted in increased mortality due to decreased expression of TNF-α (Han et al., 2009a). This suggests that TLR2 is not only required to sense microbial ligands derived from Gram-positive bacteria but it would appear to differentially regulate the production of protective cytokines depending on the nature of the infection.

During live S. pyogenes infection, one of the key pro-inflammatory cytokines detected in septic patients is IL-1β. Streptolysin O (SLO) has been shown to activate the PRR, Nlrp3 and the inflammasome to produce this cytokine contributing to the so-called “cytokine storm” (Harder et al., 2009). S. pyogenes infection can also induce TLR2, 3, 7 and 8 expression after 24h stimulation with human macrophages, culminating in downstream expression of type I interferons (Miettinen et al., 2008). Models of poly-microbial sepsis have also demonstrated a pathogenic role for TLR9 induced signaling, leading to excessive production of pro-inflammatory cytokines and cell death (Plitas et al., 2008).

Other GAS associated virulence factors may also interact with TLRs. For example, in vitro SEA stimulation of splenocytes results in up-regulation of MyD88 and corresponding cytokine production (e.g. IL-1β and TNF-α) (Kisser et al., 2010). The same findings were attained when SEA was injected into C57BL/6 mice, indicating that this staphylococcal superantigen is activating the MyD88 signaling pathway to induce pro-inflammatory cytokine production (Kisser et al., 2010). However, the contrary is also true. Staphylococcal ligands binding to TLR2 expressed on monocytes may prevent T cell activation through the induced secretion of IL-10 (Chau et al., 2009).
Overall, engagement of various TLRs during bacterial infection and sepsis has differing effects on the survival outcome. This suggests complexities in the control of the immune response by these receptors. However, very little work has focused on \textit{S. pyogenes} and TLR stimulation \textit{in vivo} and remains to be investigated.
1.10 Aims of this thesis

There are many complexities to how the immune system responds to systemic \textit{S. pyogenes} infection to lead to the development of sepsis. Different innate and adaptive immune cells have been implicated as either pathogenic or protective in different models of GAS infection or sepsis. There is evidence that septic patients exhibit elevated frequencies of T\textsubscript{reg}s with potent suppressive function but whether this is also true during acute \textit{S. pyogenes} infection is unknown. Prof. Daniel Altmann’s laboratory, in collaboration with Prof. Shiranee Sriskandan has previously studied the effects of streptococcal superantigens on T cell immunity; however, the precise effect of superantigens on T\textsubscript{reg}s \textit{in vivo} has not been properly addressed. Therefore, in this thesis I set out to address the following questions;

- To investigate the function of T cells during acute \textit{S. pyogenes} infection, in particular, the precise role of T\textsubscript{reg}s during GAS infection.

- To characterise the phenotype of effector cell types important in limiting GAS pathology.

- To create streptococcal superantigen knockout mutants to further investigate the role of this class of virulence factors on T cell immunity.

- To characterise the kinetics of T\textsubscript{reg}s during acute \textit{S. pyogenes} infection and determine whether streptococcal superantigens can influence the induction of this suppressive cell type.
Chapter 2

2 Materials and methods

2.1 Non-commercial reagents

2.1.1 Genotyping murine strains

- Mammalian tissue lysis buffer

  Used for digestion of murine tail biopsies to extract genomic DNA for genotyping purposes as described in Section 2.4.2
  6.057g Tris
  0.9306g Ethylenediaminetetraacetic Acid (EDTA)
  5.844g NaCl
  1.0g Sodium Dodecyl Sulfate (SDS)
  Resuspend in 500ml ddH$_2$O.

2.1.2 Microbiological culture and streptococcal mutagenesis

- Luria-Bertani (LB) broth

  Used for liquid culture of E. coli strains as described in Section 2.5.16
  10g Tryphtone (Merck, Germany)
  5g yeast extract (OXOID, UK)
  10g NaCl (Sigma-Adrich, UK)
  Resuspend in 1L H$_2$O before autoclaving to sterilise.
- LB agar

Used for plating out *E. coli* strains from glycerol stocks as described in Section 2.5.16
200ml ddH2O
7.4g LB agar (Merck, Germany)
The solution is autoclaved and cooled to 55°C before adding appropriate antibiotics (Table 2.4) and poured into sterile Petri dishes.

- Todd-Hewitt Broth (THB)

Used for liquid culture of *S. pyogenes* as described in Section 2.5.1
36.4g Todd-Hewitt Broth (OXOID, UK)
1L ddH2O
Autoclave solution to sterilise before use.

- *S. pyogenes* electroporation media

Use for resuspending *S. pyogenes* isolates prior to electroporation to generate superantigen knockout and complemented GAS strains as described in Section 2.5.18
50ml ddH2O (pre-autoclaved)
24.5g D-Glucose (Sigma-Aldrich, UK)
Filter-sterilise glucose solution into autoclaved 500ml 1mM MgCl2 solution and stored at 4°C until use.

- Todd-Hewitt-Yeast (THY) broth

Use for the recovery of *S. pyogenes* isolates after electroporation as described in Section 2.5.18
36.4g Todd-Hewitt Broth
2g yeast extract
1L ddH2O
Autoclave solution to sterilise before use.
• **THY agar**

Used for plating out electroporated *S. pyogenes* isolates for antibiotic selection as described in Section 2.5.18

- 7.28g Todd-Hewitt
- 0.4g yeast extract
- 4g bacterial agar (OXOID, UK)
- 200ml ddH$_2$O

The solution is autoclaved and cooled to 55°C before adding appropriate antibiotics (Table 2.5) and poured into sterile Petri dishes.

2.1.3 *Molecular biology*

• **SET buffer**

Used for extraction of genomic streptococcal DNA for PCR analysis as described in Section 2.5.20

- 750µl 5M NaCl (Sigma-Aldrich, UK)
- 2.5ml 0.5M EDTA
- 1ml 1M Tris, pH 7.5 (Sigma-Aldrich, UK)

Resuspend in 50ml molecular biology water.

• **50X TAE buffer**

Used for running nucleic acid agarose gels

- 121.0g Tris
- 28.6ml 16N acetic acid
- 50ml 0.5M EDTA pH 8.0

Resuspend in 500ml ddH$_2$O. For pouring and running agarose gels, use 1X TAE.
2.1.4 Southern blot

Reagents for used for determination of the orientation of inserted pGHostsmez plasmid into streptococcal genomic DNA as described in Section 2.5.22

• Denaturation solution

  12.5ml 10M NaOH (BDH, UK)
  75ml 5M NaCl
  Resuspend in 250ml ddH₂O.

• Neutralisation solution

  125ml 1M TrisCl (Sigma-Aldrich, UK)
  75ml 5M NaCl
  Resuspend in 250ml ddH₂O.

• 20X SSC solution

  87.65g NaCl
  44.1g Sodium citrate (Sigma-Aldrich, UK)
  400ml molecular biology water
  The pH was adjusted to 7.0 with 14N HCl (BDH, USA) and total volume made up to 500ml with molecular biology water and autoclaved. For subsequent wash steps, 2X and 0.5X SSC wash buffer are used.
2.1.5 Flow cytometry

- Red blood cell lysis buffer

Used to lyse red blood cells in whole blood and single cell splenocytes preparations prior to flow cytometric staining as described in Section 2.5.12

4.14g NH₄Cl (Sigma-Aldrich, UK)

0.5g KHCO₃ (Sigma-Aldrich, UK)

0.018g EDTA

Adjust pH to 7.35 with 0.1M NaOH and make up to 500ml with ddH₂O. Filter-sterilise before use and store at room temperature until ready for use.

2.2 Primers

2.2.1 Primers for genotyping murine strains

Table 2.1: List of primers for genotyping different strains of mice.

All transgenic and knockout murine strains used in this study (Table 2.6) were maintained by PCR genotyping of tail biopsy DNA prepared as described in Section 2.4.2 with primers listed below.

<table>
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<th>Target</th>
<th>Strain of mice</th>
<th>Sense primer sequence (5’→3’)</th>
<th>Anti-sense primer sequence (5’→3’)</th>
<th>PCR product size (bp)</th>
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</thead>
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<td>Neomycin</td>
<td>All TCR KO</td>
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<td>AGGTGAGATGACAGG</td>
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<td></td>
<td></td>
<td>CTATTC</td>
<td>AGATC</td>
<td></td>
</tr>
<tr>
<td>TCR β WT</td>
<td>All TCR KO</td>
<td>TGTCTGAAGGGCAA</td>
<td>GCTGATCCGGTGCCAT</td>
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</tr>
<tr>
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<td></td>
<td>TGACTG</td>
<td>CTATT</td>
<td></td>
</tr>
<tr>
<td>TCR δ WT</td>
<td>All TCR KO</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>CTGGTG</td>
<td>AGTTT</td>
<td></td>
</tr>
<tr>
<td>Foxp3 WT</td>
<td>Foxp3.DTR or HLA-DQ8.Aβ.DTR</td>
<td>TGGACCCTAGATGA</td>
<td>CCAGATTTTGGTG</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATTTTGGT</td>
<td>GAGTG</td>
<td></td>
</tr>
<tr>
<td>DTR</td>
<td>Foxp3.DTR or HLA-DQ8.Aβ.DTR</td>
<td>GGGACCCTAGAAGCT</td>
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<tr>
<td></td>
<td></td>
<td>GCTGCG</td>
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2.2.2 Primers for qRT-PCR

Table 2.2: List of primers and hydrolysis probes used for qRT-PCR.

To study the relative fold change in the expression of specific target genes during acute *S. pyogenes* infection, qRT-PCR was performed as described in Section 2.5.11 using primers and probes as listed below.

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<th>Gene</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
<th>Hydrolysis probe</th>
<th>Product size</th>
<th>NCBI sequence</th>
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<tr>
<td>Gene</td>
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<td>Anti-sense primer</td>
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<td>Product size</td>
<td>NCBI sequence</td>
</tr>
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<td>gapdh</td>
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<td>TGAAGCAGGCAT</td>
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<tr>
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**Transcription factors & cell lineage markers**

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<th>Hydrolysis probe</th>
<th>Product size</th>
<th>NCBI sequence</th>
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<td>GTTCTCTCTTCG AAGAGACTTCC</td>
<td>GTATCTCTCTGT AAATCTCTCTC</td>
<td>CTGGGAACAGTATT GCTGGTGACAAC C</td>
<td>111</td>
<td>NM_0311 68.1</td>
</tr>
<tr>
<td>ifn-γ</td>
<td>TCATTGAAATCAC ACCTGATTACTA CC</td>
<td>TGGACCTCAAAAC CTTGCAATAC</td>
<td>CTTCTCAAGC AA CAGCAAGC C</td>
<td>98</td>
<td>NM_0083 37.3</td>
</tr>
<tr>
<td>tnf-α</td>
<td>CTCACACTCAGA TCATCTTCTCAA A</td>
<td>CTGCCACCTGTG GTTGGCTA</td>
<td>ACAAGCCTGTA GCCAAGCTGAG AA</td>
<td>75</td>
<td>NM_0136 93.2</td>
</tr>
<tr>
<td>il-10</td>
<td>TTAATGCAGGAC TTAAAGGGTTAC TT</td>
<td>ATTCAATGCTC TTTGCTTACT</td>
<td>CCAAGCCTTATC GGAATGATCCA GTT</td>
<td>123</td>
<td>NM_0105 48.1</td>
</tr>
<tr>
<td>il-17a</td>
<td>CTGCTGTTGTGA TGCTGTTGCT</td>
<td>AAGGGGATTTAA GCACCTGT GGTGTTGTAAG</td>
<td>AGCCTCCAGGG CCAAAACACTGAG</td>
<td>149</td>
<td>NM_0105 52.3</td>
</tr>
<tr>
<td>il-4</td>
<td>AACGAAGAACAC CACAGAGAGTG</td>
<td>AGTTTCTTCTCA AGCATGGAGTTT</td>
<td>TCGTCTGTAGGG CTCCAAGGGTTG T</td>
<td>101</td>
<td>NM_0228 3.2</td>
</tr>
<tr>
<td>il-9</td>
<td>GTCCTCACTGTG ATTGTACCAC A</td>
<td>CGTAGTCTCTTCT AGAACCTCAACT A</td>
<td>TGATTTGCTGTT GCAATTGT CCTG</td>
<td>135</td>
<td>NM_0083 73.1</td>
</tr>
<tr>
<td>mpo</td>
<td>GGTCCAGATCAT CACAATACAG</td>
<td>GAGGGAGCTGA ATCGTGTGTAAG A</td>
<td>CCAACACCAAGG GCAGGAATG</td>
<td>107</td>
<td>NM_0108 24.2</td>
</tr>
<tr>
<td><strong>TLRs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tlr2</td>
<td>CAGACAAAGCCGT CAAATCTCAG</td>
<td>CGTTTTCCTGAAG AGGACTGTTAT</td>
<td>TTTGCTCCTTCTG GATCTTGGGCC</td>
<td>87</td>
<td>NM_0119 0.5</td>
</tr>
</tbody>
</table>

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2.2.3 Primers for *S. pyogenes* mutagenesis

Table 2.3: List of primers used for streptococcal superantigen mutagenesis.

To determine whether *S. pyogenes* isolates had been successfully mutated by the insertion of plasmids into host chromosome by electroporation, PCR analysis was performed using extracted streptococcal genomic DNA as described in Section 2.5.21 with primers below.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ → 3’)</th>
<th>Tm (°C)</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>aphl (F)</td>
<td>ATAGGATGGCAAGATCCTGG</td>
<td>62.7</td>
<td>kanamycin cassette</td>
</tr>
<tr>
<td>aphl (R)</td>
<td>GCCATATTCAACGGGAAACG</td>
<td>66.1</td>
<td>kanamycin cassette</td>
</tr>
<tr>
<td>rRNA (F)</td>
<td>CGGTAACCTAACGAAAGGG</td>
<td>60.0</td>
<td>rRNA</td>
</tr>
<tr>
<td>rRNA (R)</td>
<td>CGTTGTACCAACCATTGTAGC</td>
<td>61.8</td>
<td>rRNA</td>
</tr>
<tr>
<td>smeZ1</td>
<td>GTCAGCAAGTATATCTAC</td>
<td>42.9</td>
<td>smeZ promoter</td>
</tr>
<tr>
<td>smeZR600</td>
<td>TTCTATATCTAAATGCCC</td>
<td>48.8</td>
<td>smeZ (internal)</td>
</tr>
<tr>
<td>S1</td>
<td>GCGGATCCGCGATAAACCCAGCGAACCAT</td>
<td>82.4</td>
<td>speA promoter</td>
</tr>
<tr>
<td>A1</td>
<td>GCGATCAGTTATGAGGAAAAACATAAAAAGTATTG</td>
<td>72.4</td>
<td>speA (internal)</td>
</tr>
<tr>
<td>smeZF</td>
<td>TCCCTCTAAGGAAATATCTATAGTACG</td>
<td>59.9</td>
<td>Internal smeZ probe</td>
</tr>
<tr>
<td>smeZR</td>
<td>GTTCCAATCAAATGGGAGGAGGA</td>
<td>69.8</td>
<td>Internal smeZ probe</td>
</tr>
<tr>
<td>spea-EcoRI-</td>
<td>GCGAAATTCGATTCTTCCTTTAAACTCATCTTA</td>
<td>71.2</td>
<td>speA promoter</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>spea-EcoRI-</td>
<td>GCGAATTTCGATAGTAAGGTGCCAA</td>
<td>70.6</td>
<td>end of coding speA</td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3 Bacterial strains

2.3.1 E. coli bacterial strains

Table 2.4: List of E. coli strains

E. coli strains containing shuttle plasmids used for mutating superantigen genes in S. pyogenes isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Plasmid</th>
<th>Antibiotic selection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H323</td>
<td>pDL278</td>
<td>Spectinomycin (50µg/ml) (Sigma-Aldrich, UK)</td>
<td>(LeBlanc et al., 1992)</td>
</tr>
<tr>
<td>H376</td>
<td>pGHosmz</td>
<td>Kanamycin (40µg/ml) (Sigma-Aldrich, UK)</td>
<td>(Unnikrishnan et al., 2002)</td>
</tr>
<tr>
<td>H431</td>
<td>pDL278smez</td>
<td>Spectinomycin (50µg/ml)</td>
<td>(Russell and Sriskandan, 2008)</td>
</tr>
<tr>
<td>H722</td>
<td>pDL278smezspea</td>
<td>Spectinomycin (50µg/ml)</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.2 S. pyogenes bacterial strains

Table 2.5: List of S. pyogenes strains

S. pyogenes isolates generated and used for in vivo infection as described in Section 2.5.1

<table>
<thead>
<tr>
<th>Isolate</th>
<th>M serotype</th>
<th>Superantigen genotype</th>
<th>Mutagenesis</th>
<th>Antibiotic selection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H293</td>
<td>89</td>
<td>spea’ smez’</td>
<td>-</td>
<td>-</td>
<td>(Unnikrishnan et al., 2002)</td>
</tr>
<tr>
<td>H305</td>
<td>1</td>
<td>spea’ smez’</td>
<td>-</td>
<td>-</td>
<td>(Sriskandan et al., 1999)</td>
</tr>
<tr>
<td>H326</td>
<td>1</td>
<td>spea’ smez’</td>
<td>Allelic replacement with pUCMUT 2.2</td>
<td>Kanamycin (400µl/ml)</td>
<td>(Sriskandan et al., 1999)</td>
</tr>
<tr>
<td>H361</td>
<td>1</td>
<td>spea’ smez’</td>
<td>Complemented with pDL278spea</td>
<td>Spectinomycin (50µl/ml)</td>
<td>(Unnikrishnan et al., 2001)</td>
</tr>
<tr>
<td>H623</td>
<td>1</td>
<td>spea’ smez’</td>
<td>Insertional duplication with pGHosmz</td>
<td>Erythromycin (1µl/ml) (Sigma-Aldrich, UK)</td>
<td>-</td>
</tr>
<tr>
<td>Isolate</td>
<td>M serotype</td>
<td>Superantigen genotype</td>
<td>Mutagenesis</td>
<td>Antibiotic selection</td>
<td>Reference</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
<td>-----------------------</td>
<td>-------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>H656</td>
<td>1</td>
<td>spe⁻⁺ sme⁻/+</td>
<td>Complemented with pDL278smez</td>
<td>Spectinomycin (50µl/ml)</td>
<td>-</td>
</tr>
<tr>
<td>H658</td>
<td>1</td>
<td>spe⁻⁻ sme⁻⁻</td>
<td>Insertional duplication with pGhostsmez</td>
<td>Erythromycin (1µl/ml)</td>
<td>-</td>
</tr>
<tr>
<td>H786</td>
<td>1</td>
<td>spe⁻⁻⁺ sme⁻⁻⁺</td>
<td>Complemented with pDL278smezspea</td>
<td>Spectinomycin (50µl/ml)</td>
<td>-</td>
</tr>
</tbody>
</table>
2.4 Strains of mice

2.4.1 Animal Husbandry

Table 2.6: List of murine strains used in this thesis

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Phenotype</th>
<th>Background strain</th>
<th>Supplier</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Wild-type (WT)</td>
<td>-</td>
<td>Charles River</td>
<td>-</td>
</tr>
<tr>
<td>TCR β KO</td>
<td>TCR β−−</td>
<td>C57BL/6</td>
<td>Jackson Laboratory</td>
<td>(Mombaerts et al., 1991)</td>
</tr>
<tr>
<td>TCR δ KO</td>
<td>TCR δ−−</td>
<td>C57BL/6</td>
<td>Jackson Laboratory</td>
<td>(Itohara et al., 1993)</td>
</tr>
<tr>
<td>TCR δβ KO</td>
<td>TCR β−− and TCR δ−−</td>
<td>C57BL/6</td>
<td>Jackson Laboratory</td>
<td>(Mombaerts et al., 1992)</td>
</tr>
<tr>
<td>Foxp3.DTR</td>
<td>Inducible knockout of Foxp3-GFP+ cells</td>
<td>C57BL/6</td>
<td>Kind gift from A. Rudensky</td>
<td>(Kim et al., 2007)</td>
</tr>
<tr>
<td>IL-17A KO</td>
<td>IL-17A−−</td>
<td>C57BL/6</td>
<td>Kind gift from Y. Iwakura</td>
<td>(Nakae et al., 2002)</td>
</tr>
<tr>
<td>β2m KO</td>
<td>β2m−−</td>
<td>C57BL/6</td>
<td>Jackson Laboratory</td>
<td>(Koller and Smithies, 1985)</td>
</tr>
<tr>
<td>HLA-DQ8.Aβ0</td>
<td>HLA-DQ8 transgenic, lacking endogenous mouse H2</td>
<td>C57BL/6</td>
<td>Made by D. Altmann</td>
<td>(Boyton et al., 1998)</td>
</tr>
<tr>
<td>HLA-DQ8.Aβ0.DTR</td>
<td>HLA-DQ8 transgenic, lacking endogenous mouse H2, inducible knockout of Foxp3-GFP+ cells</td>
<td>C57BL/6</td>
<td>Crossed for project</td>
<td>-</td>
</tr>
<tr>
<td>TLR2 KO</td>
<td>TLR2−−</td>
<td>C57BL/6</td>
<td>Jackson Laboratory</td>
<td>(Wooten et al., 2002)</td>
</tr>
</tbody>
</table>

All strains of mice used in this thesis were either purchased from recognised commercial suppliers or given as kind gifts from the original producing labs (Table 2.6). Mice were bred on site and maintained in accordance with UK government Home Office guidelines under closed IVC housing conditions. Prior pilot experiments showed the mice bred on site were not initially infected with S.
pyogenes, nor did health screens of the breeding rooms show any evidence of pre-existing infection with any streptococcal strains.

2.4.2 Genomic DNA extraction for genotyping

For each strain of mice, the genotype was routinely determined by PCR technique using extracted genomic DNA from tail biopsies. Tissue was digested overnight at 56°C in 400µl mammalian tissue lysis buffer (Section 2.1.1) and 10µl Proteinase K (100µg/ml; Sigma-Aldrich, UK). Next day, 200µl saturated NaCl solution was added and samples vigorously shaken for 30 sec before centrifugation for 15 min, 15,000g. The supernatant was transferred into a fresh eppendorf and 600µl iso-propanol added. The precipitated genomic DNA was pelleted by centrifugation at 9000g for 10 min. The pellet was washed in 750µl 70% ethanol by spinning again at 9000g for 10 min before air-drying. Genomic DNA was resuspended in 100µl molecular biology water. The concentration of genomic DNA was measured on a NanoDrop spectrophotometer (NanoDrop, USA) and ND1000 V3.1.2 software. Samples were diluted to 40ng/µl for amplification by PCR.

2.4.3 Genotyping PCR

The genotypes of the murine strains were determined from each litter by amplification of specific genes with appropriate primers (Table 2.1) using a BIOTAQ DNA polymerase (Table 2.7) with specific PCR thermal profiles (Table 2.8). PCR products were run on a 2% agarose gel made with 1X TAE buffer (Section 2.1.3) containing Sybr-Safe (Invitrogen, UK) at 120V for approximately 45 min and visualized under UV light. Expected pattern of PCR products for each strain of mice are indicated in Table 2.9.
Table 2.7: Master-mix for BIOTAQ based genotyping PCR (for 1 reaction)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer (BIOLINE Ltd, UK)</td>
<td>2.5</td>
</tr>
<tr>
<td>Magnesium (15mM) (BIOLINE Ltd, UK)</td>
<td>5</td>
</tr>
<tr>
<td>dNTPs (1mM) (BIOLINE Ltd, UK)</td>
<td>5</td>
</tr>
<tr>
<td>Molecular biology H₂O</td>
<td>7.4</td>
</tr>
<tr>
<td>BIOTAQ polymerase (5U/µl) (BIOLINE Ltd, UK)</td>
<td>0.1</td>
</tr>
<tr>
<td>Sense primer (100µg/ml)</td>
<td>1.25</td>
</tr>
<tr>
<td>Anti-sense primer (100µg/ml)</td>
<td>1.25</td>
</tr>
<tr>
<td>Sample genomic DNA (40ng/µl)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 2.8: Thermal profiles for genotyping PCRs.

<table>
<thead>
<tr>
<th>Target</th>
<th>PCR Thermal profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>94°C 3 min, 35 cycles of (94°C 30 sec, 64°C 1 min, 72°C 1 min), 72°C 2 min</td>
</tr>
<tr>
<td>TCR β WT</td>
<td>94°C 3 min, 35 cycles of (94°C 30 sec, 64°C 1 min, 72°C 1 min), 72°C 2 min</td>
</tr>
<tr>
<td>TCR δ WT</td>
<td>94°C 3 min, 37 cycles of (94°C 20 sec, 58°C 30 sec, 72°C 35 sec), 72°C 2 min</td>
</tr>
<tr>
<td>Foxp3 WT</td>
<td>94°C 3 min, 35 cycles of (94°C 40 sec, 53°C 40 sec, 72°C 30 sec), 72°C 5 min</td>
</tr>
<tr>
<td>DTR</td>
<td>94°C 5 min, 35 cycles of (94°C 15 sec, 59°C 15 sec, 72°C 45 sec), 72°C 2 min</td>
</tr>
<tr>
<td>IL-17A WT</td>
<td>94°C 2 min, 40 cycles of (94°C 15 sec, 64°C 30 sec, 72°C 1 min), 72°C 10 min</td>
</tr>
<tr>
<td>IL-17A KO</td>
<td>94°C 2 min, 40 cycles of (94°C 15 sec, 64°C 30 sec, 72°C 1 min), 72°C 10 min</td>
</tr>
<tr>
<td>β₂m WT</td>
<td>94°C 3 min, 35 cycles of (94°C 20 sec, 58°C 30 sec, 72°C 30 sec), 72°C 2 min</td>
</tr>
<tr>
<td>β₂m KO</td>
<td>94°C 3 min, 35 cycles of (94°C 20 sec, 58°C 30 sec, 72°C 30 sec), 72°C 2 min</td>
</tr>
<tr>
<td>DQαt</td>
<td>95°C 5 min, 32 cycles of (94°C 1 min, 55°C 1 min, 72°C 1 min), 72°C 8 min</td>
</tr>
<tr>
<td>DQβ</td>
<td>95°C 5 min, 32 cycles of (94°C 1 min, 55°C 1 min, 72°C 1 min), 72°C 8 min</td>
</tr>
<tr>
<td>H2 WT</td>
<td>95°C 5 min, 30 cycles of (94°C 45 sec, 60°C 45 sec, 72°C 1 min), 72°C 8 min</td>
</tr>
<tr>
<td>H2 KO</td>
<td>95°C 5 min, 30 cycles of (94°C 45 sec, 60°C 45 sec, 72°C 1 min), 72°C 8 min</td>
</tr>
<tr>
<td>TLR2 WT</td>
<td>94°C 3 min, 35 cycles of (94°C 30 sec, 65°C 45 sec, 72°C 45 sec), 72°C 2 min</td>
</tr>
<tr>
<td>TLR2 KO</td>
<td>94°C 3 min, 35 cycles of (94°C 30 sec, 65°C 45 sec, 72°C 45 sec), 72°C 2 min</td>
</tr>
</tbody>
</table>
Table 2.9: Expected PCR products for genotyping transgenic mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Expected PCR product result</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR β KO</td>
<td>Neomycin’, TCR β WT’, TCR δ WT’</td>
</tr>
<tr>
<td>TCR δ KO</td>
<td>Neomycin’, TCR β WT’, TCR δ WT’</td>
</tr>
<tr>
<td>TCR δβ KO</td>
<td>Neomycin’, TCR β WT’, TCR δ WT’</td>
</tr>
<tr>
<td>Foxp3.DTR</td>
<td>Foxp3 WT’, DTR’</td>
</tr>
<tr>
<td>IL-17A KO</td>
<td>IL-17A WT’, IL-17A KO’</td>
</tr>
<tr>
<td>β2m KO</td>
<td>β2m WT’, β2m KO’</td>
</tr>
<tr>
<td>DQ8.Aβ0</td>
<td>DQα’, DQβ’, H2 WT’, H2 KO’</td>
</tr>
<tr>
<td>TLR2 KO</td>
<td>TLR2 WT’, TLR2 KO’</td>
</tr>
</tbody>
</table>
2.5 Methods

2.5.1 Acute sepsis infection model

*S. pyogenes* isolates (Table 2.5) were streaked from glycerol stocks stored at -80°C on to Columbia Blood Agar plates (Oxoid, UK) and grown overnight at 37°C, 5% CO₂. Next day, four falcon tubes of 50ml Todd Hewitt Broth (Section 2.1.2) were each inoculated with a single streptococcal colony and cultured overnight at 37°C, 5% CO₂. The cultures were washed the next day three times in sterile Phosphate Buffered Saline (PBS) (Gibco; Invitrogen, UK) by centrifugation at 3000g, 15 min at 4°C. The bacterial pellets were pooled together and resuspended in 2ml sterile PBS. To study immune responses during acute sepsis, 50µl of the bacterial suspension or sterile PBS for sham controls was given via the intramuscular (i.m) route into the right thigh of female mice at t=0h. Female mice were used in all experiments unless stated otherwise as previously found to be more susceptible to GAS infection compared to males (Faulkner et al., 2007). The bacterial inoculum was quantified by serial plating on to blood agar plates and was between $10^8$-$10^9$ CFU per 50µl dose. Sterile PBS inoculum was also plated out and no bacterial contamination was detected.

For all standard experiments described in Chapter 3 and 4, 8-12 female C57BL/6, TCR KOs, β₂m KO, IL-17A KO and Foxp3.DTR mice were infected for 24h with H305 (NCTC8198), a M1T1 serotype WT clinical scarlet fever isolate (Sriskandan et al., 1999) and culled for microbial (Section 2.5.3), qRT-PCR (Section 2.5.7-11), flow cytometric (Section 2.5.12), serum cytokine (Section 2.5.4) and histological (Section 2.5.13-15) analysis.

For survival experiments, 6-8 female mice (C57BL/6, TCR KOs, β₂m KO, IL-17A KO, Foxp3.DTR or TLR2 KO) were infected as described above and monitored for signs of systemic infection and culled when reached defined end-points (loss of more than 20% body weight, development of respiratory difficulties, loss of mobility and
motility, hunched posture and piloerrection). In some survival experiments, mice were tail-bled at 8-9h post infection to collect serum for cytokine analysis by ELISA.

For longer time courses of H305 WT streptococcal infection as discussed in Chapter 6, groups of 4 or 5 HLA-DQ8.αβ0 female mice were infected as described above and culled at 24, 36 and 48h for serum, microbial and flow cytometric analysis. For the short time courses of H305 WT S. pyogenes infection, at defined time-points (t=4, 8, 12 and 24h) post infection, groups of 10 infected mice or groups of 5 sham treated mice were culled for qRT-PCR analysis as part of 2 independent experiments. For flow cytometric analysis, groups of 7 infected HLA-DQ8.αβ0 female mice were culled at 4, 8, 12 and 24h post infection along with groups of 6 sham-infected mice. For subsequent superantigen knockout isolate infections, groups of 8 female HLA-DQ8.αβ0 or HLA-DQ8.αβ0.DTR female mice were infected with H305, H326, H623 and H658 (Table 2.5) for 24h before harvesting for microbial, serum, qRT-PCR, flow cytometric analysis as part of two independent experiments.

2.5.2 Antibody mediated and inducible depletion of target cells in vivo

To study the role of specific cell types of interest such as CD25\(^+\), CD8\(^+\) and NK cells during acute S. pyogenes infection, antibody mediated depletion in vivo was carried out prior to intra-muscular streptococcal infection. The antibodies used for depletion were anti-CD25 (clone PC61; made by S. Steinbach), anti-CD8\(^\alpha\) (clone YTS 169; a kind gift from Prof. H. Waldmann, Dunn School of Pathology, Oxford), anti-NK1.1 (clone PK136; eBioscience, UK). Pilot depletion experiments were carried out in C57BL/6 female mice to determine the dose of antibody required to successfully deplete the target cell and duration of depletion after a single administration. The presence of target cells after depletion was monitored by flow cytometry with antibodies directed against other epitopes of the target receptor or other key specific receptors for the target cell (Table 2.13). It was determined that 100µg PC61 (Figure 3.8), 100µg YTS 169 (Figure 4.10) and 200µg PK136 (Figure 4.18) were adequate to deplete target cells for a minimum of 24h. Antibodies were administrated to mice via
an intra-peritoneal (i.p) route prior and also on the day of infection to maximise full knockdown of target cells.

To deplete Foxp3+ cells in Foxp3.DTR or HLA-DQ8.Aβ0.DTR mice as described in Chapter 3 and 6, various dose of Diphtheria Toxin (DT; Sigma-Aldrich, UK) was given to mice i.p and the presence of CD4+CD25+Foxp3+ cells measured by flow cytometry from blood, spleen and inguinal draining lymph node. A single dose of 1.25µg DT was sufficient to deplete CD4+CD25+Foxp3+ cells for approximately 48h as shown by flow cytometric analysis (Figure 3.13). For infection experiments, Foxp3.DTR or HLA-DQ8.Aβ0.DTR mice were depleted of Foxp3+ cells with 1.25µg DT every day two days prior and also on the day of infection in order to maintain full knockdown of target cells.

2.5.3 Microbial analysis of infection

To determine the bacterial burden incurred during streptococcal infection, tissue was harvested for bacterial counts. Mice were culled humanely in a CO2 chamber before cardiac puncture to obtain whole blood. 10µl whole blood was immediately added to 10µl sodium heparin (Sigma-Aldrich, UK) and 35µl sterile PBS for bacterial counts. The rest of the blood was allowed to clot to collect serum for cytokine analysis by ELISA (Section 2.5.4). Half of the spleen was harvested and 10µl PBS per mg added for homogenisation. Thigh muscle and liver were dissected and 5µl sterile PBS per mg tissue was added and homogenised. 50µl of the sodium heparinised blood, spleen and liver were plated on to blood agar. 50µl of a 10^6 dilution of homogenised thigh tissue was plated on to blood agar. Plates were incubated overnight at 37°C, 5% CO2 and β-haemolytic colonies counted the next day. Bacterial counts are expressed as Colony Forming Unit (CFU) per mg or ml of tissue or blood.
2.5.4 Serum cytokine analysis by Enzyme-linked Immunosorbent Assay (ELISA)

Cytokine levels were determined post infection from serum by Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available kits. Blood was collected from naïve, infected or sham-infected mice by cardiac puncture and allowed to clot before centrifugation at 1000g for 15 min. The serum was collected and stored at -20°C. Optimised sandwich ELISAs determined serum cytokines concentrations with IL-17A and IFN-γ antibody detection and capture pairs (MABTECH AB, Sweden) and IL-6 antibody pairs (Invitrogen, UK). In brief, capture antibody was diluted in the manufacturer’s recommended buffer (Table 2.10) and 100µl added to 96-well immuno-plate (Greiner Bio-one, UK) overnight at 4°C. Next day, the plate was washed twice with 200µl PBS before blocking with 200µl 1% BSA/PBS for 1h at RT. The plates were washed twice with 200µl washing buffer (0.05% Tween/PBS) before 100µl of pre-diluted serum samples (1:50 in blocking buffer) added in duplicate. A 2-fold dilution series using recombinant cytokine was also added to the plate starting with the highest concentration as 1000pg/ml and lowest as 15.625pg/ml to form a standard curve to determine the concentration of the unknown samples. A negative control of 100µl blocking buffer was included to determine the background optical density. The plate was incubated overnight at 4°C. The following day, the ELISA plate was rinsed 5 times with washing buffer, before 100µl cytokine detection antibody diluted in blocking buffer (Table 2.10) added for 1h at RT. The plate was washed 5 times before 100µl Streptavidin-HRP (Invitrogen, UK) was added for 30 min at RT. The plate was washed 6 times before 100µl 3,3’, 5,5”-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, UK) was added for detection and the colour change monitored. The enzymatic reaction was stopped by the addition of 100µl 1M H2SO4. Optical densities of samples against a recombinant protein standard curve were measured using an ELISA plate reader (µQuant BIO-Tek Instruments, Inc.) and KC Junior software was used to determine the concentration of the cytokines in samples, normalised against the background optical density of negative control.
Table 2.10: Concentration of reagents for ELISA.

Final concentrations of antibodies and enzymes for serum cytokine ELISAs.

<table>
<thead>
<tr>
<th>Antibody or enzyme</th>
<th>Concentration</th>
<th>Dilution buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17A capture antibody</td>
<td>1µg/ml</td>
<td>PBS, pH 7.4</td>
</tr>
<tr>
<td>IL-6 capture antibody</td>
<td>1.25µg/ml</td>
<td>0.1M NaHCO₃, pH 8.3</td>
</tr>
<tr>
<td>IFN-γ capture antibody</td>
<td>1µg/ml</td>
<td>0.1M NaHCO₃, pH 8.3</td>
</tr>
<tr>
<td>IL-17A detection antibody</td>
<td>0.5µg/ml</td>
<td>1% BSA/PBS</td>
</tr>
<tr>
<td>IL-6 detection antibody</td>
<td>0.1µg/ml</td>
<td>1% BSA/PBS</td>
</tr>
<tr>
<td>IFN-γ detection antibody</td>
<td>0.5µg/ml</td>
<td>1% BSA/PBS</td>
</tr>
<tr>
<td>Streptavidin-HRP</td>
<td>1000X</td>
<td>1% BSA/PBS</td>
</tr>
</tbody>
</table>

2.5.5 Preparation of streptococcal stimulation factors

For stimulation of either murine splenocytes or human PBMCs, different streptococcal preparations were used. In some instances, heat-killed *S. pyogenes* was used to stimulate murine splenocytes at a 1:1000 dilution (Section 2.5.6). Heat-killed GAS was prepared from the bacterial inoculum made as described in Section 2.5.1 and boiled at 80°C for 1h.

To investigate the role of secreted streptococcal superantigens, overnight cultures of *S. pyogenes* isolates (Table 2.5) were grown in 20ml RPMI 1640 (Gibco, Invitrogen, UK) containing 10% heat-inactivated Fetal Calf Serum (FCS) and 2mM L-Glutamine (L-Glut; all from Gibco, Invitrogen, USA) and bacterial counts were calculated to be similar (between 2-8 x10⁷ CFU/ml) indicating similar growth rate. The cultures were filter-sterilised and stored at -20°C. The supernatants of these cultures contained the secreted superantigens from the given strain, which were used to stimulate human PBMCs and murine splenocytes (Section 2.5.6 and 2.5.25).
2.5.6 Cytometric Bead Assay (CBA)

In order to characterise multiple cytokine responses during in vitro stimulation of murine splenocytes, Cytometric Bead Assay (CBA) was carried out. Foxp3.DTR female transgenic mice (n=5 per treatment group) were given two doses of DT or PBS for 2 days before spleens were harvested. Single cell suspensions were made in DMEM containing 10% FCS, Penicillin and Streptomycin (P/S; all from Gibco; Invitrogen, UK). 10^6 splenocytes were stimulated either with 100µl heat-killed H305 S. pyogenes (Section 2.5.5) or cell-free streptococcal supernatants (Section 2.5.5) overnight at 37°C, 5% CO_2. A positive control of splenocytes stimulated with Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (10ng/ml & 500ng/ml respectively; Sigma-Aldrich, UK) was used, along with a negative control of splenocytes incubated in media only to determine the basal levels of cytokines. Next day, supernatants were collected and stored at -20°C until ready for CBA analysis of TNF, IFN-γ, IL-17A, IL-9, IL-4 and IL-10 cytokine concentrations by mouse CBA Flex sets according to the manufacturer’s protocols (BD Biosciences, UK). Supernatants were diluted 1:5 in assay diluent and data acquired on a BD FACSArray (Becton Dickinson, UK).

2.5.7 Total RNA extraction

To assess the fold change in expression of specific target genes during acute infection, total RNA from tissue was extracted to make complementary DNA (cDNA) and quantitative Real-Time PCRR (qRT-PCR) analysis employed. Spleen and inguinal draining lymph nodes were harvested from naïve, sham-infected or infected mice and stored in 300µl RNALater™ RNA Stabilisation Reagent (Qiagen, UK) at -80°C until ready for use. Total RNA was extracted using acid phenol method with TRIzol (Invitrogen, UK). In brief, tissue was homogenised in 1ml TRIzol before 200µl chloroform added and samples vigorously mixed. The samples were allowed to incubate at RT for 3 min before centrifugation at 12,000g for 15 min at 4°C. The top colourless aqueous layer containing total RNA was transferred into 0.5ml iso-
propanol and incubated for 10 min at RT. After another centrifugation step of 12,000g for 10 min at 4°C, the supernatant was discarded and 1ml 75% ethanol added and spun down at 7500g for 5 min at 4°C to wash the RNA pellet. The supernatant was discarded and the pellet allowed to air-dry before resuspension in 30μl RNase-free water containing 1.5μl RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, UK) at 58°C for 10 min to prevent degradation of RNA. RNA concentration and nucleic acid/protein purity ratio were analysed using a NanoDrop spectrophotometer. Total RNA was diluted to 200ng/μl and stored at -80°C until ready for use. The integrity of total RNA was determined by running 1μg on a 2% agarose gel for 30 min at 100V before visualizing under UV light to identify 40S and 60S mammalian ribosomal subunits (Figure 2.1).

Figure 2.1: Representative total RNA integrity agarose gel.

Total RNA was extracted from spleens or inguinal draining lymph nodes harvested from either sham (n=5) or *S. pyogenes* infected (n=10) HLA-DQ8.Aβ<sup>0</sup> mice (12h time point). 1μg total RNA was run on a 2% agarose gel. The two bright bands of the mammalian ribosomal subunits: 40S and 60S along with the smear of mRNA can be seen under UV light as shown above.
2.5.8 Reverse transcription

1µg of total RNA was reversed transcribed into cDNA using Superscript® III Reverse Transcriptase (Invitrogen, UK) in a two-step reaction. In brief, for one reaction mix, 1µl random primers (250ng/µl; Invitrogen, UK), 1µl dNTPs (10mM) and 6µl RNase-free water was added to 5µl total RNA (200ng/µl) and incubated at 65°C for 5 min. The RNA mix was cooled on ice for 1 min before adding 1µl 0.1M DTT (Invitrogen, UK), 1µl Superscript III Reverse Transcriptase, 1µl RNase-out inhibitor and 4µl 5X first strand buffer (Invitrogen, UK). Reverse transcription was carried out at 50°C for 1h, followed by the final incubation at 70°C for 15 min to inactivate enzyme. cDNA was stored at -20°C until further analysis.

2.5.9 Design of qRT-PCR primers and probes

Primers and hydrolysis probes for qRT-PCR were designed using Primer3 software with the help from Daniel Lowther. Sequences for target genes of interest (Table 2.2) were obtained from NCBI and intron-exon boundaries identified. In order to ensure amplification of cDNA and not contaminating genomic DNA in the sample, the sense, anti-sense primers or the hydrolysis probe was designed over an exon-exon boundary. Sense and anti-sense primers were selected to be 20-27bp in length, between 30-70% GC content and have a melting temperature 60°C±3°C. The hydrolysis probe was chosen to be similar to the primers but with a higher melting temperature of 70°C±3°C and have dual fluorescence label attached (5’-FAM, 3’-TAMRA). Secondary structures of primers and hydrolysis probes were checked with NetPrimer software to minimise the effect of primer dimers, cross dimers and hairpin loops secondary structures. Oligonucleotides were purchased from SigmaGenosys and resuspended in RNase-free water and stored at -20°C.
Table 2.11: Master-mix for BIOTAQ PCR for testing qRT-PCR primers (for 1 reaction)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>2</td>
</tr>
<tr>
<td>Magnesium chloride (50mM)</td>
<td>2</td>
</tr>
<tr>
<td>dNTPs (1mM)</td>
<td>4</td>
</tr>
<tr>
<td>Molecular biology H2O</td>
<td>9.3</td>
</tr>
<tr>
<td>BIOTAQ polymerase (5U/µl)</td>
<td>0.2</td>
</tr>
<tr>
<td>Sense primer (10µM)</td>
<td>1</td>
</tr>
<tr>
<td>Anti-sense primer (10µM)</td>
<td>1</td>
</tr>
<tr>
<td>Sample cDNA (50ng/µl)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Primers were initially checked for specificity by conventional PCR analysis. Stock cDNA was prepared from naïve HLA-DQ8.Aβ0 female mice. Mice were humanely sacrificed and spleens harvested into RPMI tissue culture media containing 10% FCS, 1% P/S and 1% L-Glut. A single cell suspension was prepared by passing the spleen through a 70µM cell sieve (Becton-Dickinson, UK). Cells were diluted to 3x10^6 cells/ml and 1ml added to a 24-well flat bottom plate. Half of the wells were left un-stimulated, while the other half were stimulated with concanavalin A (Sigma-Aldrich, UK) at a final concentration of 5µg/ml for 2 days at 37°C, 5% CO₂. Afterwards, cells were harvested and total RNA and cDNA synthesis carried out as described in Section 2.5.7-8. The stock cDNA of either unstimulated or stimulated splenocytes was used to carry out conventional PCR, testing the qRT-PCR sense and anti-sense primers using BIOTAQ DNA polymerase (Table 2.11) using the following thermal PCR profile; 50°C 2 min, 95°C 10 min followed by 40 cycles of 95°C 30 sec, 60°C 30 sec and 72°C 30 sec. PCR product was run on 2% agarose gel to determine the effectiveness and specificity of the designed primers.
2.5.10 Primer efficiency

A

Figure 2.2: Representative qRT-PCR primer/hydrolysis probe efficiency standard curve.

A 5-fold serial dilution of stock cDNA was run by qRT-PCR for MPO primer and probe pairs and from the amplification plot (A), Ct values for each cDNA concentration were read at a threshold of 2000. A standard curve of log cDNA concentration versus Ct value (B) was used to calculate the primer pair/hydrolysis probe efficiency as calculated by MX3000Pro software. (Stratagene, USA)

To calculate the fold change in gene expression, the efficiency of the qRT-PCR primer pairs and hydrolysis probes was determined using a standard curve of diluted
cDNA. A five-fold serial dilution of either un-stimulated or stimulated stock cDNA (Section 2.5.9) was run by qRT-PCR in triplicate for all reference and target genes. A linear standard curve was generated by plotting the log of the cDNA concentration against Ct (Cycle threshold) value read at a threshold of 2000 for each dilution. The efficiency of the primers and the R² value (correlation coefficient) were acquired from the linear standard curve (Figure 2.2).

2.5.11 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was run in triplicate on naïve, sham or S. pyogenes infected spleen and draining lymph node cDNA samples as prepared in Section 2.5.6, using in-house designed target gene specific primers and hydrolysis probes (Table 2.2) on a MX3000P real-time PCR thermocycler (Stratagene, USA). Target genes were run with two reference genes from a choice of eight (b2m, gapdh, hrpt1, rpl-13a, tbp, tfrc, sdha and ywhaz), validated by running qRT-PCR on cDNA from sham and infected HLA-DQ8.Aβ0 mice. The two reference genes that varied the least in terms of Ct value read at a threshold of 2000 for each different tissue were chosen as tissue specific reference genes. qRT-PCR reactions were performed in 20µl total volume as shown in Table 2.12 with a thermal profile of 50°C for 2 min, followed by 10 min at 95°C and then 50 cycles of 15 sec at 95°C, 15 sec at 60°C and 15 sec at 72°C.

Table 2.12: Master-mix for qRT-PCR (for 1 reaction)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X TaqMan Gene Expression Mastermix (Stratagene, USA)</td>
<td>10</td>
</tr>
<tr>
<td>Molecular biology H2O</td>
<td>4</td>
</tr>
<tr>
<td>Hydrolysis probe (1µM)</td>
<td>2</td>
</tr>
<tr>
<td>Sense primer (10µM)</td>
<td>1</td>
</tr>
<tr>
<td>Anti-sense primer (10µM)</td>
<td>1</td>
</tr>
<tr>
<td>Sample cDNA (40ng/µl)</td>
<td>2</td>
</tr>
</tbody>
</table>
The fold change in gene expression, relative to time matched sham-infected samples were normalised to two tissue specific reference genes and calculated using the $\Delta\Delta C_T$ method (Pfaffl, 2001; Pfaffl et al., 2002) by the Relative Expression Software Tool (REST), taking into account primer pair efficiency as calculated in Section 2.5.10 and randomisation statistical tests.

2.5.12 Flow cytometry

In order to quantify percentages of cells of interest during GAS infection, flow cytometry was used. Female mice inoculated with $S.\ pyogenes$, PBS or untreated were culled at defined time points and inguinal draining lymph nodes and spleens harvested and homogenised into a single cell suspension in PBS containing 10% FCS using 70µm cell sieves. Red blood cells were lysed in splenocyte preparations using 0.5ml lysis buffer (Section 2.1.5) for 5 min. All cells were washed in ice-cold PBS (10% FCS) twice before blocking with Fc Block (1:1000 dilution; eBioscience, USA) for 10 min on ice. Surface staining was carried out with antibodies indicated in Table 2.13 for 20 min at 4°C. Cells were washed and resuspended in 200µl 1X Fix/Perm solution (eBioscience, USA) for 30 min at 4°C. If intracellular staining was required, samples were washed twice in 1X permeabilisation buffer (eBioscience, USA) and intracellular antibodies (Table 2.13) are added for 30 min at 4°C before washing and fixation in 200µl 4% paraformaldehyde. Fluorescence Minus One (FMO) controls or isotope controls were used to determine positive stained populations and samples were run on a FACS Aria II™ flow sorter and analysed using FlowJo software (Treestar, USA). Median fluorescence intensity (MFI) and percentages of lymphocytes or total cell populations were calculated using FlowJo software. Absolute numbers of stained cells was calculated by multiplying the percentage of total cells (calculated by FlowJo) by the total number cells from the tissue as obtained by cell counts with trypan blue staining.
Table 2.13: List of antibodies used for flow cytometry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Company</th>
<th>Type of staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>γδ TCR</td>
<td>γδ T cells</td>
<td>GL4</td>
<td>PE</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4⁺ cells</td>
<td>GK1.5</td>
<td>APC-H7</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4⁺ cells</td>
<td>GK1.5</td>
<td>APC</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD8a</td>
<td>CD8⁺ cells</td>
<td>53-6.7</td>
<td>V450</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD8a</td>
<td>CD8⁺ cells</td>
<td>53-6.7</td>
<td>FITC</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD3e</td>
<td>T and NKT cells</td>
<td>500A2</td>
<td>V500</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>NK1.1</td>
<td>NK cells</td>
<td>PK136</td>
<td>PeCy7</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD282</td>
<td>TLR2⁺ cells</td>
<td>6C2</td>
<td>PE</td>
<td>eBioscience, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD335</td>
<td>NKp46</td>
<td>29A1.4</td>
<td>eFluor 450</td>
<td>BD Phamingen, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>CD49b</td>
<td>NK cells</td>
<td>DX5</td>
<td>FITC</td>
<td>eBioscience, UK</td>
<td>Surface</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Foxp3⁺ cells</td>
<td>FJK-16S</td>
<td>Alexa fluor 488</td>
<td>BD Phamingen, UK</td>
<td>Intracellular</td>
</tr>
<tr>
<td>IL-17A</td>
<td>IL-17A⁺ cells</td>
<td>TC11-18H10</td>
<td>Alexa fluor 647</td>
<td>BD Phamingen, UK</td>
<td>Intracellular</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>IFN-γ⁻ cells</td>
<td>XMG1.2</td>
<td>Alexa fluor 700</td>
<td>BD Phamingen, UK</td>
<td>Intracellular</td>
</tr>
<tr>
<td>CD282</td>
<td>TLR2⁺ cells</td>
<td>T2.5</td>
<td>Alexa fluor 647</td>
<td>eBioscience, UK</td>
<td>Intracellular</td>
</tr>
</tbody>
</table>

For *ex vivo* cytokine flow cytometry, mice were given Brefeldin A (BFA; Sigma-Aldrich, UK) i.p to prevent the release of cytokines from cells during the infection (Liu and Whitton, 2005). The dose of BFA and length of incubation within the mouse was optimised for IL-17A cytokine release (Figure 2.3) and 50µg BFA given in the last 4h of the infection gave the maximal detection of cytokine by flow cytometry (Figure 2.3).
Figure 2.3: Ex vivo cytokine optimisation flow cytometry.

C57BL/6 female mice (n=3 per group) were infected with $10^8$ CFU H305 S. pyogenes for 24h. In the last 4h of infection, mice were given various doses of BFA (25, 50, 100, 150µg) i.p (A). Spleens were harvested and stained for IL-17A expression and representative histograms are shown above. The shaded histogram represents Fluorescence Minus One (FMO) control and the open histogram represents the IL-17A staining with the number in the top left corner indicating IL-17A MFI. In an independent infection experiment, mice were given 50µg BFA for different lengths of time: 2, 4, 6 and 7h before harvesting spleens for IL-17A staining and representative histograms are shown above (B).
To study the effects of *S. pyogenes* on TLR2 expression in whole blood, H305 and H293 exponential phase bacteria were obtained by adding 1ml overnight THB culture into 9ml fresh THB and grown until OD$_{600}$=0.1. 5µl bacterial culture was added to 50ml sterile PBS (~125 CFU/ml). Blood from HLA-DQ8.Aβ0 mice (n=16) was obtained by cardiac puncture and pooled. 1ml pooled blood was co-cultured with either 5µl diluted H305 or H293 cultures or 5µl PBS for 4h, 37°C, 5% CO$_2$. Red blood cells were lysed in 5ml lysis buffer for 10 min at RT and cells centrifuged for 10 min at 1200rpm. Supernatants were saved for viable bacterial counts (~250 CFU/ml) and the pellets washed in 5ml PBS containing 1% P/S twice by centrifugation at 1200rpm, 10 min at RT and resuspended in PBS (10% FCS) before TLR2 surface and intracellular staining as described above.

2.5.13 **Haematoxylin & Eosin (H&E) staining**

H305 WT *S. pyogenes* or sham-infected thigh muscle was harvested from one female Foxp3.DTR mouse and snap-frozen in iso-pentene before storage at -80°C. Serial sections were cut at a thickness of 12µm using a cyrostat (Leica, UK) and sections collected on Superfrost plus microscope slides (VWR, UK) and stored at -80°C before subsequent staining to define histological changes in tissue during GAS infection.

For Haematoxylin and Eosin (H&E) staining, sections were defrosted and incubated in ice-cold 1% hydrogen peroxide in methanol solution for 15 min before rinsing in tap water to wash off the alcohol. The slides were next immersed in 1% Mayer’s Haematoxylin solution (VWR, UK) for approximately 8 sec for nuclear staining before rinsing in running tap water for 5 min. The sections were fixed in 1% acetic acid in ethanol for 1 min before counter-staining with Eosin solution for 12 sec (VWR, UK). The slides were rinsed briefly in tap water and dehydrated in increasing concentrations of xylene (VWR, UK) before incubation in 100% xylene for 4 min. The section was mounted in DPX (VWR, UK) and a cover-slide added on top,
excluding air-bubbles. The sections were left to dry overnight before images taken on a light microscope (Leica, UK) at various objectives.

2.5.14 Gram staining

The presence of Gram-positive bacterium in Foxp3.DTR *S. pyogenes* or sham-infected thigh tissue was detected by Gram staining. In brief, the snap frozen sections were stained in 1 min with Crystal violet (HARLECO, Fisher Scientific, UK) and rinsed in tap water. Gram iodine (HARLECO, Fisher Scientific, UK) was added for 30 sec as a mordant to fix the Crystal violet. The slide was flooded with ethanol/acetone decolouriser (HARLECO, Fisher Scientific, UK) before counterstaining with safranin (HARLECO, Fisher Scientific, UK) for 30 sec. The section was washed under a running tap. Gram-positive bacteria are stained purple, whereas Gram-negative bacteria are stained pink. Images of the section were immediately taken on a light microscope (Leica, UK) at various objectives.

2.5.15 Immunohistochemistry (IHC)

To visualise the presence of Group A carbohydrate, specific for Group A *Streptococcus* and myeloperoxidase (MPO), reflective of the presence of activated neutrophils in snap-frozen *S. pyogenes* or sham-infected Foxp3.DTR thigh muscle, Immunohistochemistry (IHC) was performed. In brief, tissue sections were incubated in ice-cold 1% hydrogen peroxide in methanol solution for 15 min before rinsing in PBS. Primary antibodies diluted in 1% serum/PBS (Table 2.14) were added to cover the entire section and incubated overnight at RT. The next day, the slide was rinsed in PBS twice and secondary biotinylated antibody (1:200 in 1% serum/PBS; Table 2.14) applied for 45 min at RT before washing in PBS. Avidin-Biotin Complex (ABC) solution was made up according to the manufacturer’s instruction (Vector Laboratories, UK) and incubated on the slide for 1h at RT. For visualising, chromagen (Vector Laboratories, UK) was applied for approximately 3 min and the
reaction stopped by immersion in PBS. The nuclei were counterstained with Haematoxylin solution before dehydration in xylene and mounting in DPX was described in Section 2.5.13.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Host</th>
<th>Secondary antibody</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Dilution buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Group A Carbohydrate</td>
<td>Goat, polyclonal</td>
<td>Anti-goat</td>
<td>Group A Streptococcus</td>
<td>1:50</td>
<td>Donkey serum</td>
</tr>
<tr>
<td>Anti-MPO</td>
<td>Rabbit, polyclonal</td>
<td>Anti-rabbit</td>
<td>Myeloperoxidase</td>
<td>1:150</td>
<td>Goat serum</td>
</tr>
</tbody>
</table>

2.5.16 Bacterial plasmid extraction and restriction digest

In order to assess the relative contribution of streptococcal superantigens towards the disease associated pathology and interactions with T cells mediated immunity, it was necessary to create a panel of *S. pyogenes* knockout isolates from the M1T1 serotype H305 WT strain. Genetically knocking out superantigens in GAS isolates would allow the dissociation of immune responses mounted to the bacterial antigens from the more dominant and potent responses due to the presence of secreted superantigens.

Sriskandan and colleagues have previously created superantigen knockout isolates from a M89 (H293) or M1 (H305) serotype WT *S. pyogenes* isolates using insertional duplication or allelic replacement mutagenesis (Unnikrishnan et al., 2002; Sriskandan et al., 1999). Similar experimental approaches were utilised to extend the panel of M1 superantigen knockout strains for use in this study. *E. coli* hosts (H323, H376 or H431) (Table 2.4) containing the pre-constructed plasmids (pDL278, pGHostsmez and pDL278smez) used to knock out and complement *smez* in H293 (Unnikrishnan et al., 2002; Russell and Sriskandan, 2008) and H722 containing pDL278smezspea (Section 2.5.23) were plated out on LB agar plates (Section 2.1.2) containing
appropriate antibiotics (Table 2.4) and grown at 37°C, 5% CO₂ overnight. Ten 50ml falcon tubes containing 10ml LB media (Section 2.1.2) with antibiotics were each inoculated with a single bacterial colony and grown overnight at 37°C with shaking (200rpm). Plasmids were extracted from bacterial host using a Midi-prep kit (Qiagen, USA) according to manufacturer’s protocol. In brief, overnight bacterial cultures were spun down and the pellet lysed to release plasmid DNA into supernatant, which was transferred and bound to spin-columns for washing steps. Plasmid was eluted from spin-columns and precipitated out of solution using 3.5ml iso-propanol and aliquoted into 1.5ml eppendorf tubes and spun down at 15,000g for 10 minutes to increase plasmid yield. The DNA pellets were washed with 300µl 70% ethanol and centrifuged at 15,000g for 10 minutes. The supernatant was discarded and the pellet air-dried for 20 minutes at RT before being resuspended in 25µl molecular grade water and pooled together. The concentrations of plasmid were determined using a Nanodrop spectrophotometer and found to be between 700-1000ng/µl.

![Image](image.jpg)

Figure 2.4: Restriction digest of pGhostsmez plasmid.

After plasmid extraction from *E. coli* with a midiprep extraction kit, pGhostsmez was digested with EcoRI for 2h at 37°C before running on a 1% agarose gel. Uncut plasmid was also run as a control. Restriction digest of plasmid produced two band; linearised plasmid: 8Kb and 300bp *smez*’ triplet.

The purity of the plasmids was verified by restriction digest. pGhostsmez was cut with EcoRI (New England Biolabs, USA) (Figure 2.4) to cut out the cloned in *smez*’
triplet (Figure 5.1) (Unnikrishnan et al., 2002), while pDL278 and pDL278smez (Russell and Sriskandan, 2008) were digested with BamHI (GIBCO BRL, USA), either to linearised the plasmid or cut out cloned in smez coding region (Figure 2.5). 4µl of digested product was analysed on a 1% agarose TAE gel and run for 1h at 100V before visualization under UV light.

![Restriction digest of pDL278 and pDL278smez.](image)

Figure 2.5: Restriction digest of pDL278 and pDL278smez.

After plasmid extraction from E. coli, pDL278 and pDL278smez were digested with BamHI enzyme for 2h at 37°C before running on a 1% agarose gel. Uncut plasmid was also run as a control. Restriction digest of pDL278 produced the linearised plasmid: 6Kb along with the 900bp smez product in pDL278smez.

2.5.17 Preparation of S. pyogenes electrocompetent cells

*S. pyogenes* isolates for transformation to knockout or complement superantigens (H305, H326, H623 and H658) were plated on to blood agar and grown overnight at 37°C, 5% CO₂. 10ml THB was inoculated with a single colony and incubated overnight in the same conditions. Next day, the overnight culture was added to 90ml fresh THB and grown until OD₆₀₀ was between 0.15 and 0.175. The bacterial cells were harvested by centrifugation at 3500g, 10 min at 4°C. The cells were washed twice in 10ml ice-cold electroporation media (Section 2.1.2) and resuspended in 750µl ice-cold electroporation media. The cells were either used immediately for transformation or kept on ice until further use.
2.5.18 Bacterial electroporation

75µl ice-cold electrocompetent bacterial cells were added to 5µl, 3µl or 1µl extracted plasmid (Section 2.5.16) in sterile eppendorf tubes before being transferred into ice-cold 1mm E-shot™ standard electroporation cuvettes (Invitrogen, USA). The bacterial cells and DNA were electroporated at 1KV, 129ohms of resistance, 50µf capacitance using a Gene Pulser XCell™ electroporator (BIO-RAD, USA). A negative control of 5µl sterile water was also transformed into S. pyogenes isolates. 1ml THY (Section 2.1.2) was added to the cuvette immediately after electroporation and transferred to 9ml fresh THY and incubated at 30°C for 3h. Cells were harvested at 2500g, 10 min at 4°C and resuspended in 500µl fresh THY and 100µl plated on to THY agar plates (Section 2.1.2) containing the appropriate antibiotics for positive clone selection (Table 2.5).

H305/pGhostsmez and H326/pGhostsmez were plated on to THY plates containing erythromycin and incubated for 24-48 hours at 30°C, 5% CO₂ to allow for plasmid replication. Bacterial cells transformed with either pDL278, pDL278smez or pDL278smezspea were plated on to THY plates containing spectinomycin and incubated at 37°C, 5% CO₂ for 24 hours. The transformation efficiency was estimated to be 10⁴ CFU/µg pDL278. Negative controls did not grow on antibiotic plates.

2.5.19 pGhostsmez host chromosome integration

Clones from H305/pGhostsmez or H326/pGhostsmez transformations were streaked on to blood agar plates to verify β-haemolytic activities, indicative of GAS isolates. 5ml THY cultures containing erythromycin were made from the clones and incubated at 30°C, 5% CO₂ overnight. 20% glycerol stocks were made from these cultures and stored at -80°C. 1ml of overnight culture was inoculated into 9ml fresh THY and incubated for 2h at 30°C and then moved to 37°C overnight for host chromosome integration. Next day, the cultures were spun down at 2500g, 10 min at 4°C and
resuspended in 500μl fresh THY. 100μl was plated out on to THY containing erythromycin plates and also blood agar plates to check for β-haemolytic activity and incubated overnight at 37°C. Clones from these plates were then used for glycerol stocks for permanent isolate storage.

2.5.20 *Streptococcal genomic DNA extraction*

Genomic DNA was extracted from transformed streptococcal cells for PCR verification of plasmid insertion. 50ml THB was inoculated with a single colony from a freshly plated out *S. pyogenes* isolate and incubated overnight at 37°C, 5% CO₂. The cells were harvested by centrifugation at 2500g, 10 min at 4°C and resuspended in 1ml sterile PBS. 20μl mutanolysis (10,000U/ml; Sigma-Aldrich, UK) and 20μl lysozyme (100mg/ml; Sigma-Aldrich, UK) were added and incubated at 37°C for 10 min. The samples were spun down at 200g for 10 min and resuspended in 500μl SET buffer (Section 2.1.3). 5μl 10% SDS solution (BDH, UK) and 0.5mg/ml Proteinase K (Sigma-Aldrich, UK) were added and incubated for 2h at 55°C with occasional mixing. 175μl 5M NaCl and 500μl chloroform were added and left to incubate at RT for 30 min. The samples were centrifuged at 4500g for 15 min and the aqueous layer transferred into clean eppendorf tubes and DNA precipitated with 500μl iso-propanol. DNA was pelleted at 16,000g for 5 min and washed with 1ml 70% ethanol at 16,000g for 3 min. The pellet was air-dried for 20 min and dissolved in 200μl molecular biology water. The concentration of genomic DNA was determined using a Nanodrop spectrophotometer.

2.5.21 *Streptococcal genomic DNA PCR*

The amplification of DNA sequences by PCRs was carried out to determine whether superantigen genes were knocked out in electroporated streptococcal isolates. Primers: aphI (F), aphI (R), rRNA (F), rRNA (R), S1 and A1 were used for *smez* KO
mutagenesis are shown in Table 2.3. Amplification were carried out using Mega-mix Blue (Microzone, UK) for products less than 1Kb in size and the master-mix for one reaction was made up as follows: 20.5µl Mega-mix Blue, 1.25µl sense primer (100µM), 1.25µl anti-sense primer (100µM) and 2µl genomic DNA (1:10 dilution ~100ng/µl DNA). The PCR samples were run on the following thermal profile:

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
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<tbody>
<tr>
<td>95</td>
<td>5 min</td>
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<td>95</td>
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<td>Annealing temperature</td>
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<td>72</td>
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<td>4</td>
<td>HOLD</td>
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Annealing temperatures were calculated as 2°C below the lowest Tₘ of the primer pair being used. 10µl of PCR reaction was run on a 2% agarose gel and run for 1h at 100V before visualization under UV light.

Amplification of DNA products more than 1Kb were carried out with Bio-X-Act Short DNA polymerase (Bioline Ltd, UK). Primers (smezP1 and smezR600) were used for smez KO mutagenesis are shown in Table 2.3. Master-mix for 1 reaction were prepared as follows: 10µl 10X Optibuffer (Bioline Ltd, UK), 6µl 50mM MgCl₂, 10µl 10mM dNTPs, 1µl sense primer (100µM), 1µl anti-sense primer (100µM), 1µl Bio-X-Act Short DNA polymerase, 70µl molecular biology water, 2µl genomic DNA (1:10 dilution ~100ng/µl). Annealing temperatures were calculated as 2°C below the lowest Tₘ of the primer pair being used. The thermal profile below was used for product amplification before 4µl product was run on a 1% agarose gel for 1h at 100V.
2.5.22 Southern blot

Southern blotting was performed to confirm the orientation of pGhostsmez plasmid in the H305 host genome and hence disruption of functional smez transcription. An internal smez probe was constructed from a PCR product made from primers smezF and smezR (Table 2.3) at the start and end of smez from H305 genomic DNA. PCR was carried out using GoTaq DNA polymerase (Promega, USA) and the master-mix for one reaction was made up as follows: 0.5µl GoTaq DNA polymerase (5U/µl), 20µl 5X Taq colourless buffer (Promega, USA), 10µl 1mM dNTPs, 1µl smezF primer (100µM), 1µl smezR primer (100µM), 65.5µl molecular biology water, 2µl H305 genomic DNA (1:10 dilution ∼100ng/µl DNA). The thermal profile below was used for product amplification.

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<tr>
<th>Temperature (°C)</th>
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<th>Number of cycles</th>
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The PCR product was purified using QIAquick PCR Purification Kit to remove contaminating PCR reagents (Qiagen Ltd, UK). 5 µl purified PCR product was boiled with 11 µl molecular grade water for 10 min before adding 4 µl DIG high prime (Roche, Germany) and incubating for 3 h at 37°C. The probe for Southern blotting was boiled again for 10 min before being added to 1 ml DIG easy hyb (Roche, Germany).

5 µg genomic DNA from H305 (WT) and H305/pGhostsmez transformation clones were digested with XbaI enzyme (Promega, USA) in a total reaction volume of 20 µl. 5 µl 1X loading buffer was added to all samples and then run on a 0.9% agarose gel without ethidium bromide for 2 h at 120 V along with 7 µl DIG labeled DNA ladder III (Roche, Germany). The gel was covered in 1X TAE buffer containing 5 µl ethidium bromide for 30 min and then visualised under UV light. The gel was washed in Denaturation solution (Section 2.1.4) twice for 15 minutes at RT with gentle shaking and rinsed in ddH₂O. The gel was washed twice in neutralisation solution (Section 2.1.4) for 15 min at RT with gentle shaking before equilibrated in 20X SSC buffer (Section 2.1.4) for 10 min. The gel was set to blot on to nitrocellulose membrane overnight at RT.

Next day, the membrane was UV crossed-linked at 1200 J (UVC500 UV crosslinker, Hoefer) and allowed to air-dry. The membrane was blocked with 50 ml DIG easy hyb at 42°C for 1 h rotating. smez probe in DIG easy hyb was next added to the membrane and left rotating overnight at 42°C.

The membrane was washed twice for 5 min in 2X wash buffer (Section 2.1.4) and then twice in 0.5X wash buffer (Section 2.1.4) (pre-heated to 68°C for 15 min with rotation). The blot was equilibrated in washing buffer (Roche, Germany) for 1 min before being submerged in blocking buffer (Roche, Germany) for 1 h with gentle shaking. 1.5 µl anti-DIG antibody (Roche, Germany) was diluted in 30 ml blocking solution and added to the membrane once the blocking buffer had been discarded and incubated for 1 h. The blot was washed three times in washing buffer for 15 min each and equilibrated in 20 ml detection buffer (Roche, Germany) for 3 min. The blot was
developed by adding CSPD* substrate (Roche, Germany) and exposed to photographic film and developed using a Curix60 developer (AGFA).

2.5.23 Cloning of pDL278smezspea plasmid

In order to complement the double superantigen knockout phenotype in H658, it was necessary to construct a self-replicating plasmid containing both the coding regions of smeZ and speA. This was achieved by adding the speA coding sequence extracted from WT S. pyogenes genome into pDL278smeZ plasmid (Russell and Sriskandan, 2008) (Figure 2.6).

Figure 2.6: pDL278smezspea shuttle vector.

pDL278smeZ plasmid was modified with the addition of speA gene into EcoRI restriction site to create a self-replicating shuttle vector expressing both smeZ and speA superantigen coding regions, retaining endogenous promoter sequences.

In brief, speA gene including the promoter region was amplified from WT S. pyogenes genomic DNA (H305) by PCR with the addition of EcoRI restriction sites at the start and end of the product using primers speA-EcoRI-F and speA-EcoRI-R.
Proof-reading BIO-X-Act-short DNA polymerase was used with the thermal profile as described in Section 2.5.20 with an annealing temperature of 70°C for 30 sec and extension at 68°C for 2 min. pDL278smez plasmid was extracted from H376 with a midiprep kit (Qiagen, UK) and the concentration and purity determined as described in Section 2.5.16. 16μl plasmid and PCR product were digested with EcoRI enzyme for 2h at 37°C in a total volume of 20μl in duplicate. The enzyme was heat-inactivated by heating at 65°C for 20 min. Arctic phosphatase (New England Biolabs, USA) was added to the cut plasmid and incubated for 1h at 37°C to prevent self-ligation of the plasmid before heat-inactivation of the enzyme at 65°C for 5 min. The plasmid and PCR product were run on a 0.9% agarose gel for 2h at 100V before linearised plasmid and cut spea product were cut out. The cut pDL278smez and spea were extracted from the agarose gel using a QIAquick gel extraction kit (Qiagen, UK) and each duplicate was pooled together before elution in 30μl molecular biology water to increase yield of DNA. 5μl of cut plasmid and PCR were run on 0.9% agarose gel to check purity before quick ligation 3:1 ratio of insert to vector using Quick T4 DNA ligase (New England Biolabs, USA) in a total volume of 20μl. Ligation reaction was incubated at 14°C overnight. Next day, 1μl ligation reaction was transformed into 100μl Max Efficiency DH5α chemical competent E. coli cells (Invitrogen, UK) by incubated of DNA and cells on ice for 30 min before heat-shock at 42°C for 45 sec. A positive control of 1μl of pDL278 and a negative control of 1μl water were also transformed into DH5α cells. The cells were placed on ice immediately after heat-shock for 2 min before 0.9ml RT SOC media (Invitrogen, UK) was added and incubated for 1h at 37°C with shaking (225rpm) to allow recovery after transformation. 200μl was plated on LB agar plates containing spectinomycin (50μg/ml) and incubated overnight at 37°C. Next day, colonies were picked and overnight cultures prepared for glycerol stocks and plasmid extraction using miniprep kit (Qiagen, UK). Extracted plasmids were digested with EcoRI and BamHI to verify the presence of the cloned spea and pre-existing smez fragments. Clone 18 and 20 were found to contain both smez and spea coding regions and clone 20 was re-designated as H772 (Figure 2.7). Sequencing of cloned spea product in H772 was performed with M13 primer pairs to confirm the absence of mutations creation by the cloning process before electroporation into H658 according to Section 2.5.16 to create H786.
Figure 2.7: Restriction digest of clone 18 and 20.

Plasmids purified from clone 18 and 20 and pDL278smez were digested with BamHI (A) and EcoRI (B) for 2h at 37°C before run on a 0.9% agarose gel. Both clones 18 and 20 contained inserted 900bp smez product along with pDL278smez when cut with BamHI. Only clones 18 and 20 contained 1.1Kb spea product upon digestion with EcoRI confirming the presence of the cloned in product. Uncut clones 18 and 20 (C) were found to be approximately 8Kb in size, confirming that only one copy of spea had inserted into the linearised pDL278smez vector.

2.5.24 Preparation of human PBMCs and murine splenocytes

Transgenic HLA-DQ8.Aβ^0 (n=3-6) mice were humanely sacrificed and spleens were dissected out and passed through a 70µm cell sieve to obtain a single cell suspension. The splenocytes were resuspended in RPMI (10% FCS, L-Glut, P/S), ready for use in proliferation assays as described in the next section.

In order to complement and confirm proliferation data obtained utilising murine splenocytes, 20ml of human blood was collected from a small cohort of healthy donors (n=2) and diluted 1:1 in PBS. Human PBMCs were separated out of whole blood by overlaying on top of an equal volume of Ficoll-Paque (Sigma-Aldrich, UK) and centrifuged at 800g for 25 min. The interface, rich in lymphocytes was washed in 10ml RPMI 1640 (10% FCS, L-Glut, P/S) twice and resuspended in 5ml media mix.
Proliferation assays were carried out on murine splenocytes or human PBMCs to assess the presence or absence of specific secreted streptococcal superantigens in the isolates prepared as described in Section 2.5.5. In brief, cell counts were carried out on both murine splenocytes and human PBMCs by diluting cells 1:10 in 0.4% Trypan Blue (Sigma-Aldrich, UK) and counting cells in a KOVA Glasstic® slide 10 with grids (Hycor, Biomedical Inc., USA). 100µl of prepared murine splenocytes (concentration of $3.5 \times 10^5$ cells/well) or human PBMCs (concentration $2 \times 10^5$ cells/well) were seeded in triplicate into a flat bottomed 96-well plate and stimulated with 100µl of 10-fold dilutions of cell free bacterial supernatants or GAS infected HLA-DQ8.Aβ0 sera (1:20) at 37°C, 5% CO2. Negative control of 100µl media was also included. 1µ-Curie $^3$H-thymidine (Amersham, GE healthcare, USA) was added to each well after 88h for murine splenocytes and 64h for human PBMCs and cells were harvested after 8h of incubation onto filter mats (Wallac, Finland) in a 1295-004 Betaplate 96-well cell harvester (Wallac and Berthold, UK).

Streptococcal growth curves and opsonophagocytosis assay

To determine the rate of growth of the various superantigen knockout mutants in liquid broth, overnight cultures of *S. pyogenes* isolates were diluted 1:10 with fresh THB and incubated at 37°C, 5% CO2. Every hour, the optical density of the cultures was sampled until stationary phase. This was repeated as part of three independent experiments.

To determine the rate of growth of the superantigen knockout isolates in whole blood, an opsonophagocytosis assay was carried out. Overnight THB cultures were diluted 1:10 with fresh broth and grown until the optical density was 0.17 at 600nm. 5µl of the exponential phase cultures was added to 50ml sterile PBS and 30µl was added to 270µl human healthy donor blood (n=4) or female HLA-DQ8.Aβ0 murine blood (n=3) containing sodium heparin in triplicates. The infected blood cultures were
incubated for 3h at 37°C rotating before 10µl was plated out on to blood agar along with 10µl of the inoculum. The multiplication factor of CFU recovered from infected blood over CFU of the inoculum was calculated.

2.5.27 Statistical analysis

Appropriate statistical tests were carried out according to the nature of the data set using GraphPad Prism software (GraphPad Software Inc, USA) and data presented as mean ± SEM. Non-parametric Kruskal-Wallis statistical test was carried out on bacterial counts, proliferation assay, percentage weight loss during infection as data points did not follow normal Gaussian distribution and did not pass D’Agostino and Pearson normality testing. Significant differences in survival experiments were calculated using Log-Rank (Mantel-Cox) statistical test. Results for ELISA, CBA and opsonophagocytosis assays passed the D’Agostino and Pearson normality testing and parametric 1-Way ANOVA statistical testing was performed. For flow cytometry analysis, percentage of lymphocytes data sets were subjected to non-parametric Kruskal-Wallis statistical test due to the nature of ranked data points. For statistical analysis of absolute numbers and MFI of flow cytometric data, 1-Way ANOVA was carried out as results followed normal Gaussian distribution according to D’Agostino and Pearson normality testing. Bacterial growth curves were analysed using linear regression to determine any statistical differences. Significance differences in qRT-PCR data was found using REST software based on a mathematical model of primer efficiencies and calculated Ct values before the expression ratio between sample and control groups are subjected to randomisation testing to determine significant differences. Significant differences between infected and sham controls for qRT-PCR are indicated as asterisks above the bars.
Chapter 3

3 The role of $\alpha\beta$ and $\gamma\delta$ T cells during acute *Streptococcus pyogenes* infection.

3.1 Introduction

It is important for the host immune system to mount appropriate responses in the face of invading pathogens. If pathogens do penetrate and invade the host tissue, innate immunity is initiated rapidly to neutralise microorganisms. Adaptive immunity in the form of T and B cells is lastly mounted after subsequent activation and clonal expansion to coordinate and enhance effector cell function.

The role of T cells during infection has been demonstrated to be important in many different disease models. T cells can be divided into two populations based on their T Cell Receptor (TCR) expression; $\alpha\beta$ and $\gamma\delta$ T cells. $\alpha\beta$ T cells are the predominant population, consisting of 95% of the total T cell population. They are capable of recognising a large array of antigens presented by MHC class II molecules on APCs, such as DCs, macrophages and B cells (Kupfer *et al.*, 1987). $\gamma\delta$ T cells on the other hand are fewer in number in circulating blood (5% of total T cell population) and tend to be more abundant in the gut mucosa (Born *et al.*, 2010). The antigenic source of $\gamma\delta$ T cell activation is still unknown, although a group of molecules collectively known as phosphoantigens have been proposed. It has also been suggested that this class of T cells are not restricted to activation by conventional antigen processing and presentation by APCs like their $\alpha\beta$ T cell counterparts. Rather it has been stipulated they an be activated by direct $\gamma\delta$ TCR binding to non-MHC molecules, such as viral glycoproteins (Sciammas *et al.*, 1994).
A role of $\alpha\beta$ and $\gamma\delta$ T cells during sepsis has been highlighted by analysis of patients with Streptococcal Toxic Shock Syndrome (SSTS). One clinical study showed an expansion of $\gamma\delta$ T cells in PBMCs taken from patients with SSTS (Arvand et al., 1996). $\alpha\beta$ T cells from the same cohort of GAS infected patients were also shown to proliferate upon stimulation in vitro with supernatants from the streptococcal isolates extracted from the same patients (Arvand et al., 1996). $\gamma\delta$ T cells stimulated with heat-killed GAS produce IFN-$\gamma$ (Follows et al., 1992), demonstrating that both $\alpha\beta$ and $\gamma\delta$ T cells activities are modulated either directly by the bacterium or via secreted virulence factors such as superantigens.

The function of $\alpha\beta$ and $\gamma\delta$ T cells during sepsis has further been addressed experimentally using TCR knockout (KO) mice. TCR $\beta$ knockout mice that have undergone Cecal Ligation Puncture (CLP) surgery, a poly-microbial model of sepsis, exhibited increased survival compared to operated wild-type (WT) control mice (Enoh et al., 2007). TCR $\delta$ KO mice however, exhibited no significant difference in survival relative to WT mice after CLP surgery (Enoh et al., 2007). This would suggest that in this experimental model of sepsis, $\alpha\beta$ T cells are detrimental to the host during poly-microbial infection, whereas $\gamma\delta$ T cells play no significant role in disease outcome. The use of these TCR knockout mice has not been previously used for studies of acute $S. pyogenes$ infection to investigate the relative roles of $\alpha\beta$ and $\gamma\delta$ T cells.

The pro-inflammatory cytokine, IL-17A, has been extensively linked to autoimmunity (Crome et al., 2010; Bettelli et al., 2008) and has also been examined in many different infectious disease models (Curtis and Way, 2009). Most research has focused on CD4$^+$ T cells producing this cytokine, known as $T_{h17}$ cells, with evidence demonstrating that IL-17A production by CD4$^+$ T cells during $S. pneumoniae$ or $Klebsiella pneumoniae$ infection is beneficial due to increased neutrophil recruitment, aiding bacterial clearance in the lung (Zhang et al., 2009c; Ye et al., 2001b). These results were further confirmed through antibody-mediated depletion of IL-17A or CD4$^+$ T cells shown to reverse the recruitment of neutrophils and monocytes to the site of infection and lead to increased bacterial colonisation and poor survival outcome (Zhang et al., 2009c). Over-expression of IL-17A in the
pulmonary compartments has similarly been shown to be beneficial, enhancing survival outcome during lethal *K. pneumoniae* infection (Ye *et al.*, 2001a).

Whether IL-17A has a role during *S. pyogenes* infection is not well understood with only a handful papers published to date. Tonsil cultures from patients with recurrent GAS-associated tonsillitis can be stimulated with heat-killed M1 serotype GAS to produce IL-17A together with TGF-β, suggestive of differentiation of Th17 cells during infection (Wang *et al.*, 2010a). Purified superantigens from *S. pyogenes* and superantigen-contaminated preparations of Peptidoglycan are potent inducers of IL-17A from T cells (Li *et al.*, 2008). However, the direct contribution of IL-17A has not been examined during acute *S. pyogenes* infection either in mice or humans.

In order to investigate the specific role of T cells during infectious diseases, various techniques have been employed. Early studies concentrated on the use of antibodies to specifically deplete a chosen cell population in vivo (Vignali *et al*., 1989; Müller *et al*., 1987). This has both advantages and disadvantages; for example, most antibodies used for depletion are specific and will only remove the chosen cell type in vivo. The use of antibodies to deplete specific targets has been so successful that it has been exploited in the field of translational medicine in the form of therapeutic treatments for certain human diseases. For example, Infliximab, a monoclonal antibody targeted against TNF-α has been used to treat autoimmune and other inflammatory diseases, such as rheumatoid arthritis and Crohn’s disease (Silva *et al*., 2010). However, the efficiency of antibody-mediated depletion in vivo in experimental models can vary depending on antibody affinity with the target and the chosen dosing regimen to achieve a full knockdown can be time consuming and expensive.

The use of antibodies to exclusively deplete cell types or cytokines during infection or autoimmunity has helped to enhance our understanding of the immune response. For example, BALB/c mice depleted of γδ T cells with a monoclonal anti-γδ TCR antibody (clone GL3) prior to coxsackievirus B3 infection showed delayed progression to myocarditis due to decreased Th1 cells and increased Treg numbers in the spleen (Huber, 2009). The pathogenic nature of IL-17A was also revealed in a murine model of Experimental Autoimmune Uveoretinitis (EAU) as shown by
amelioration of the disease upon antibody mediated IL-17A blockade (Amadi-Obi et al., 2007). These two examples demonstrate that antibody mediated depletion or neutralisation can be used effectively to show the pathogenic and protective nature of T cells and cytokines in different disease models.

The use of antibodies has not always proved practical in studies focusing on one particular target cell or cytokine. Anti-asialo GM$_1$ antibody has been extensively used to deplete NK cells in many different murine models. NK cell depletion with this antibody is usually achieved to a high specificity (Badgwell et al., 2002), however this antibody also exhibits cross reactivity towards monocytes and macrophages (Wiltrout et al., 1985). In this context, it would appear that the use of antibodies to mediate cell depletion is a useful technique to dissect out the role of a particular cell type or cytokine but caution must be taken in interpreting results if the antibody is non-specific. It should also be noted that the effects are transient and will only last as long as the antibody is given.

Given the limitations of antibody mediated depletion, the popularity of transgenic murine models with subsets of T cells or cytokines genetically removed have risen over the past few years within the field of immunology (Moon and McSorley, 2009; Shultz et al., 2007). Their application ensures the knowledge and confidence that any results attained will be due to complete depletion, without the fear of partial and transient effects as observed through the use with neutralising antibodies. This field has also been expanded by the creation of conditional and inducible transgenic knockout models, allowing researchers to study cell types or transcription factors that have proved to be fatal or severely affect development if knocked out in conventional transgenic animals.

Conventional transgenic models have been employed successfully to study the relative contribution of different T cell subsets and cytokines during bacterial infection. For example, the role of γδ T cells during L. monocytogenes infection was addressed in TCR δ$^{-}$ mice (Hamada et al., 2008). It was deduced that this particular subpopulation of T cells secreted IL-17A during the early stages of infection in the liver, which lead to the recruitment of neutrophils and hence decreased bacterial
burden (Hamada et al., 2008). The protective role of IL-17A was also further confirmed in *L. monocytogenes* infected IL-17A$^{-/-}$ mice displaying exacerbation of the disease (Hamada et al., 2008). A similar pattern of immune responses was also observed during *E. coli* and *Salmonella enterica* serovar enteritidis infection (Shibata et al., 2007; Siegemund et al., 2009). These results would suggest that $\gamma\delta$ T cells and through their subsequent IL-17A production correlated with a protective outcome during certain bacterial infection.

The use of conventional knockout mice has not always been suitable for all experimental systems. Sometimes, the creation of knockout mice for target genes has shown these mutations are fatal to the survival and development of the animal. To overcome this problem, conditional knockout mice can be generated to allow the researcher to switch off the gene in a specific target organ or cell type in the adult animal using Cre-Lox recombination (Sauer, 1998), without the detrimental effects as exhibited by genetic knockout mice (Bockamp et al., 2008).

Inducible knockout transgenic mice exploit the use of suicide reporters to selectively deplete a chosen population at any given time. One example of an inducible knockout model include the CD11c-DT transgenic mice, whereby CD11c$^+$ cells are marked by Diphtheria Toxin Receptor expression and upon administration of Diphtheria Toxin (DT) into the mouse leads to selective apoptosis of CD11c$^+$ cells (Loof et al., 2007). This has been used to study the effects of depletion of DCs during *S. pyogenes* infection, indicating that this cell type is key in IL-12 production during GAS infection (Loof et al., 2007).

The aim of this chapter was to determine the relative contribution of $\alpha\beta$ and $\gamma\delta$ T cells during acute *S. pyogenes* infection using conventional TCR knockout transgenic models. I sought out to further dissect the role of the two different sub-populations of $\alpha\beta$ T cells; regulatory and effector $\alpha\beta$ T cells during systemic infection through the use of antibody mediated depletion and also inducible knockout mice. Given that a key issue in this example of bacterial sepsis is the relative contributions of direct bacterial pathology from immune/inflammatory collateral effects, I aimed to determine whether these cell types are protective or pathogenic during GAS infection.
Cytokine production from different cell types during acute *S. pyogenes* infection was also studied to evaluate whether IL-17A or IFN-γ responses are employed to bring about these immunological effects.
3.2 The contribution of $\alpha\beta$ and $\gamma\delta$ T cells during acute sepsis

Immunity mounted by infectious pathogens such as *E. coli* (Shibata *et al.*, 2007) and *L. monocytogenes* (Usami *et al.*, 1995) revealed the complexity of T cell mediated responses. The relative contribution of $\alpha\beta$ and $\gamma\delta$ T cells during infectious diseases can vary, with both T cell population been implicated as either pathogenic or protective. The precise role of $\alpha\beta$ and $\gamma\delta$ T cells during *S. pyogenes* infection is still unclear with only a handful papers published regarding this matter in the CLP model of poly-microbial sepsis. I sought out in this chapter to investigate the relative contributions of $\alpha\beta$ and $\gamma\delta$ T cells during acute *S. pyogenes* infection with the use of TCR knockout transgenic mice.

Various TCR knockout transgenic strains were employed to help study whether $\alpha\beta$ or $\gamma\delta$ T cells are required during acute sepsis as induced by *S. pyogenes* infection. The percentage survival of either TCR $\beta\delta$ or $\beta$ knockout mice given a lethal dose of *S. pyogenes* via an intra-muscular route was compared to infected C57BL/6 controls (Figure 3.1). The lack of both $\alpha\beta$ and $\gamma\delta$ T cells in $\beta\delta$ knockout TCR mice proved to be protective as demonstrated by increased survival compared to WT controls post lethal infection with *S. pyogenes* (Figure 3.1A). The lack of $\alpha\beta$ T cells in TCR $\beta$ knockout mice also proved to be advantageous and increased the percentage survival compared to infected C57BL/6 mice (Figure 3.1A). These results were also in line with serum IL-6 and IL-17A concentrations taken 8h post infection. TCR $\beta\delta$ knockout mice had the lowest level of serum IL-6, a surrogate marker of systemic disease severity (Damas *et al.*, 1992) (Figure 3.1B). Interestingly, TCR $\beta$ knockout mice had elevated levels of IL-17A in the blood, presumably from the remaining $\gamma\delta$ T cell population (Figure 3.1B). Overall, this set of data is suggestive that $\alpha\beta$ T cells, and to a lesser extent $\gamma\delta$ T cells contain a detrimental T cell population during lethal *S. pyogenes* infection.
Figure 3.1: Increased survival post lethal infection with *S. pyogenes* in the absence of αβ and γδ T cells.

A. Survival curve of infected C57BL/6, TCR β KO and TCR βδ KO mice (n=8 males and females). Mice were given 7x10^7 CFU H305 *S. pyogenes* (WT) via an intra-muscular route and monitored for defined end points and culled. The curves are significantly different (p=0.0139) as calculated by Log-Rank (Mantel-Cox) statistical test.

B. Serum IL-6 and IL-17A analysis. Serum was collected 8h post infection and concentration of IL-6 and IL-17A determined by ELISA. Mean and SEM are represented and 1-way ANOVA statistical test was carried out, no significant differences were found.

Sub-lethally infected TCR βδ or β knockout mice also showed a similar pattern of disease severity. 24h post infection, thigh, liver, spleen and blood were harvested to determine the bacterial burden. TCR βδ knockout mice had the lowest levels of recovered bacteria at the site of infection (thigh) and systemically (liver) compared to infected WT C57BL/6 controls (Figure 3.2A). TCR β knockout mice have intermediate levels of bacterial counts compared to WT and TCR βδ knockout mice (Figure 3.2A). There was however, no difference in the percentage weight loss between the three strains of infected mice (Figure 3.2B). The reduced bacterial counts in the TCR knockout mice also correlated with decreased serum IL-6 levels (Figure 3.2C) and there appeared to be a marginal increase in serum IL-17A upon the
depletion of αβ T cells in TCR β knockout and depletion of both αβ and γδ T cells in TCR βδ knockout mice compared to infected WT controls (Figure 3.2C).

**Figure 3.2:** The effect on bacterial burden upon removal of αβ and γδ T cells during acute *S. pyogenes* infection.

A. Bacterial counts after 24h infection. C57BL/6, TCR β KO and TCR βδ KO mice (n=8 females per strain) were infected with 2.1x10⁸ CFU H305 *S. pyogenes* via intra-muscular route. Bacterial counts in the thigh, blood, spleen and liver was determined. Mean and SEM are represented and 1-way ANOVA statistical test was carried out and no significant differences was found.

B. Percentage total weight loss after 24h infection. Start body weight and cull weight of each mouse was recorded and percentage total weight loss was calculated.

C. Serum ELISA analysis of IL-6 and IL-17A. Serum was collected after 24h of infection and concentration of IL-6 and IL-17A measured by ELISA.

From these sets of experiments, the presence of αβ T cells would appear to be most detrimental during systemic infection with *S. pyogenes* through the use of TCR β knockout mice (Figure 3.1). From the survival experiment, increased IL-17A in the blood of infected TCR β knockout transgenic mice was detected (Figure 3.1); suggestive that the remaining γδ T cells in this transgenic may be responsible for the production of this pro-inflammatory cytokine. The production of IL-17A may also be associated with the increased resistance to lethal infection with *S. pyogenes*. In order to determine whether γδ T cells are protective or pathogenic during GAS infection,
TCR δ knockout transgenic mice lacking γδ T cells were used for further experiments.

Figure 3.3: Survival outcome post lethal infection with *S. pyogenes* upon removal of γδ T cells.

A. Survival curve of infected C57BL/6 (n=7 females) and TCR δ KO mice (n=6 females). Mice were given $6 \times 10^8$ CFU H305 *S. pyogenes* (WT) i.m and monitored for defined end points and culled.

B. Bacterial counts after 24h infection. C57BL/6 and TCR δ KO mice (n=8 females per strain) were infected with $1.4 \times 10^9$ CFU H305 *S. pyogenes* i.m. Bacterial counts in the thigh, blood, spleen and liver was determined. Mean and SEM are represented and 1-way ANOVA statistical test was carried out and no significant differences was found.

C. Serum ELISA analysis of IL-6, IL-17A and IFN-γ. Serum was collected after 24h of infection and concentration of IL-6, IL-17A and IFN-γ measured by ELISA and no significant differences was found.
When TCR δ knockout transgenic mice were infected with a lethal dose of WT S. pyogenes, there was no significant difference between the survival rate between WT and TCR δ knockout animals (Figure 3.3A). However, during acute infection when tissue was harvested 24h post infection to determine bacterial burden in WT and TCR δ knockout transgenic mice, there was a general trend of increased numbers of recovered bacteria in infected TCR δ knockout transgenic mice (Figure 3.3B). It was curious to note, there was no statistical difference between levels of IL-6 and IL-17A in the blood of infected WT and TCR δ knockout mice (Figure 3.3C). However there was a modest increase of serum IFN-γ in infected TCR δ knockout mice (Figure 3.3C).

Overall, there may be a modest protective element associated with the presence of γδ T cells during acute S. pyogenes infection as demonstrated by a trend of increased bacterial burden in the early stages of infection in TCR δ knockout mice (Figure 3.3B). This protective effect was however, not due to increased production of serum IL-17A as indicated in Figure 3.1. Increased serum IFN-γ as observed in infected TCR δ knockout mice (Figure 3.3C) and this cytokine may be detrimental towards bacterial clearance and contribute to the hyper-inflammatory response presented by septic patients. However, during lethal infection, it would appear that γδ T cells do not play a significant role in the survival outcome (Figure 3.3A).

3.3 Gene expression changes upon S. pyogenes infection in TCR β and βδ knockout mice

qRT-PCR analysis was carried out on draining inguinal lymph nodes and spleens harvested from S. pyogenes infected TCR βδ or β knockout mice to identify any changes in gene expression upon depletion of αβ and γδ T cells. foxp3 gene expression in the draining lymph node (DLN) was significantly down-regulated in both the TCR βδ and β knockout transgenic mice compared to infected C57BL/6
mice, confirming these mice presumably lack Foxp3$^+$ Tregs due to the loss of αβ T cells (Figure 3.4). Interestingly, there was also a significant difference in foxp3 expression between infected TCR βδ and β knockout transgenic mice (Figure 3.4), suggestive that γδ T cells may also express foxp3. A similar pattern of down-regulation gitr, ctla-4 and lag-3 expression (genes also associated with Tregs) was observed in infected TCR βδ and β knockout transgenic mice compared to infected controls (Figure 3.4). t-bet expression was decreased in TCR β knockout and even lower in TCR βδ knockout transgenic mice (Figure 3.4) upon infection with S. pyogenes, presumably due to the loss of αβ T cells. gata-3 and rorf expression was significantly up-regulated in TCR β knockout mice compared to infected WT controls (Figure 3.4). This is surprising as these transcription factors are generally connected with Th2 and Th17 αβ T cells, which are absent in TCR β knockout mice. Perhaps, other immune cell types such as NK and iNKT cells are the source of these changes in transcription factor expression (Kim et al., 2006; Doisne et al., 2009).

qRT-PCR analysis of cytokine gene expression in infected draining lymph nodes from TCR βδ and β knockout transgenic mice revealed only subtle changes in expression pattern compared to infected WT controls. il-10 and tnf-α gene expression was significantly down-regulated, whereas tgf-β was significantly up-regulated in TCR β knockout mice compared to infected WT controls (Figure 3.5). This is striking data as TGF-β and IL-10 are cytokine associated with Treg suppressive function and it would appear αβ T cells expressing IL-10 during GAS infection are depleted in TCR β knockout mice tgf-β expression in other cell types was increased. In TCR βδ knockout mice, ifn-γ and il-17a were significantly up-regulated relative to infected WT controls (Figure 3.5). Up-regulation of ifn-γ and il-17a upon the depletion of both T cell subsets could be attributed to other cell types, such as NK cells, that are known to secrete these pro-inflammatory cytokines and may have suppressive control liberated from them.
Figure 3.4: qRT-PCR analysis of transcription factor gene expression in DLNs from infected TCR β and βδ knockout mice.

C57BL/6, TCR β KO and TCR βδ KO mice (n=8 females per strain) were infected with 2.1x10⁸ CFU H305 S. pyogenes i.m. DLNs were harvested for qRT-PCR analysis of transcription factor gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
C57BL/6, TCR β KO and TCR βδ KO mice (n=8 females per strain) were infected with 2.1x10^8 CFU H305 S. pyogenes i.m. DLNs were harvested for qRT-PCR analysis of cytokine gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
In the spleen, changes in transcription factor gene expression were less impressive than in the draining inguinal lymph node. foxp3 expression was yet again significantly down-regulated in GAS infected TCR βδ and β knockout transgenic mice compared to infected WT controls (Figure 3.6A). No other differences were observed in transcription factor genes between infected WT and TCR knockout transgenic mice (data not shown). In order to study whether γδ+ TCR cells also express Foxp3; preliminary flow cytometric staining of splenocytes from infected C57BL/6 mice was carried out (Figure 3.6B). Indeed, only a very small percentage of γδ+ TCR cells were found to be Foxp3+ as well (approximately 1.62% of γδ+ cells) (Figure 3.6B). More samples will need to be tested in order to confirm this finding.

Figure 3.6: Analysis of Foxp3 expression in spleens from infected TCR β and βδ knockout mice.

A. C57BL/6, TCR β KO and TCR βδ KO mice (n=8 females per strain) were infected with 2.1x10^8 CFU H305 S. pyogenes i.m for 24h. Spleens were harvested for qRT-PCR analysis of foxp3 expression. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in foxp3 expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: **p<0.01, ***p<0.001.

B. Splenocytes from GAS infected C57BL/6 mice (n=1 female) were stained for Foxp3 and γδ TCR by flow cytometry. Gated γδ+ cells (PE stained) were preliminarily examined for Foxp3 (FITC stained) expression as shown above in the FACS plot. Percentage of Foxp3-γδ+ cells is shown in pink.
Figure 3.7: qRT-PCR analysis of cytokine expression in spleens from infected TCR β and βδ knockout mice.

C57BL/6, TCR β KO and TCR δβ KO mice (n=8 females per strain) were infected with 2.1x10^8 CFU H305 S. pyogenes i.m. Spleens were harvested for qRT-PCR analysis of cytokine gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Cytokine gene expression in the spleen had switched from the profile observed in the draining lymph node with predominant differences in \textit{il-10} and \textit{ifn-\textgamma} expression between infected WT and TCR knockout mice detected (Figure 3.7). \textit{il-10} was up-regulated, with a significant difference between both TCR knockout transgenic compared to infected WT controls (Figure 3.7). \textit{il-6} and \textit{tnf-\alpha} were interestingly expressed at a much lower level than in the draining lymph node (Figure 3.7). \textit{ifn-\textgamma} was up-regulated with a significant difference between both TCR knockout transgenic compared to infected WT controls (Figure 3.7). There was no significant difference in \textit{il-17a} gene expression in either of the infected TCR knockout transgenic strains (Figure 3.7). T\textsubscript{H}2 cytokine genes such as \textit{il-4} and \textit{il-9} were only marginally up-regulated compared to infected WT controls (data not shown), suggestive that these cytokines may not have a major role in the spleen during acute GAS infection.

In summary, from the data extracted from infected TCR \textbeta\delta and \textbeta knockout transgenic mice, it would appear that \textalpha\textbeta T cells are on balance, detrimental during acute GAS infection as shown by increased survival towards lethal infection with \textit{S. pyogenes} (Figure 3.1A) and decreased bacterial burden 24h post infection (Figure 3.2A). qRT-PCR analysis confirmed the loss of \textalpha\textbeta T cells in infected TCR \textbeta\delta and \textbeta knockout transgenic mice correlated with decrease expression of transcription factors associated with T\textsubscript{regs}, T\textsubscript{H1} and T\textsubscript{H17} cells in the draining lymph node (Figure 3.4). Perhaps these cell types are key in contributing to the increased bacterial burden observed in TCR knockout transgenic mice. Cytokine gene expression suggested high expression of \textit{il-10} and low expression of \textit{ifn-\textgamma} in the draining lymph node and spleen may play a role in the decreased bacterial burden in TCR \textbeta\delta and \textbeta knockout transgenic mice compared to infected WT controls (Figure 3.2A).
3.4 Regulatory and effector αβ T cells during S. pyogenes infection

As experiments thus far have suggested that αβ T cells may be detrimental during GAS infection, two subsets of αβ T cells were further investigated to determine whether regulatory αβ T cells or effector αβ T cells were responsible for this phenotype. To achieve this, it was deemed necessary to utilise an experimental technique to specifically deplete regulatory αβ T cells, generally characterised by CD4⁺CD25⁺Foxp3⁺ cells to study if Tregs are the pathogenic cell population of αβ T cells during GAS infection.

Figure 3.8: PC61 antibody mediated depletion optimisation.

Foxp3.DTR mice were given 100µl PBS (untreated, top panel) or 50, 100, 200µl PC61 (1mg/ml, bottom panel) i.p for 24h. Spleens were harvest for flow cytometric analysis of CD4⁺CD25⁺Foxp3⁺ cells. Gated CD4⁺ cells (APC-H7 stained) were examined for CD25 (PE stained) and Foxp3 (GFP) expression as shown above. Experiment carried out with the help of Dr. R. Ingram.

Antibody mediated depletion was chosen initially to deplete regulatory T cells during acute GAS infection. Tregs express CD25, also known as the alpha chain of the IL-2...
receptor. An anti-CD25 monoclonal antibody (clone PC61) has been used successfully to deplete CD25^+ cells by blocking IL-2 binding and has previously been used in studies of tumour rejection and autoimmunity (Onizuka et al., 1999; McHugh and Shevach, 2002). This depletion has been confirmed to have high specificity towards the knockdown of T_{regs}, corresponding to nearly 80% depletion of CD4^+CD25^+Foxp3^+ through the administration of 200µg/ml PC61 antibody in my hands (Figure 3.8).

Antibody clone PC61 was used to deplete CD25^+ cells prior to infection with S. pyogenes for 24h to determine whether T_{reg} cells were responsible for the pathogenic effects of αβ T cells. Upon removal of CD25^+ cells, it was observed there was no difference in bacterial burden compared to infected mice given isotype matched control antibody (Figure 3.9A). This would suggest that, by this outcome measure, regulatory T cells play no role in S. pyogenes infection. There was however, a decrease in serum IL-6 and IL-17A in infected mice depleted of CD25^+ cells (Figure 3.9B), indicating decreased disease severity in the absence of T_{regs}. 
A. Bacterial count upon PC61 antibody mediated depletion of CD25+ cells. C57BL/6 females (n=12 per treatment group) were given 100µl PBS (untreated) or 100µl PC61 (1mg/ml) i.p 24h before and on the day of infection with $10^8$ CFU H305 S. pyogenes i.m 24h post infection, thigh, blood, spleen and liver were harvested to determine bacterial burden. No bacterial growth as found in sham infected mice (data not shown). Experiment carried out with the help of Dr. R. Ingram.

B. Serum IL-6 and IL-17A. Serum was collected from H305 infected PBS or PC61 treated mice (n=6 per treatment group) 24h post infection and serum IL-6 and IL-17A was measured by ELISA. Experiment carried out with the help of Dr. R. Ingram.
Figure 3.10: qRT-PCR analysis of transcription factor gene expression in spleen from infected PC61 treated mice.

C57BL/6 females (n=12 per treatment group) were given 100µl PBS (untreated) or 100µl PC61 (1mg/ml) i.p 24h before and on the day of infection with $10^8$ CFU H305 S. pyogenes i.m. Spleens were harvested for qRT-PCR analysis of transcription factor gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.

qRT-PCR analysis was performed on draining lymph nodes and spleens from either isotype-matched controls or PC61 antibody treated infected mice. No difference in gene expression was observed in the draining lymph node in any transcripts analysed in infected PC61 and isotype control treated mice (data not shown). A decrease in foxp3 gene expression was observed in the infected spleens of PC61 treated mice compared to isotype-matched controls as expected due to CD25+ cell and hence Tregs depletion (Figure 3.10). This reduction in Treg associated transcriptional marker was also accompanied unexpectedly by significant down-regulation in transcription factors linked to other T cell subsets, such as t-bet, gata-3 and rorγt upon PC61 treatment (Figure 3.10), indicating an effect on transcription in other T cell subsets also expressing CD25.
Figure 3.11: qRT-PCR analysis of cytokine gene expression in spleen from infected PC61 treated mice.

C57BL/6 females (n=12 per treatment group) were given 100µl PBS (untreated) or 100µl PC61 (1mg/ml) i.p 24h before and on the day of infection with $10^8$ CFU H305 S. pyogenes i.m. Spleens were harvested for qRT-PCR analysis of cytokine gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.

qRT-PCR analysis of cytokine gene expression in the spleen revealed upon treatment with PC61 before sub-lethal infection, there was a statistical difference between infected PC61 treated mice and isotype-matched control mice in all transcripts analysed (Figure 3.11). This was unexpected as the use of PC61 was originally thought only to deplete regulatory CD4+CD25+Foxp3+ cells, but it would appear that this antibody had a wider effect in the spleen.
Figure 3.12: Expression of CD25 on γδ T cells.

A. Preliminary flow cytometric staining of C57BL/6 splenocytes showing IL-17A expression of gated CD25⁺γδ TCR⁺ cell. Isotype control (Alexa fluor 647) is grey line, IL-17A stained (Alexa fluor 647) cells is black line. Experiment carried out with the help of Dr. R. Ingram.

B. Preliminary analysis of percentage of IL-17A⁺CD25⁻ and CD25⁺ γδ TCR⁺ cells (left panel) and IL-17A expression per cell (MFI) of CD25⁻ and CD25⁺ γδ TCR⁺ cells (right panel) (n=3 mice).

The results produced through the use of PC61 antibody were unexpected, as infection experiments with TCR knockout transgenic mice suggested that αβ T cells were associated with more severe pathogenesis during S. pyogenes challenge, whereas no difference was found in bacterial burden upon CD25⁺ cell depletion. CD25 may be considered a reliable marker for Tregs but it is also an activation marker for T cells in general (Tenorio et al., 2011). Therefore, it was conceivable that PC61 was depleting regulatory CD4⁺CD25⁺Foxp3⁺ cells as well as activated T effector cells, which may be protective during GAS infection, including γδ T cells as suggested in Figure 3.3B. Preliminary flow cytometric analysis of splenocytes harvested from GAS infected C57BL/6 mice indicated that γδ T cells also express CD25 (Figure 3.12A). It was also noted that this CD25⁺ population of γδ T cells were also potent producers of IL-17A (Figure 3.12B-C), previously shown to be elevated in infected TCR β knockout transgenic mice (Figure 3.1B).
From these experiments, it was concluded that the use of antibody clone PC61 was not sufficiently specific to determine whether T$_{\text{regs}}$ were beneficial or pathogenic subset of $\alpha\beta$ T cells. PC61 administration to WT mice was found to deplete both regulatory and effector cell populations, for example $\gamma\delta$ T cells, masking the true contribution of T$_{\text{regs}}$ during acute sepsis.

3.5 Specific depletion of Foxp3$^+$ cells during acute sepsis

Another method of depleting regulatory T cells was needed in replacement of PC61 antibody mediated depletion. As Foxp3 is considered to the prototypic marker of T$_{\text{regs}}$, a Foxp3 knockout murine model would be ideal for studies into the role of T$_{\text{regs}}$ during GAS infection. A strain of Foxp3 knockout mice called scurfy already exist but this strain of mice has been shown to have profound autoimmune defects and developmental problems due to the lack of functional Foxp3 and do not survive into adulthood (Brunkow et al., 2001). The use of scurfy mice therefore would not lend itself experimentally in this model of acute sepsis.

Conditional inducible Foxp3 knockout mice (Foxp3.DTR) have previously been demonstrated to have specific depletion of Foxp3$^+$ cells (Kim et al., 2007). A targeted construct containing cDNA encoding Diphtheria Toxin Receptor (DTR), followed by IRES and GFP sequences was inserted into the 3' UTR of foxp3. Therefore, cells expressing Foxp3 would also express DTR and upon the administration of Diphtheria Toxin (DT) into the mouse would lead to specific depletion of Foxp3$^+$ cells (Kim et al., 2007). The addition of a GFP tag also allowed the Foxp3$^+$ cells to be tracked by flow cytometry.

I decided to use Foxp3.DTR mice to investigate the role of Foxp3$^+$ regulatory cells during acute sepsis. The dosing regimen of DT administration was first optimised to achieve full Foxp3$^+$ knockdown in this strain of mice (Figure 3.13). Repeated daily doses of DT were given via an intra-peritoneal route into Foxp3.DTR mice for 3
consecutive days (Figure 3.13A) and percentage of Foxp3-GFP$^+$ cells of the CD4$^+$ population was analysed by flow cytometry from groups of mice culled either after 1, 2 or 3 doses (Figure 3.13B). Foxp3$^+$ cell depletion was achieved after 1 dose of DT and was maintained low by repeated doses, indicating efficient knockdown of target cells (Figure 3.13C).

**Figure 3.13: Diphtheria Toxin mediated Foxp3$^+$ cell depletion dose optimisation.**

A. Dose response of DT mediated Foxp3$^+$ cell depletion. Foxp3.DTR (n=3 males per time point) were given 100µl DT (12.5µg/ml) i.p once a day for three consecutive days as shown in the schematic.

B. Representative staining of Foxp3-GFP$^+$ and CD4$^+$ cells (APC-H7 stained) in the spleen of untreated (left) and day 1 DT treated mice (right).

C. Percentage of CD25$^+$Foxp3$^+$ cells of gated CD4$^+$ T cells upon Foxp3 depletion. Day 0 were untreated mice, day 1-3 were Foxp3 depleted, representative of the number of DT doses received. Spleen, inguinal lymph node and blood were harvested and analysed by flow cytometry for the mean percentage of CD25$^+$Foxp3$^+$ cells of gated CD4$^+$ T cells as represented.
Figure 3.14: FoXP3 recovery post Diphtheria Toxin mediated FoXP3⁺ cell depletion.

A. Recovery of FoXP3⁻ cells post DT mediated depletion. FoXP3.DTR (n=4 males per time point) were given 100µl DT (12.5µg/ml) i.p once a day on day 0 and 1 as shown in the schematic.

B. Percentage of CD25⁺FoxP3⁺ cells of gated CD4⁺ T cells post FoxP3 depletion. FoxP3.DTR (n=4 males per time point) were given 100µl DT (12.5µg/ml) i.p as shown in the schematic A. Day 0 were untreated mice, day 2-10 were groups of mice allowed to recover their FoxP3⁺ cells. Spleen, inguinal lymph node and blood were harvest and analysed by flow cytometry for the mean percentage of CD25⁺FoxP3⁺ cells of gated CD4⁺ cells represented.

Having determined that DT mediated FoxP3⁺ depletion was achieved within 24h, it was next necessary to investigate how long this effect would last and how quickly the FoxP3⁺ cell population would recover post DT treatment. FoXP3.DTR mice were all given 2 daily doses of DT and then every two days thereafter, groups of mice were culled and percentage of FoxP3-GFP⁺ cells analysed by flow cytometry (Figure 3.14A). Knockdown of FoxP3-GFP⁺ cells was achieved as expected two days after DT treatment (Figure 3.14B). However, the percentage of FoxP3-GFP⁺ cells of the CD4⁺ population rebounded quickly and was near WT levels by day 8 (Figure 3.14B). This indicated that in order to maintain FoxP3⁺ cell depletion, DT would need to be given every other day to keep FoxP3⁺ cell levels low.
3.6 Pathogenic effects of Foxp3\(^+\) cells during acute \textit{S. pyogenes} infection

Figure 3.15: Effect of DT mediated Foxp3\(^+\) cell depletion during acute \textit{S. pyogenes} infection.

A. Bacterial counts after 24h infection. Foxp3.DTR mice were given 100µl PBS or DT i.p (n=9 females per treatment group) once every day for 2 days before and on the day of infection with 4.2x10\(^8\) CFU H305 or PBS (sham infection) \textit{S. pyogenes} i.m for 24h. Bacterial counts in the thigh, blood, spleen and liver was determined. No bacterial growth was detected in sham-infected mice (data not shown). Mean and SEM represented and 1-way ANOVA statistical test was carried out and any significant differences indicated: *p<0.05.

B. Serum ELISA analysis of IL-6 and IL-17A. Serum was collected after 24h of infection and concentration of IL-6 and IL-17A measured by ELISA. 1-way ANOVA statistical analysis was carried out and any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Once the DT mediated depletion of Foxp3+ cells was optimised, Foxp3.DTR mice were given two daily doses of DT to deplete Foxp3+ cells prior and also one extra dose on the day of *S. pyogenes* infection to study whether Tregs are pathogenic during acute sepsis. Upon removal of Foxp3+ cells, there was decreased systemic bacterial burden in Foxp3.DTR mice after 24h of infection (Figure 3.15A). There was a slight decrease in numbers of recovered bacteria in the thigh; the site of infection in the DT treated mice (Figure 3.15A). This decrease in bacterial counts in DT treated mice was accompanied with a reduction of serum IL-6 compared to infected PBS treated mice (Figure 3.15B). The removal of Foxp3+ cells also resulted in increased serum IL-17A and decreased levels of IFN-γ (Figure 3.15B). This implies the decreased bacterial burden may be due to increased IL-17A production and this pro-inflammatory cytokine may be protective and IFN-γ may be pathogenic as suggested in experiments with infected TCR δ knockout transgenic mice (Figure 3.3C).

![Figure 3.16: Initial analysis of pathology of infected thigh muscle upon removal of Foxp3+ cells.](image)

Foxp3.DTR mice were given 100µl PBS or DT i.p (n=1 female per treatment group) once every day for 2 days before and on the day of infection with 4.2x10⁸ CFU H305 *S. pyogenes* or PBS (sham) i.m for 24h. Thigh muscle was snap-frozen in is-pentane and stored at -20°C. 5µm sections were cut using a cryostat for preliminary Gram staining (A) and staining with anti-Group A carbohydrate by Immunohistochemistry (B). Images were taken at 10X objective.
The slight decreased number of recovered bacteria at the site of infection in DT treated Foxp3.DTR mice was mirrored in a preliminary examination of snap frozen thigh tissue Gram-stained for the presence of Gram-positive bacterium (Figure 3.16A). Gram-positive bacteria, stained purple were identified in PBS treated mice infected with *S. pyogenes* (Sham infected), whereas there was less staining in DT treated infected mice (Figure 3.16A). There was no staining in un-infected sham (sham sham) Foxp3.DTR mice (Figure 3.16A). This result was also mirrored by IHC staining for group A carbohydrate expressed on the surface of *S. pyogenes* (Figure 3.16B). Sham infected thigh tissue contained the most GAS, stained brown compared to sham sham and DT infected treatment groups (Figure 3.16B). Due to the small number of samples, further work will need to be carried out to confirm these findings.

![Sham sham, Sham infected, DT infected](image)

**Figure 3.17: Preliminary histology of infected thigh muscle upon removal of Foxp3+ cells.**

Foxp3.DTR mice were given 100µl PBS or DT i.p (n=1 female per treatment group) once every day for 2 days before and also on the day of infection with 4.2x10^8 CFU H305 *S. pyogenes* or PBS (sham) i.m for 24h. Thigh muscle was snap-frozen in iso-pentane and stored at -20°C. 5µm sections were cut using a cryostat before preliminary H&E staining (A) and anti-MPO by IHC (B). Images were taken at 10X objective.

Preliminary H&E staining of thigh muscle harvested post *S. pyogenes* infection revealed intensive staining, indicative of increased inflammation in PBS treated Foxp3.DTR mice infected with *S. pyogenes* (Sham infected) (Figure 3.17A) compared to uninfected PBS treated (sham sham) and infected DT treated mice. Pilot
IHC analysis also showed increased MPO staining (brown) in sham infected thigh tissue (Figure 3.17B), reflective of increased neutrophil activation in PBS treated Foxp3.DTR mice infected with \textit{S. pyogenes}. Infiltrating activated neutrophils may contribute to the increased inflammation in infected Foxp3.DTR with their Foxp3$^+$ cells intact. Mice with Foxp3$^-$ cells depleted showed signs of less inflammation and neutrophil activation (Figure 3.17A).

![Graph A](image)

**Figure 3.18: Survival outcome post lethal dose of \textit{S. pyogenes} upon removal of Foxp3$^+$ cells.**

A. Survival curve of infected PBS (Foxp3$^+$) or DT (Foxp3$^-$) treated Foxp3.DTR mice (n=8 females per group). Mice were given 100µl PBS or DT once a day for 2 days before and on the day of infection with 7x10$^7$ CFU H305 \textit{S. pyogenes} (WT) and monitored for defined end points and culled. PBS and DT treatment was continued every day after infection.

B. Serum was collected 8h post infection during the survival experiment. Concentration of serum IL-6 and IL-17A determined by ELISA. Mean and SEM are represented and 1-way ANOVA statistical test was carried out, no significant differences were found.

As removal of Foxp3$^+$ cells prior to sub-lethal infection with \textit{S. pyogenes} resulted in decreased bacterial burden (Figure 3.15A), this was suggestive that Foxp3$^+$ cells may be pathogenic during GAS infection. The survival outcome of Foxp3.DTR mice receiving a lethal dose of \textit{S. pyogenes} treated with either PBS or DT was next determined (Figure 3.18A). Disappointingly, no significant difference in survival
outcome was noted in Foxp3.DTR mice with or without Foxp3⁺ cells (Figure 3.18A). Serum IL-6 and IL-17A levels 8h post infection were similar in infected PBS and DT treated mice (Figure 3.18B). This data implies that Foxp3⁺ cells may not have a significant role during long-term lethal bacterial challenge but may only be pathogenic during the early stages of infection.

3.7 Gene expression changes in draining lymph node and spleen upon removal of Foxp3⁺ cells during acute GAS infection

![Graphs showing gene expression changes](image)

Figure 3.19: qRT-PCR analysis of transcription factor gene expression in DLN from infected DT treated mice.

Foxp3.DTR mice were either given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before and on the day of infection with 5x10⁷ CFU H305 S. pyogenes or PBS (sham) i.m for 24h. DLN were harvested for qRT-PCR analysis of transcription factor gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Draining lymph nodes and spleens were harvested for qRT-PCR analysis to identify any changes in gene expression in infected PBS and DT treated infected Foxp3.DTR mice. *foxp3* gene expression was statistically decreased in draining lymph nodes from DT treated mice compared to PBS treated infected mice (Figure 3.19). Interestingly, *gata-3* expression was also significantly down-regulated in the DT treatment group (Figure 3.19). Whether T\(_H\)2 cells also express Foxp3 is debatable (Wang et al., 2010b).

Cytokine gene expression in the draining lymph node was also investigated by qRT-PCR analysis. All transcripts studied were significantly up-regulated in general in response to infection with *S. pyogenes* compared to sham-treated mice (data not shown). The only statistical difference between infected PBS and DT treated mice was *il-9* expression (Figure 3.20). It is unclear whether IL-9, a known T\(_H\)2 cytokine is important during GAS infection, despite *gata-3*, the transcriptional marker for T\(_H\)2 cells being down-regulated upon DT treatment during infection (Figure 3.19).

![Figure 3.20: qRT-PCR analysis of *il-9* gene expression in DLN from infected DT treated mice.](image)

Foxp3.DTR mice were either given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before infection with 5x10\(^7\) CFU H305 *S. pyogenes* or PBS (sham) i.m for 24h. DLNs were harvested for qRT-PCR analysis of *il-9* gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in *il-9* expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
qRT-PCR analysis of transcription factor genes in the spleen mirrored similar expression pattern as in the draining lymph node for *foxp3* and *rorγ* (Figure 3.21). However, there was no difference in *gata-3* expression upon removal of Foxp3<sup>+</sup> cells in the spleen, previously shown to be down-regulated in the draining inguinal lymph node (Figure 3.21). *t-bet* expression was significantly elevated in the spleen in DT treated mice relative to PBS treated controls (Figure 3.21). Depletion of Foxp3<sup>+</sup> cells may have removed T<sub>reg</sub> mediated suppression over T<sub>H1</sub> cells to allow this subset to proliferate.

![Graphs showing expression levels of Foxp3, t-bet, gata-3, and rorγ in spleen samples](image)

Figure 3.21: qRT-PCR analysis of transcription factor gene expression in spleens from infected DT treated mice.

Foxp3.DTR mice were either given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before and on the day of infection with 5x10<sup>7</sup> CFU H305 <i>S. pyogenes</i> or PBS (sham) i.m for 24h. Spleens were harvested for qRT-PCR analysis of transcription factor gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Figure 3.22: qRT-PCR analysis of cytokine expression in spleens from infected DT treated mice.

Foxp3.DTR mice were either given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before infection with 5x10^7 CFU H305 or PBS (sham) i.m for 24h. Spleens were harvested for qRT-PCR analysis of cytokine gene expression changes. Infected samples were normalized to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Gene expression of cytokines in the spleen of either sham or GAS infected Foxp3.DTR mice revealed that upon removal of Foxp3\(^{+}\) cells, there was a significant decrease in \textit{il-17a} and \textit{il-4} transcription compared to PBS treated mice infected with \textit{S. pyogenes} (Figure 3.22). This is in contrast to the belief that T\(_{\text{regs}}\) suppress T\(_{\text{H17}}\) and T\(_{\text{H2}}\) cytokine responses and may indicate depletion of Foxp3\(^{+}\)\(\gamma\delta\) cells as discussed above. Down-regulation of \textit{il-17a} expression was in contrast to high levels of IL-17A protein in the serum of GAS infected DT treated mice (Figure 3.15B). On the other hand, \textit{il-9} expression was again elevated in the spleen of infected DT treated mice (Figure 3.22).

Overall, from the qRT-PCR analysis of DT or PBS treated Foxp3.DTR mice infected with \textit{S. pyogenes}, there was only a moderate change in gene expression in either the draining lymph node or spleen. Interestingly, increased \textit{il-9} expression was consistently observed in both the draining lymph node and spleen upon DT treatment. Whether this cytokine is responsible for the increased protection during acute \textit{S. pyogenes} infection is unknown and will require further investigation.

### 3.8 Cytokine responses mediated by Foxp3\(^{+}\) cells during GAS infection

Depletion of Foxp3\(^{+}\) cells prior to acute \textit{S. pyogenes} infection resulted in increased IL-17A and decreased IFN-\(\gamma\) serum levels (Figure 3.15B), indicating that the balance of T\(_{\text{eff}}\) cytokine production may be controlled by Foxp3\(^{+}\) cells following \textit{S. pyogenes} infection. \textit{In vitro} and \textit{ex vivo} cytokine responses were studied further in infected Foxp3.DTR mice to determine the extent of T\(_{\text{reg}}\) mediated control over cytokine production.
Figure 3.23: Cytokine production post heat-killed *S. pyogenes* stimulation upon removal of Foxp3\(^+\) cells.

Foxp3.DTR mice (n=5 mice per treatment group) were given 100µl PBS or DT (12.5µg/ml) for 2 days before spleens were harvested. 10\(^6\) splenocytes were stimulated with heat-killed H305 WT *S. pyogenes* isolate, PMA/Ionomycin (positive control) and media only (negative control) for 24h. Supernatant was collected and CBA was carried out to measure the concentration of IFN-γ, TNF, IL-17A, IL-4, IL-9 and IL-10. Mean and SEM of concentrations minus background from media stimulated cells are represented and 1-way ANOVA statistical analysis was carried out and any significant differences indicated: ***p<0.001.

Multi-cytokine analysis was carried out on heat-killed *S. pyogenes* stimulated splenocytes harvested from Foxp3.DTR mice given PBS to maintain Foxp3\(^+\) cells or DT to deplete Foxp3\(^+\) cells. TNF, IL-4, IFN-γ, IL-9, IL-10 and IL-17A levels were measured in the culture supernatant by Cytometric Bead Assay (CBA) (Figure 3.23). Stimulation of splenocytes devoid of Foxp3\(^+\) cells in general resulted in increased production of all cytokines measured (Figure 3.23). IFN-γ production was significantly unleashed in the absence of Foxp3\(^+\) cells (Figure 3.23). This is in line with evidence that Foxp3\(^+\) T\(_{\text{reg}}\) cells can suppress proliferation and cytokine production from effector T cells (Thornton and Shevach, 1998).
Results from *in vitro* stimulation of splenocytes in the presence of heat-killed WT *S. pyogenes* were compared with *ex vivo* cytokine flow cytometric analysis of draining lymph nodes and spleens harvested from Foxp3.DTR mice either given PBS or DT before 24h infection with H305. Flow cytometric analysis of cell types in the draining inguinal lymph node revealed that only Foxp3^+^ cells were significantly reduced upon the administration of DT as expected (Figure 3.24) and there was no obvious changes in the percentage of IFN-γ^+^ or IL-17A^+^ cells of the lymphocyte population (Figure 3.24).

![Cell types present in DLN upon removal Foxp3^+^ cells during S. pyogenes infection.](image)

**Figure 3.24**: Cell types present in DLN upon removal Foxp3^+^ cells during *S. pyogenes* infection.

Foxp3.DTR mice were either given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before infection with 4.2x10^8 CFU H305 *S. pyogenes* or PBS (sham i.m for 24h). Draining inguinal lymph nodes were harvested for flow cytometric analysis of cell types and IL-17A and IFN-γ production. Mean and SEM of absolute numbers (top) of cell types and percentage of IFN-γ^+^ and IL-17A^-^ of lymphocytes are represented. 1-way ANOVA statistical analysis was carried out on absolute numbers of cell types in the lymph node and any significant differences indicated: *p<0.05. Kruskal-Wallis statistical analysis was carried out on percentage of cytokine^-^ cells of lymphocytes data, no significant difference was found.
Figure 3.25: IL-17A and IFN-γ production in DLN upon removal of Foxp3+ cells during acute sepsis.

Foxp3.DTR mice were given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before and on the day of infection with 4.2x10^8 CFU H305 S. pyogenes or PBS i.m for 24h. DLN were harvested for flow cytometric analysis of IL-17A (A) and IFN-γ (B) production by various cell types. Mean and SEM of percentage of cytokine+ cells of lymphocytes (top) and absolute numbers of cytokine+ cells (bottom) are represented. 1-way ANOVA statistical analysis was carried out on absolute numbers and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001. Kruskal-Wallis statistical analysis was carried out on percentage of cytokine+ cells of lymphocytes data and of total cell population and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.

Further analysis of IFN-γ+ or IL-17A+ producing cell types in the draining lymph node showed that upon S. pyogenes infection, IFN-γ responses were paradoxically more likely to be modulated in the absence of Foxp3+ cell mediated control (Figure 3.25B) with CD8+ and CD4+ cells of the lymphocyte population producing less IFN-γ upon DT treatment during infection (Figure 3.25B). IL-17A responses were more
robust and upon Foxp3$^+$ cell depletion with little difference between infected mice with or without Foxp3$^+$ cells. It is interesting to see that a very small percentage of Foxp3$^+$ cells were found to produce either IL-17A or IFN-γ, cytokines typical connected with T$_{H}$$^{17}$ and T$_{H}$$^{1}$ cells respectively (Figure 3.25).

Figure 3.26: Cell types present in spleen upon depletion of Foxp3$^+$ cells during acute sepsis.

Foxp3.DTR mice were either given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before infection with 4.2x10$^8$ CFU H305 or PBS (sham) i.m for 24h. Spleens were harvested for flow cytometric analysis of cell types and IL-17A and IFN-γ production. Mean and SEM of absolute numbers (top) of cell types and percentage of IFN-γ$^+$ and IL-17A$^+$ of lymphocytes are represented. 1-way ANOVA statistical analysis was carried out on absolute numbers of cell types in the lymph node and any significant differences indicated: *p<0.05. Kruskal-Wallis statistical analysis was carried out on percentage of cytokine$^+$ cells of lymphocytes data, no significant difference was found.
Foxp3.DTR mice were either given 100µl PBS or DT i.p (n=8 females per treatment group) once every day for 2 days before and on the day of infection with 4.2x10⁸ CFU H305 S. pyogenes or PBS (sham) i.m for 24h. Spleens were harvested for flow cytometric analysis of IL-17A (A) and IFN-γ (B) production by various cell types. Mean and SEM of percentage of cytokine⁺ cells of lymphocytes (top) and absolute numbers of cytokine⁺ cells (bottom) are represented. 1-way ANOVA statistical analysis was carried out on absolute numbers and any significant differences indicated: *p<0.05, ***p<0.001. Kruskal-Wallis statistical analysis was carried out on percentage of cytokine⁺ cells of lymphocytes data and of total cell population and any significant differences indicated: *p<0.05, ***p<0.001.

Cytokine production in the spleen of S. pyogenes infected Foxp3.DTR mice with or without Foxp³⁺ cells also exhibited a similar result to the draining lymph node. Only Foxp³⁺ cell were significantly reduced upon DT treatment (Figure 3.26). An expansion in the percentage of NK cells in the lymphocyte population during infection and DT treatment was also observed (Figure 3.26). There was no significant
change in the percentage of cell types in the spleen (Figure 3.26), or the percentage of IL-17A or IFN-γ producing lymphocytes (Figure 3.26).

In contrast to the draining lymph node, where dominant changes in IFN-γ production were observed, significant differences in IL-17A production in γδ T and NK cells upon the removal of Foxp3+ cells during GAS infection were characterised (Figure 3.27A). Yet again, decrease in the percentage of IFN-γ+ CD4+ T cells of the lymphocyte population was seen upon infection in Foxp3+ depleted mice compared to infected PBS treated controls (Figure 3.27B).

In conclusion, from flow cytometric analysis of IL-17A and IFN-γ production during acute sepsis in mice with or without Foxp3+ cells, there was a differential role of each of these cytokines. Increased IL-17A response from γδ and NK cells in the spleen and reduced IFN-γ responses from CD8+ and CD4+ cells in the draining inguinal lymph node upon depletion of Foxp3+ cells during acute S. pyogenes may be responsible for the decreased bacterial burden in Foxp3.DTR mice with their Foxp3+ cells depleted.

To further understand whether suppressed IL-17A responses in the spleen is involved in the pathogenic nature of Foxp3+ cells during acute sepsis, IL-17A knockout transgenic mice (Nakae et al., 2002) were utilised to determine if this pro-inflammatory cytokine is protective during GAS infection.
Figure 3.28: Bacterial outcome post *S. pyogenes* infection upon depletion of IL-17A.

**A.** Survival curve of infected C57BL/6 (*n*=7 females) and IL-17A−/− mice (*n*=6 females). Mice were given 6x10⁸ CFU H305 i.m and monitored for defined end points and culled.

**B.** Bacterial counts after 24h infection. C57BL/6 and IL-17A KO mice (*n*=8 females per strain) were infected with 1.4x10⁹ CFU H305 *S. pyogenes* i.m. Bacterial counts in the thigh, blood, spleen and liver was determined. Mean and SEM are represented and 1-way ANOVA statistical test was carried out, no significant differences was found.

**C.** Serum ELISA analysis of IL-6. Serum was collected after 24h of infection and concentration of IL-6 measured by ELISA. No significant difference was found.
There was no difference in survival of IL-17A knockout mice compared to infected WT controls after a lethal dose of WT S. pyogenes (Figure 3.28A). Bacterial counts from either WT or IL-17A knockout mice after 24h infection with H305 revealed a trend of increased bacterial burden, as shown by higher number of recovered bacteria in the blood, spleen and liver in IL-17A knockout mice relative to infected WT controls (Figure 3.28B). This was matched with a slight increase in serum IL-6 in infected IL-17A knockout mice (Figure 3.28C). However, no difference was found in serum IFN-γ in either strain of mice (data not shown).

In summary, it would appear there is only weak evidence to confirm a protective role of IL-17A during acute S. pyogenes infection, as demonstrated here by increased systemic bacterial burden in IL-17A knockout mice compared to WT controls.
3.9 Discussion

From the data presented in this chapter, it can be concluded that some T cell subsets are detrimental during acute *S. pyogenes* infection. This was found in particular for αβ T cells and γδ T cells, as shown by increased survival in TCR βδ knockout mice post lethal GAS infection (Figure 3.1). This was further confirmed by decreased bacterial counts in the thigh, spleen, liver and blood after 24h of infection with WT *S. pyogenes* (Figure 3.2B) and decreased levels of IL-6 in the serum (Figure 3.2C) in TCR βδ knockout mice.

qRT-PCR analysis of draining lymph node and spleen harvested from infected TCR β and βδ knockout mice revealed some interesting changes in gene expression pattern. Removal of αβ T cell subset in TCR β knockout mice resulted in the down-regulation of *foxp3* and *t-bet* gene expression (Figure 3.4). This was not surprising as these transcription factors are associated with αβ T cells. However, it was interesting to note that upon depletion of γδ T cells in TCR βδ knockout mice also further decreased *foxp3* expression (Figure 3.4 & 3.6A). The nature of regulatory γδ T cells in human PBMCs has previously been published (Kühl *et al.*, 2010), although this study stated this population was Foxp3-. This is in contrast to Cassetti and colleagues (Casetti *et al.*, 2009), who found a particular subset of γδ T cells (Vδ2) expressing Foxp3 and fully capable of suppressing αβ T cells. Flow cytometry analysis of *S. pyogenes* infected C57BL/6 mice revealed a very small percentage of γδ T cells expressing Foxp3 (Figure 3.6B), whether these regulatory γδ T cells have a role during acute sepsis is still unclear.

The lack of αβ and γδ T cells in infected TCR βδ knockout mice also demonstrated a down-regulation in some key cytokines associated with regulatory subsets. For example, *il-10* was decreased in TCR βδ knockout mice compared to infected WT mice in the draining lymph node (Figure 3.5), whereas it was significantly up-regulation in the spleen (Figure 3.7). The precise cellular source of *il-10* transcription is unknown as only global expression in the target organ was examined and IL-10 can be made by several cell-types such as T cells, B cells and monocytes (Vieira *et al.*, 2010).
2004; Mosmann et al., 1990; O'Garra et al., 1990; de Waal Malefyt et al., 1991). Therefore, this differential expression pattern may be due to the removal of αβ+ and/or γδ+ regulatory T cells in the draining lymph node, which are known to express IL-10 (Roncarolo et al., 2001; Kühl et al., 2010). The removal of suppressive control by αβ+ and/or γδ+ regulatory T cells in the spleen may explain the unleashed expression of il-10 by an unknown innate cell type, such as macrophages (Zhang et al., 2011).

Overall, this set of data suggests that T cells may be contributing to the hyper-inflammation presented in septic patients. This is in line with published data using the TCR β knockout mice in CLP models of poly-microbial sepsis exhibiting increased resistance to mortality (Enoh et al., 2007). In the same paper, the authors found no difference in mortality in TCR δ knockout transgenic mice compared to WT controls post CLP surgery (Enoh et al., 2007), which is in line to my finding using the same strain of mice infected with a lethal dose of S. pyogenes (Figure 3.3A). However, there was a general trend of increased recovered bacteria 24h post infection in TCR δ knockout mice compared to infected WT controls (Figure 3.3B), which does not agree with data from Enoh and colleagues. Decreased bacterial counts in CLP treated TCR δ knockout mice was detected in their model (Enoh et al., 2007). The different model of induced sepsis being utilised could explain this discrepancy.

The precise subset of pathogenic αβ+ T cells was next examined through the depletion of regulatory T cells with anti-CD25 antibody (clone PC61). This antibody was used for most early studies investigating T regs due to high specificity of depletion (Onizuka et al., 1999; McHugh and Shevach, 2002). This was also true in my hands as well (Figure 3.8). Disappointingly, the depletion of CD25+ cells prior to sub-lethal S. pyogenes infection did not yield any significant difference in bacterial counts between PC61 or isotype matched control antibody pre-treated mice (Figure 3.9A). There was a slight decrease in serum IL-6 and IL-17A in the PC61 treated mice in relation to isotype control treated mice (Figure 3.9B). qRT-PCR analysis confirmed that PC61 treatment decreased foxp3 gene expression as envisaged (Figure 3.10) and also all other transcripts examined (Figure 3.10-11). It was deduced the PC61 antibody might also be depleting the effector T subsets, such as γδ T cells along with
the regulatory population. This was confirmed by flow cytometric analysis showing CD25\(^+\) \(\gamma\delta\) T cells were also high producers of IL-17A (Figure 3.12). CD25\(^+\) \(\gamma\delta\) T cells have previously been identified as potent IL-17 producers in mice (Shibata et al., 2008). It was speculated that this population of cells might be responsible for serum IL-17A production observed in lethally infected TCR \(\beta\) knockout mice (Figure 3.1B).

A more specific protocol to deplete regulatory cells was required to understand whether \(\alpha\beta\) T\(_{\text{regs}}\) are pathogenic in this model of acute sepsis. Inducible conditional Foxp3.DTR mice were used due to specific depletion of Foxp3\(^+\) cells upon the administration of Diphtheria Toxin (Kim et al., 2007). The depletion regimen in this transgenic model was optimised (Figure 3.13-14), before sub-lethal infection with \(S.\) pyogenes in mice with either their Foxp3\(^+\) cell population intact or depleted. Removal of Foxp3\(^+\) cells during sub-lethal GAS infection resulted in decreased systemic bacterial burden (Figure 3.15A). This correlated with a decrease in serum IL-6 and IFN-\(\gamma\) (Figure 3.15B), whereas serum IL-17A was elevated upon the depletion of Foxp3\(^+\) cells in infected mice (Figure 3.15B), suggestive that the presence of Foxp3\(^+\) cells might be detrimental during acute GAS infection.

Removal of Foxp3\(^+\) cells appeared to limit the bacterial spread to the thigh, the site of infection (Figure 3.15A). Staining of snap-frozen thigh muscle revealed the presence of Gram-positive bacterium in infected mice with Foxp3\(^+\) cells with much less being detected in infected mice devoid of Foxp3\(^+\) cells (Figure 3.16A-B). Less inflammation was observed in infected DT treated mice (Figure 3.17A) due to perhaps enhanced bacterial containment and clearance, although not mediated by neutrophils as shown by decreased MPO IHC staining (Figure 3.17B). This is in contrast to published data now emerging relating to T\(_{\text{reg}}\) suppression of innate immune populations such as neutrophils. Richards and colleagues (Richards et al., 2010) have shown T\(_{\text{reg}}\) can limit neutrophils responses in the context of a murine tumour model. However, this mechanism could not be extrapolated to this model of acute sepsis and further quantification of MPO will need to be carried out to support my findings.
Despite a statistical difference in bacterial burden between mice containing Foxp3$^+$ cells and those with this cell population depleted during a sub-lethal infection with *S. pyogenes*, lethal infection showed no difference in survival between the two treatment groups (Figure 3.18). Perhaps, the sheer long-term effects of GAS infection overwhelm the immune response and therefore make no difference if Foxp3$^+$ cells are present or not during lethal infection. To date, the precise role of T$_{reg}$s during invasive *S. pyogenes* infection has not been fully addressed although my data suggests that T$_{reg}$s are detrimental. T$_{reg}$s were found not to be induced during intra-nasal infection with *S. pyogenes* (Costalonga *et al.*, 2008). However, this was based on a model of *S. pyogenes* colonisation in the Nasal Associated Lymphoid Tissue (NALT) and not associated with invasive disease.

The role of T$_{reg}$s during infection remains controversial and has been reviewed by Belkaid & Tarbell, (Belkaid and Tarbell, 2009). T$_{reg}$s are pathogenic during the early stage of *M. tuberculosis* infection by limiting effector CD4$^+$ and CD8$^+$ T cells to prolong the bacterial expansion phase (Shafiani *et al.*, 2010). On the other hand, T$_{reg}$s may be beneficial during infection by limiting collateral damage caused by the ensuing immune response (Lund *et al.*, 2008).

From my finding presented in this chapter, the pathogenic role of T$_{reg}$s during acute *S. pyogenes* may be due to limiting effector functions, reminiscent of the mechanism proposed in a tuberculosis model of infection (Shafiani *et al.*, 2010). qRT-PCR analysis of draining lymph nodes and spleens from infected PBS or DT treated mice revealed very modest changes in cytokine gene expression changes, contrary to difference in pathogenesis being observed. *il-9* expression was significantly up-regulated in both the draining lymph node and spleen (Figure 3.20 & 3.22). IL-9 has been suggested to protect mice from Gram-negative bacterial infection (Grohmann *et al.*, 2000). Whether IL-9 or perhaps even T$_{H9}$ cells have a role during acute *S. pyogenes* infection has never previously been investigated and will be the target for future experiments.

*iil-17a* gene expression was up-regulated and *ifn-γ* expression was down-regulated in the spleen of infected mice devoid of Foxp3$^+$ cells compared to their infected PBS treated counterparts (Figure 3.22). This was suggestive for protective and pathogenic
roles for IL-17A and IFN-γ respectively during sepsis that may contribute to the observed decreased systemic bacterial burden in GAS infected DT Foxp3.DTR mice.

*In vitro* stimulation of splenocytes from either PBS or DT pre-treated Foxp3.DTR mice with heat-killed WT *S. pyogenes* revealed that upon removal of Foxp3⁺ cells, there was increased production of TNF in the culture supernatant (Figure 3.23). IFN-γ, IL-9, IL-10 and IL-4 were also increased in this splenocyte treatment group although not significant (Figure 3.23). IL-17A was only marginally lower than stimulated PBS pre-treated splenocytes (Figure 3.23). This would suggest that depletion of Foxp3⁺ cells has effects on *in vitro* cytokine production.

Upon *in vivo* *S. pyogenes* infection of either PBS or DT pre-treated Foxp3.DTR transgenic mice, draining lymph nodes or spleens showed no difference in the percentage of either IL-17A or IFN-γ producing cells of the lymphocyte population (Figure 3.24 & 3.26). In the inguinal draining lymph node, IFN-γ responses were paradoxically suppressed in the absence of Foxp3⁺ cells during infection, particular in CD4⁺ and CD8⁺ T cell populations (Figure 3.25). The opposite was true in the spleen, where IL-17A responses were unleashed in γδ T and NK cells upon removal of Foxp3⁺ control during acute GAS infection (Figure 3.27). This set of data correlated with serum ELISA results (Figure 3.15B), where increased IL-17A levels and decreased IFN-γ levels in infected DT treated mice was measured. This is suggestive of differential expression of these two pro-inflammatory cytokines by either adaptive or innate cell types may be contributing to the decreased bacterial burden detected in the GAS infected Foxp3⁺ depleted mice.

IFN-γ has previously been suggested to be limit dissemination of *S. pyogenes* from the NALT to the draining lymph node as shown through the use of IFN-γ⁻/⁻ knockout mice (Hyland *et al.*, 2009). Mice depleted of their NK cell population exhibit increased resistance to fatal infection with GAS and slower progression to severe disease (Goldmann *et al.*, 2005a). This phenotype was also accompanied with decreased serum levels of IFN-γ, associated with the so-called cytokine storm induced during sepsis. This highlights that IFN-γ is both protective and pathogenic depending on the experimental model. Upon depletion of Foxp3⁺ cells in Foxp3.DTR
mice, there may be some unknown regulatory mechanism to suppress CD4$^+$ and CD8$^+$ T cells from producing IFN-γ to decrease bacterial burden in infected DT treated mice. In this model, it would appear that IFN-γ is pathogenic, consistent with evidence from Goldmann and colleagues (Goldmann et al., 2005a).

The true effect of IL-17A during acute sepsis was further examined through the use of IL-17A knockout transgenic mice (Nakae et al., 2002). During lethal infection with WT \textit{S. pyogenes} in WT or IL-17A knockout mice, no difference in the survival between these two strains of mice was found (Figure 3.28A). However, sub-lethal infection with GAS in WT or IL-17A knockout mice suggested a trend of increased bacterial burden in IL-17A knockout transgenic (Figure 3.28B). These findings are reminiscent of \textit{in vivo} systemic versus local \textit{S. aureus} infection in IL-17A$^{-/-}$ mice, whereby no difference in bacterial clearance was noted during systemic infection between knockout and WT strains (Henningsson et al., 2010). However, local infection of the knee-joint with \textit{S. aureus} showed that IL-17A$^{-/-}$ mice exhibited severe arthritis and reduced bacterial clearance compared to infected WT mice (Henningsson et al., 2010). This shows, perhaps IL-17A may be redundant during the lethal \textit{S. pyogenes} infection but may have a protective role during the early stages of local infection.

The protective nature of IL-17A during other infectious disease models has been extensively demonstrated. For example, during \textit{S. pneumoniae} or \textit{K. pneumoniae} infection, T Helper 17 cells are protective due to enhanced neutrophil recruitment aiding bacterial clearance (Zhang et al., 2009c; Ye et al., 2001a). IL-17A is produced by γδ T cells during \textit{E. coli} infection and upon cytokine depletion, increased bacterial burden was observed (Shibata et al., 2007). These papers support my findings that this pro-inflammatory cytokine may be also protective during the early stages of acute \textit{S. pyogenes} infection.

Overall, through the extensive use of various transgenic models, there is some evidence that γδ T cells may be beneficial during the early stages of GAS infection. I have shown that αβ T cells are pathogenic during acute \textit{S. pyogenes} infection. In particular, the Foxp3$^+$ regulatory subset of αβ T cells are detrimental to the
pathogenic outcome during the early stages of infection. Removal of Foxp3\(^+\) cells increased IL-17A and decreased IFN-\(\gamma\) levels in the serum and changed the cytokine expression pattern of \(\gamma\delta\), NK, CD8\(^+\) and CD4\(^+\) T cells, suggesting differential protective and pathogenic roles for these pro-inflammatory cytokines during sepsis.
Chapter 4

4 The role of MHC class I dependent mechanisms during acute S. pyogenes infection

4.1 Introduction

Adaptive immunity has evolved to efficiently clear and limit infection against diverse pathogenic microorganisms. As mediators of adaptive immunity, T cells can be defined based on their expression of their co-receptors, CD4 or CD8. CD4+ T cells are broadly associated with immune responses directed against extracellular bacterial infection due to their ability to facilitate other immune cell effector mechanisms, such as induce B cell isotype class switching and maturation and activation of cytotoxic T cells (CTLs) and macrophages (Berzins et al., 1988; Wagner and Röllinghoff, 1978; Cantor and Boyse, 1975). On the other hand, upon activation with antigen, CD8+ T cells can differentiate into CTLs with effector functions generally associated with responses against viral or intracellular pathogens that require the infected cell to be killed. During the early 1980s when the routes of antigen processing were being elucidated, it became clear that while the MHC class II/CD4 axis of immunity was primarily functioning to sample antigens phagocytosed from the external milieu, MHC class I/CD8 immunity was sampling endogenous peptides loaded in the endoplasmic reticulum, as would be the case in viral infection (Townsend et al., 1986).

Since the first reports during the early 1990s of mice deficient in either CD8 or β2-microglobulin, it has been evident that this component of adaptive immunity is highly significant (though often not the sole mechanism) in adaptive immunity to intracellular pathogen infections (Bender et al., 1992; Harty et al., 1992; Fung-Leung et al., 1991). This is strong evidence that CD8+ T cells are key in mediating immunity
during viral infections. CD8⁺ T cells upon activation, differentiate into CTLs and exert anti-viral effects, such as the production of IFN-γ or TNF-α. This has been shown to control viral replication as demonstrated in a mouse model of Hepatitis B viral infection (Guidotti et al., 1996). A role for CD8⁺ T cells has also been implicated in other viral infection. For example, effector CTLs are activated infection with Herpes Simplex Virus (HSV)-2; however in this model, CD4⁺ T cells are also required for CD8⁺ T cell migration to the site of infection (Nakanishi et al., 2009). This highlights the importance of both types of T cells in mounting an effective immune response. While CTLs was implicated as protective in this study, other groups have found the CD8⁺ T cell mediated response to be part of the disease pathology. This has been demonstrated with Lymphocytic Choriomeningitis Virus (LCMV), whereby intra-cerebral infection of either WT mice showed neurological impairment and died of choriomeningitis, while CD8 T cell depleted mice showed mild signs of illness but later recovered and survived infection (Fung-Leung et al., 1991).

CD8⁺ T cell immunity is also implicated in the response infection of cells by intracellular bacteria, such as L. monocytogenes. Antigens from this Gram-positive bacterium can be processed and presented by MHC class I molecules to CD8⁺ T cells (Grauling-Halama et al., 2009). The role of CD8⁺ T cells during intracellular bacterial infection has been exploited in vaccine candidates against M. tuberculosis and L. monocytogenes designed to induce protective CD8⁺ T cell mediated immunity (Nagata and Koide, 2010).

The studies of MHC class I deficient mice also helped to define the role of class I in the function of other effector cell-types, NK cells and γδ cells (Liao et al., 1991; Pereira et al., 1992). NK cells were initially described in terms of the ‘missing self’ hypothesis, whereby tumorigenesis or viral infection subverts normal class I expression (Ljunggren and Kärre, 1990). This was demonstrated in vitro by NK cell killing of tumour cells lacking the expression of MHC class I molecules (Ljunggren and Kärre, 1990). NK cells are now perceived to act through a complex balance of inhibitory and activating receptors, and seem able to detect a wide range of pathogens including bacteria and viruses (Bessoles et al., 2011; Jost et al., 2011). Since most of
these pathogens are not generally thought to cause class I down-regulation, other possibilities include recognition of class I-bound peptides or effects of induced stress proteins.

NK cells can produce IL-17A and IFN-γ, important pro-inflammatory cytokines briefly discussed in Chapter 3. NK cells can be stimulated in the presence of IL-6 to produce IL-17A during Toxoplasma gondii infection (Passos et al., 2010). Host cells infected by viruses can also be killed by NK cells to limit further spread of disease (Lee et al., 2007). C57BL/6J mice with the beige mutation contain NK cells with decreased functions natural killing, and are more susceptible to Murine Cytomegalovirus (MCMV) infection, tending to have a poorer outcome (Bancroft et al., 1981).

CD1d, a non-classical MHC class I-related molecule, can present antigens in the form of glycolipids to a specific class of restricted T cells called invariant NKT (iNKT) cells. These cells share characteristics of both NK and T cells, such as the expression of semi-invariant TCR and also markers associated with NK cells such as NK1.1 (mouse only), bridging both innate and adaptive immunity (Van Kaer et al., 2011). Upon activation iNKT cells can produce a large array of different cytokines, rapidly and in large amounts, such as IL-4, IL-10, IL-17A, IFN-γ and TGF-β and are implicated in the response to infections (Leung and Harris, 2010). iNKT cells and CD1d molecules are important in pulmonary elimination of P. aeruginosa (Nieuwenhuis et al., 2002) and it has also been shown that iNKT can prevent the dissemination of Borrelia burgdorferi from the blood into host tissue (Lee et al., 2010b).

S. pyogenes is classically defined as an extracellular bacterium, exerting its pathogenic effects outside the cell. CD4⁺ T cell mediated immunity is key in eliminating this bacterium and S. pyogenes has seemingly evolved the secretion of virulence factors such as superantigens to modulate ensuing CD4⁺ T cell responses (Bueno et al., 2007). There is now emerging data to suggest this Gram-positive bacterium may be able to survive within cells such as neutrophils (Staali et al., 2003) and macrophages (Thulin et al., 2006), allowing further opportunity for infection.
Other virulence factors, such as Streptolysin O expressed by *S. pyogenes* can prevent the degradation of the bacterium inside the cell by the lysosome, prolonging survival within the host cell (Håkansson *et al.*, 2005; Logsdon *et al.*, 2011).

If *S. pyogenes* can survive within host cells, it is conceivable that cytotoxic effector cells, such as CD8$^+$ and NK cells may be required to elicit target cell killing. It has been shown that mice depleted of CD8$^+$ T cells and NK cells are more resistant to CLP induced poly-microbial sepsis (Sherwood *et al.*, 2004). However, the direct effect of CD8$^+$ T cells and NK cells have not been properly addressed in acute *S. pyogenes* infection models.

As shown by data and discussed in Chapter 3, there appeared to be a role for CD8$^+$ T cells and NK cells during acute *S. pyogenes* infection. Infection experiments conducted using Foxp3.DTR transgenic mice would suggest that upon the removal of Foxp3$^+$ T$_{reg}$ mediated suppression there was significantly decreased IFN-γ production by both CD8$^+$ T cells and NK cells in the draining lymph node (Figure 3.25) and significant increased percentage of IL-17$^+$ NK cells in the spleen (Figure 3.27). There was a very small increase in the absolute numbers of NKT cells both in the draining lymph node and spleen making IL-17A upon infection in the absence of Foxp3$^+$ cells, although this was not significant. Whether these changes in cytokine production by CD8$^+$ T cells, NK cells and to a lesser extent NKT cells upon the removal of Foxp3$^+$ cells truly contribute to the decreased pathology still remains unknown.

As CD8$^+$ T cells and NK cells may be involved in my murine model of sepsis, the aim of this chapter was to further examine the role of these cell types during acute *S. pyogenes* infection. I will investigate whether CD8$^+$ T cells and NK cells are pathogenic or protective during GAS infection. Both these two cell types share in common the requirement for MHC class I molecules, either through antigen presentation to CD8$^+$ T cells or discrimination of self and non-self by NK cells, I will first examine whether MHC class I molecules is needed during acute sepsis induced by the classically regarded extracellular human pathogen *S. pyogenes*. 
4.2 β2-microgloblin knockout mice exhibit increased bacterial burden during the early stages of *S. pyogenes* infection

In order to determine whether MHC class I molecules are required during acute *S. pyogenes* infection, I decided to use a mouse model that lacked this molecule. β2-microgloblin (β2m) is a component of the MHC class I molecule along with polymorphic α polypeptide chains. Transgenic mice created lacking β2m (Koller and Smithies, 1985; Koller *et al.*, 1990) have been characterised to have no expression of MHC class I molecule due to the targeted loss of β2m (Koller *et al.*, 1990). Another published phenotype of these knockout mice was the complete absence of CD4+CD8+ T cells, correlating with the loss of MHC class I molecules (Koller *et al.*, 1990; Zijlstra *et al.*, 2010).

β2m knockout mice were infected with a lethal dose of *S. pyogenes* to investigate whether MHC class I molecule was required during infection with this Gram-positive bacterium. Survival was determined in C57BL/6 WT versus β2m knockout mice over the course of four days. There was no significant difference in survival post lethal infection with *S. pyogenes* in WT or knockout animals (Figure 4.1). This was suggestive that, MHC class I presentation is redundant during acute infection with GAS, at least as measured by survival.

![Figure 4.1: Percentage survival in β2m−/− mice post lethal *S. pyogenes* infection.](image)

Survival curve of infected C57BL/6 and β2m KO mice (n=7 females per strain). Mice were given 6x10⁸ CFU H305 *S. pyogenes* (WT) i.m and monitored for defined end points and culled.
During a shorter course of infection of 24h, there was increased systemic bacterial burden in $\beta_2m$ knockout mice compared to WT controls, with more bacteria recovered in the thigh, blood, spleen and liver in the knockout mice (Figure 4.2). However, this increased bacterial spread in $\beta_2m$ knockout mice was accompanied curiously with a slight decrease in serum IL-6, a surrogate marker of disease severity compared to infected WT mice (Figure 4.3). Infected $\beta_2m$ knockout mice exhibited a trend of elevated serum IL-17A and decreased IFN-$\gamma$ concentrations in the blood compared to infected WT controls (Figure 4.3). It should also be noted, there was no significant difference in the baseline levels of serum IL-6, IL-17A and IFN-$\gamma$ in uninfected C57BL/6 or $\beta_2m$ knockout mice, confirming that the lack of class I expression did not alter the resting state of the production of these pro-inflammatory cytokines (Figure 4.3).

![Figure 4.2: Bacterial burden in $\beta_2m^{-/-}$ mice.](image)

C57BL/6 and $\beta_2m^{-/-}$ mice (n=6-7 females per strain) were infected with $6.5\times10^8$ CFU H305 or PBS (sham) i.m for 24h. Bacterial counts in the thigh, blood, spleen and liver was determined. Mean and SEM are represented and Mann-Whitney U statistical test was carried out and no significant differences were found. No bacterial growth was detected in sham infected C57BL/6 and $\beta_2m^{-/-}$ mice (data not shown).
C57BL/6 and β2m KO mice (n=7 females per strain) were infected with 6.5x10^8 CFU H305 or PBS (sham) i.m for 24h. Serum was collected and concentration of IL-6, IL-17A and IFN-γ measured by ELISA. Mean and SEM are represented and 1-Way ANOVA statistical test was carried out and significant differences indicated: **p<0.01, ***p<0.001.

These results from 24h infection experiments suggest there was a role of an MHC class I pathway during the early stages of acute *S. pyogenes* infection. However, it would appear that this role in antigen presentation may have been redundant during later stages of lethal *S. pyogenes* infection.

4.3 Gene expression changes during early stages of *S. pyogenes* infection in β2m knockout mice

qRT-PCR analysis was performed on cDNA from draining lymph nodes and spleens harvested from WT and β2m knockout mice after 24h infection with WT *S. pyogenes* to investigate if there were any changes in gene expression.

No significant differences in gene expression were observed in the spleen of infected β2m knockout mice compared to infected WT controls (data not shown). However, there were changes detected in the inguinal draining lymph node (Figure 4.4). There was significant up-regulation of *gitr*, *ctla-4* and *lag-3* in infected β2m knockout mice compared to infected C57BL/6 controls (Figure 4.4). These are genes generally
associated with CD4+Foxp3+ Tregs but are also expressed by other activated cells such as CD8+ T cells, NK cells, B cells and macrophages (Nocentini and Riccardi, 2009; Workman et al., 2002). This was reflective of the presence of more activated cells during infection in β2m knockout mice. Interestingly, expression of master regulators of other CD4+ T helper subsets, such as t-bet, gata-3 and rorγt were also up-regulated in infected β2m knockout mice relative to infected controls (Figure 4.4), suggestive of increased differentiation of T_{h}1, T_{h}2 and T_{h}17 cells respectively in the absence of MHC class I expression during the response to GAS infection.

qRT-PCR analysis of cytokine gene expression in the draining lymph node revealed there was statistical increased expression of all transcripts analysed, including il-6 and ifn-γ in infected β2m knockout mice compared to infected C57BL/6 controls (Figure 4.5). Infected β2m deficient mice showed an overall up-regulation of cytokine genes. However, there may exist a post-transcriptional mechanism regulating protein production to explain the slight decrease in serum IL-6 and IFN-γ levels in GAS infected β2m knockout mice relative to infected C57BL/6 controls (Figure 4.3).
Figure 4.4: Fold changes in transcription factor gene expression in β2m−/− mice during acute GAS infection.

C57BL/6 and β2m−/− mice (n=6-8 females per strain) were infected with 2.1x10⁸ CFU H305 or PBS (sham) i.m. DLN were harvested for qRT-PCR analysis of transcription factor gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Figure 4.5: Fold changes in cytokine gene expression in β₂m⁻/⁻ mice during acute GAS infection.

C57BL/6 and β₂m⁻/⁻ mice (n=6-8 females per strain) were infected with 2.1x10⁸ CFU H305 or PBS (sham) i.m. DLN were harvested for qRT-PCR analysis of cytokine gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
4.4 *Ex vivo* cytokine production in infected β₂m knockout mice

Figure 4.6: Lack of CD8⁺ T cells and subsequent decreased IFN-γ production in infected β₂m⁻/⁻ mice.

C57BL/6 and β₂m KO mice (n=2 females per strain) were infected with 2.1x10⁸ CFU H305 *S. pyogenes* i.m for 24h. Splenocytes were harvested and stained for IFN-γ⁺ (Alexa fluor 700 stained) production in CD8⁺ (V450 stained; top panel), NK (Pe-Cy7 stained; middle panel) and NKT (gated on NK1.1⁺ cells expressing CD3-V500 stained; bottom panel) cells and analysed by flow cytometry. Representative staining is represented above.
As the qRT-PCR data revealed an overall up-regulation of all analysed cytokine transcripts, ex vivo flow cytometry was next performed to investigate whether there was a change in the cytokine profile of immune cells in infected β2m knockout mice. The phenotype of these mice was confirmed by flow cytometry to show β2m knockout mice had a severely decreased CD8\(^+\) T cell population and hence any cytokine production from this subset was also diminished particularly in the spleen (Figure 4.6). Interestingly, there was also impairment in IFN-γ production in NK and NKT cells in the spleen of β2m knockout mice as highlighted in Figure 4.6. This was not surprising, as these cells also require MHC class I expression for activation and function. β2m forms part of the CD1d complex, required for antigen presentation of glycolipids to iNKT cells (Arrenberg et al., 2008).

Flow cytometric analysis of infected β2m knockout mice compared to infected WT controls revealed there was a significant decrease in the number of CD8\(^+\) T cells in β2m knockout mice in both the draining lymph node and spleen (Figure 4.7). This was in agreement with the expected phenotype of these mice (Koller et al., 1990). There was also a significant increased in the number of CD4\(^+\) T cells, particularly in the draining lymph node in the knockout animals (Figure 4.7), suggestive of a compensatory mechanism due to the decreased numbers of CD8\(^+\) T cells. Also there was an expansion of NK cell numbers present in β2m knockout mice (Figure 4.7).

C57BL/6 and β2m KO mice (n=7 females per strain) were infected with 6.5x10\(^8\) CFU H305 S. pyogenes i.m for 24h. DLN and spleens were harvested for flow cytometric analysis of cell types present in these lymphoid tissues. Mean and SEM of absolute numbers of cell types in the DLN (left) and spleen (right) are represented. 1-way ANOVA statistical analysis was carried out and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.
IL-17A and IFN-γ production, two examples of cytokines made by CD8\(^+\), NK and NKT cells were also measured by flow cytometry during early stages of \(S.\) \textit{pyogenes} infection. In the draining lymph node, due to the general drop in CD8\(^+\) T cell numbers, decreased IL-17A and IFN-γ production by this cell type was also observed in infected β\(_2\)m knockout mice compared to WT controls (Figure 4.8). Corresponding to the increased NK cell absolute numbers in the draining lymph node of knockout mice (Figure 4.7), there was also increased numbers of IL-17A\(^+\) and IFN-γ\(^+\) NK cells (Figure 4.8). No significant difference in the production of either cytokine by NKT cells was observed (Figure 4.7).

![Figure 4.8: IL-17A and IFN-γ production in the DLN in infected β\(_2\)m\(^-\) mice.](image)

C57BL/6 and β\(_2\)m KO mice (n=7 females per strain) were infected with 6.5x10\(^8\) CFU H305 \(S.\) \textit{pyogenes} i.m for 24h. DLN were harvested for flow cytometric analysis of IL-17A (left panel) and IFN-γ (right panel) production by stained cell types. Mean and SEM of cytokine\(^+\) cells of lymphocytes (top panel) and absolute numbers (bottom panel) are represented. Kruskal-Wallis and 1-way ANOVA statistical analysis was carried out and any significant differences indicated: \(*p<0.05\), \(*\)*\(*p<0.001\).

In the spleen, a different cytokine profile for analysed cell types was observed. Decreased IL-17A or IFN-γ production was seen in CD8\(^+\) T cells of infected β\(_2\)m
knockout mice as predicted (Figure 4.9). There was also increased numbers of IL-17A+ Foxp3+, γδ+ and CD4+ cells in the spleen of infected β2m knockout mice (Figure 4.9). In terms of IFN-γ production, there was increase percentage of IFN-γ+ Foxp3+ or γδ+ cells of the lymphocyte population (Figure 4.9). The reason for these changes in cytokine profile for Foxp3+ or γδ+ cells is unclear, however, it appeared to be linked to the lack of MHC class I expression.

**Figure 4.9: IL-17A and IFN-γ production in the spleen in infected β2m-/- mice.**

C57BL/6 and β2m KO mice (n=7 females per strain) were infected with 6.5x10⁸ CFU H305 *S. pyogenes* i.m for 24h. Spleens were harvested for flow cytometric analysis of IL-17A (left panel) and IFN-γ (right panel) production by various cell types. Mean and SEM of cytokine+ cells of lymphocytes (top panel) and absolute numbers (bottom panel) are represented. Kruskal-Wallis and 1-way ANOVA statistical analysis was carried appropriately and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.

Overall, through the use of β2m knockout mice there appeared to be some requirement for MHC class I molecules during the early stages of acute *S. pyogenes* infection. The deficiency of β2m resulted in increased bacterial burden and a shift in
cytokine production by Foxp3+, γδT, CD4+ and NK cells, which may contribute to the increase pathology observed.

4.5 Effects of CD8+ T cell depletion prior infection with *S. pyogenes*

As a role for MHC class I was demonstrated during the early stages of GAS infection, with the greatest effect involving the loss of CD8+ T cells in this transgenic model, it was decided that the precise role of CD8+ T cells during *S. pyogenes* infection would be investigated through antibody-mediated depletion of this target cell.

![Spleen](image)

**Figure 4.10: Optimisation of antibody-mediated depletion of CD8+ T cells.**

C57BL/6 mice (n=2 females) were treated with either 100µg YTS 169 or isotype matched control for 24 or 48h. Splenocytes were harvested and CD8+ T cells were stained and analyzed by flow cytometry. CD4+ T cells were also depleted using 100µg anti-CD4 monoclonal antibody (clone YTS 191) as a positive control. Antibodies were a kind gift from Prof. H. Waldmann and Dr. S. Cobbold, Sir William Dunn School of Pathology, Oxford. Representative staining is shown above.
Antibody mediated neutralisation of CD8$^+$ T cells was first optimised. One dose of anti-CD8α monoclonal antibody clone YTS 169 (Kish et al., 2005) was administrated to C57BL/6 mice and the presence of CD8$^+$ T cells measured by flow cytometry with staining with clone 53-6.7 recognising another epitope of CD8 (Figure 4.10). CD8$^+$ T cell mediated depletion was achieved to a high degree of specificity up to 24 and 48h post inoculation. CD4$^+$ T cells were also depleted with clone YTS 191 to ensure no cross reactivity between YTS 169 and other cell types (Figure 4.10).

Having confirmed the CD8$^+$ T cell population could be efficiently depleted and levels remained low up to 48h after one dose of antibody, it was decided that C57BL/6 mice would be given YTS 169 or isotype matched control antibody one day before and also on the day of infection with WT S. pyogenes for 24h to study the role of CD8$^+$ T cells.

24h post infection with S. pyogenes, increased bacterial counts were detected in the blood and liver in YTS 169 treated mice compared to infected control mice, indicating increased bacterial spread (Figure 4.11). There was a significant increase in numbers of recovered bacteria in the thigh; the site of infection in mice depleted of CD8$^+$ T cells compared to isotype control treated mice (Figure 4.11). The increased bacterial burden was also supported by amplified serum IL-6 levels in the anti-CD8 antibody treatment group relative to sham infected and infected isotype control groups (Figure 4.12).
Figure 4.11: The effect on bacterial burden in CD8\(^+\) T cell depleted mice during acute *S. pyogenes* infection.

C57BL/6 mice (n=6-8 females per treatment group) were pre-treated with 100µg YTS 169 mAb or isotype matched controls one day before and on the day of infection with 6.7x10\(^8\) CFU H305 or PBS (sham) i.m for 24h. Bacterial counts in the thigh, blood, spleen and liver was determined. Mean and SEM are represented and Mann-Whitney U statistical test was carried out and any significant differences indicated: \*p<0.05. No bacterial growth was detected in sham treated mice (data not shown).

Figure 4.12: Serum cytokine levels in CD8\(^+\) T cell depleted mice during *S. pyogenes* infection.

C57BL/6 mice (n=6-8 females per treatment group) were pre-treated with 100µg YTS 169 mAb or isotype matched controls one day before and on the day of infection with 6.7x10\(^8\) CFU H305 or PBS (sham) i.m for 24h. Serum was collected and concentrations of IL-6, IL-17A and IFN-γ were measured by ELISA. Mean and SEM are represented and 1-Way ANOVA statistical test was carried out and significant differences indicated: **p<0.01, ***p<0.001.
Figure 4.13: Fold changes in the DLN in CD8+ T cell depleted mice during infection.

C57BL/6 mice (n=6-8 females per treatment group) were pre-treated with 100µg anti-CD8 antibody (clone YTS 169) or isotype-matched controls one day before and on the day of infection with 2.3x10^8 CFU H305 S. pyogenes or PBS (sham) i.m. DLNs were harvested for qRT-PCR analysis of gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.

Draining lymph nodes and spleens were harvested from infected CD8+ T cell depleted and isotype control treated mice for qRT-PCR analysis to identify any differences in gene expression pattern. Disappointingly, little significant change was found between infected mice with their CD8+ T cell population intact or those with this subset depleted. In the draining lymph node, infected YTS 169 treated mice had significantly down-regulated their expression of t-bet compared to infected isotype control mice (Figure 4.13). il-4 gene expression was also significantly down-regulated upon depletion of CD8+ T cell during infection (Figure 4.13).

In the spleen, il-10, il-4 and il-9 expression was increased in infected CD8+ T cell depleted mice relative to infected controls (Figure 4.14). This suggested that despite the increased bacterial burden observed in CD8 T cell depleted mice; the pattern of gene expression induced by infection was relatively unaffected, with only moderate changes in t-bet and T_h2 associated cytokine genes. CD8+ T cells can express the transcription factor T-bet (Intlekofer et al., 2008), but the relevance of T_h2 responses during S. pyogenes and associated superantigens remains unclear (Müller-Alouf et al., 1996).
Figure 4.14: Fold changes in the spleen in CD8$^+$ T cell depleted mice during infection.

C57BL/6 mice (n=6-8 females per treatment group) were pre-treated with 100µg anti-CD8 antibody (clone YTS 169) or isotype-matched controls one day before and on the day of infection with $2.3 \times 10^8$ CFU H305 S. pyogenes or PBS (sham) i.m. Spleens were harvested for qRT-PCR analysis of gene expression changes after 24h of infection. Infected samples were normalised to tissue specific reference genes and C57BL/6 sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01.

4.6 Ex vivo cytokine production in infected CD8$^+$ T cell depleted mice

Figure 4.15: Cell types in CD8$^+$ T cell depleted mice during S. pyogenes infection.

C57BL/6 mice (n=8 females per treatment group) were pre-treated with 100µg anti-CD8 antibody (clone YTS 169) or isotype matched controls one day before and on the day of infection with $6.7 \times 10^8$ CFU H305 S. pyogenes i.m for 24h. WT or CD8$^+$ T cell depleted mice (n=2 females per strain) were inoculated with PBS i.m for 24h as sham controls. DLNs and spleens were harvested for flow cytometric analysis of cell types present in these lymphoid tissues. Mean and SEM of absolute numbers of cell types in the DLN (left) and spleen (right) are represented. 1-way ANOVA statistical analysis was performed and any significant differences indicated: *p<0.05, **p<0.01.
As qRT-PCR revealed little difference in gene expression pattern upon the removal of CD8\(^+\) T cells during \textit{S. pyogenes} infection, \textit{ex vivo} cytokine flow cytometry was carried out to investigate if there was a switch in cytokine production in other cell types in the absence of CD8\(^+\) T cells during infection. Treatment of mice with YTS 169 specifically depleted CD8\(^+\) T cells and the absolute numbers of other cell types, such as NK and CD4\(^+\) T cells were unchanged in the draining lymph node and spleen (Figure 4.15) as shown by flow cytometry.

![Figure 4.16: IL-17A and IFN-\(\gamma\) production in the DLN in infected CD8\(^+\) T cell depleted mice.](image)

C57BL/6 mice (n=8 females per treatment group) were pre-treated with 100\(\mu\)g anti-CD8 antibody (clone YTS 169) or isotype matched controls one day before and on the day of infection with 6.7x10\(^8\) CFU H305 \textit{S. pyogenes} i.m for 24h. WT or CD8\(^+\) T cell depleted mice (n=2 females per strain) were inoculated with PBS i.m for 24h as sham controls. DLNs were harvested for flow cytometric analysis of IL-17A (left panel) and IFN-\(\gamma\) (right panel) production by stained cell types. Mean and SEM of cytokine+ cells of lymphocytes are represented. Kruskal-Wallis or 1-way ANOVA statistical analysis were carried out appropriately and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.

Due to the treatment of mice with YTS 169 antibody during GAS infection, there was an expected significant reduction in the percentage of IL-17\(^+\) and IFN-\(\gamma\)^+ CD8\(^+\) T cells within the lymphocyte population in draining lymph nodes of mice (Figure 4.16). The percentage of IFN-\(\gamma\)^+ CD4\(^+\) T cells of lymphocytes increased during infection perhaps due to a compensatory mechanism in the absence of CD8\(^+\) T cells (Figure 4.16). Surprisingly, there was also an elevation in the percentage of IFN-\(\gamma\)^+ Foxp3\(^+\) cells in CD8\(^+\) T cell depleted mice upon infection (Figure 4.16), which will be discussed.
further in Section 4.9. Whether there is an inhibitory CD8⁺ T cell population suppressing Foxp3⁺ cells from producing IFN-γ during infection remains unclear.

Figure 4.17: IL-17A and IFN-γ production in the spleen in infected CD8⁺ T cell depleted mice.

C57BL/6 mice (n=8 females per treatment group) were pre-treated with 100µg anti-CD8 antibody (clone YTS 169) or isotype matched controls one day before and on the day of infection with 6.7x10⁸ CFU H305 S. pyogenes i.m for 24h. WT or CD8⁺ T cell depleted mice (n=2 females per strain) were inoculated with PBS i.m for 24h as sham controls. Spleens were harvested for flow cytometric analysis of IL-17A (left panel) and IFN-γ (right panel) production by stained cell types. Mean and SEM of cytokine⁺ cells of lymphocytes are represented. Kruskal-Wallis and 1-way ANOVA statistical analysis was carried out and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.

In the spleen, IL-17A responses were more dominant than in the draining lymph node. Yet again, the absolute number of IFN-γ⁺ CD8⁺ T cells was reduced in the spleen of infected YTS 169 treated mice compared to infected isotype controls (Figure 4.17). The percentage of IL-17 producing cells greatly shifted in infected CD8⁺ T cell depleted mice compared to infected isotype control mice. For example, there were significantly higher percentages of Foxp3⁺, CD4⁺, NK and NKT cells making IL-17A in the absence of CD8⁺ T cells during infection (Figure 4.17).

Overall, the removal of CD8⁺ T cells was detrimental to the host during the early stages of S. pyogenes infection. Ex vivo cytokine flow cytometric analysis revealed increased production of IL-17A and IFN-γ by many different cell types in the draining lymph node. This suggested there might be a suppressive CD8⁺ T cell...
population, which would normally dampen down these cytokine responses to prevent the increased bacterial burden observed in Figure 4.11.

4.7 The role of NK cells during acute *S. pyogenes* infection

As a potential role for NK cells was implicated from the studies utilising β2m knockout mice and also from CD8⁺ T cell depletions experiments, this cell type was depleted using antibodies to investigate whether NK cells have a protective or pathogenic role during *S. pyogenes* infection.

![Figure 4.18: Optimisation of antibody mediated depletion of NK cells.](image)

C57BL/6 mice (n=2 females) were treated with either 200µg PK136 (anti-NK1.1 antibody) or isotype matched control for 48h. Splenocytes were harvested and NK cells were stained for the expression of CD49b (FITC conjugated) and NKP46 (Pacific Blue conjugated) and lack of CD3 (V500 conjugated) and analyzed by flow cytometry. Representative staining is shown above with expression of CD3 and other NK-specific markers, CD49b or NKP46 of gated lymphocyte population shown in the left and middle panel (Walzer *et al.*, 2007). The right panel shows expression of CD49b and NKP46 expression of gated CD3⁻ lymphocytes.
Most studies have employed anti-NK1.1 antibody (clone PK136) to specifically deplete NK and NK1.1+ T cells in C57BL/6 mice (Setiady et al., 2010). After optimisation experiments increasing the amount of antibody and the number of doses administrated to the mice, full depletion of NK cells could not be achieved with this reagent (Figure 4.18). 200µg anti-NK1.1 antibody given two days before harvest for flow cytometric analysis produced the greatest percentage of NK cell depletion (84%) as shown by decreased percentage of CD3−CD49b+NKp46+ cells compared to isotype matched control mice (Figure 4.18). Increasing the amount of antibody or the number of doses given to mice did not increase the percentage of depletion.

![Figure 4.19: Weight loss in largely NK cell depleted mice during GAS infection.](image)

C57BL/6 mice (n=8 females per treatment group) were pre-treated with 200µg anti-NK1.1 antibody (clone PK136) or isotype matched controls two days before and on the day of infection with 2.2x10^8 CFU H305 S. pyogenes i.m for 24h. Percentage weight loss of each infected treatment groups was determined. Mean and SEM are represented and Mann-Whitney U statistical test was carried out and any significant differences indicated: *p<0.05.

Having determined the protocol for NK cell depletion with clone PK136 in C57BL/6 mice, animals were pre-treated with PK136 or isotype matched antibody two days before and also on the day of infection with S. pyogenes for 24h. Mice largely depleted of their NK cells lose more weight during infection compared to infected isotype controls (Figure 4.19).
Figure 4.20: Bacterial burden in largely NK depleted mice during GAS infection.

C57BL/6 mice (n=8 females per treatment group) were pre-treated with 200µg anti-NK1.1 antibody (clone PK136) or isotype matched controls two days before and on the day of infection with 2.2x10^8 CFU H305 S. pyogenes i.m for 24h. Bacterial counts in the thigh, blood, spleen and liver was determined. Mean and SEM are represented and Mann-Whitney U statistical test was carried out and any significant differences indicated: *p<0.05.

Figure 4.21: Cytokine levels in serum of largely NK cell depleted mice during infection.

C57BL/6 mice (n=6-8 females per treatment group) were treated with 200µg PK136 or isotype matched controls two days before and on the day of infection with 2.2x10^8 CFU H305 S. pyogenes or PBS (sham) i.m for 24h. Serum was collected and concentrations of IL-6, IL-17A and IFN-γ measured by ELISA. Mean and SEM are represented and 1-Way ANOVA statistical test was carried out and significant differences indicated: **p<0.01, ***p<0.001.

Similar to the experiments conducted with β2m knockouts and mice depleted of their CD8^+ T cells, increased systemic bacterial burden was also observed in mice depleted
of NK cells during *S. pyogenes* infection. Increased numbers of recovered bacteria was found in the thigh, blood and liver of PK136 treated mice compared to isotype controls (Figure 4.20). Increased bacterial burden was accompanied with an increase in serum IL-6 in infected PK136 treated mice (Figure 4.21). Interestingly, the concentration of serum IL-17A and IFN-γ, cytokines known to be produced by NK cells was decreased in infected anti-NK1.1 treated mice compared to infected isotype controls (Figure 4.21).

Overall, this set of data suggest that NK cells are protective during the early stages of *S. pyogenes* infection as partial depletion resulted in increased disease severity, perhaps due to the reduction of IL-17A and IFN-γ as detected in the serum of infected PK136 treated mice.

### 4.8 Cytokine production by immune cells in the absence of NK cells during GAS infection

As depletion of NK cells during acute *S. pyogenes* resulted in a decrease in serum IL-17A and IFN-γ, *ex vivo* cytokine flow cytometry are carried out to see if this was fully attributed to the reduction in NK cell numbers. Treatment with PK136 antibody as shown in Figure 4.18 did not achieve full depletion but only a reduction in the absolute numbers of NK cells in either the draining lymph or spleen of infected mice (Figure 4.22). Absolute numbers of other cell types, including NKT cells (also expressing NK1.1) were unaffected by the treatment with this monoclonal antibody (Figure 4.22).
C57BL/6 mice (n=8 females per treatment group) were pre-treated with 200µg anti-NK1.1 antibody (clone PK136) or isotype matched controls two days before and on the day of infection with 2.2x10^8 CFU H305 i.m for 24h. WT or NK cell depleted mice (n=2 females per strain) were inoculated with PBS i.m for 24h as sham controls. DLNs and spleens were harvested for flow cytometric analysis of cell types present. Mean and SEM of absolute numbers of cell types in the draining lymph node (left) and spleen (right) are represented. 1-way ANOVA statistical analysis was carried out and no significant differences were found.

C57BL/6 mice (n=8 females per treatment group) were pre-treated with 200µg anti-NK1.1 antibody (clone PK136) or isotype matched controls two days before and on the day of infection with 2.2x10^8 CFU H305 i.m for 24h. WT or NK cell depleted mice (n=2 females per strain) were inoculated with PBS i.m for 24h as sham controls. DLNs were harvested for flow cytometric analysis of IL-17A (left panel) and IFN-γ (right panel) production by various cell types. Mean and SEM of cytokine+ cells of lymphocytes are represented. Kruskal-Wallis and 1-way ANOVA statistical analysis was carried and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.
In the draining lymph node, PK136 treatment resulted in decreased populations of IL-17A⁺ producing Foxp3⁺, γδ⁺, CD4⁺, CD8⁺, NK and NKT cells (Figure 4.23). There was no change in the percentage of IFN-γ producing cells of the lymphocyte population in the draining lymph node upon treatment with anti-NK1.1 antibody prior to *S. pyogenes* infection (Figure 4.23). In the spleen, there was a general trend of fewer IL-17A or IFN-γ producing cells (Figure 4.24). The only significant difference in the percentage of IFN-γ producing cells of lymphocytes was from CD4⁺ T cells upon treatment with PK136 antibody (Figure 4.24).

![Figure 4.24: IL-17A and IFN-γ production in the spleen in infected PK136 treated mice.](image)

C57BL/6 mice (n=8 females per treatment group) were pre-treated with 200µg anti-NK1.1 antibody (clone PK136) or isotype matched controls two days before and on the day of infection with 2.2x10⁸ CFU H305 *S. pyogenes* i.m for 24h. WT or NK cell depleted mice (n=2 females per strain) were inoculated with PBS i.m for 24h as sham controls. Spleens were harvested for flow cytometric analysis of IL-17A (left panel) and IFN-γ (right panel) production by various cell types. Mean and SEM of cytokine⁺ cells of lymphocytes are represented. Kruskal-Wallis and 1-way ANOVA statistical analysis was carried and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.

In summary, NK cells were found to be protective during the early stages of acute *S. pyogenes* infection. As full depletion of NK cells could not be achieved through the use of PK136 clone antibody, interpretation of the data must be treated with caution. The greatest effect of PK136 treatment was the significant reduction in the percentage of IL-17A⁺ cells during infection, correlating with a drop in serum IL-17A levels. This suggests the presence of NK cells might be critical for the recruitment and
activation of other immune cells, such as T cells to produce IL-17A to aid bacterial clearance.
4.9 Discussion

Pathogens have long evolved to infect and exploit host cells to replicate and evade the immune system. Many different bacterial species have been demonstrated to survive intracellularly within cells, eliciting either NK or CD8\(^+\) T cell-mediated immunity to contain and eliminate the infection. Commonly studied intracellular bacteria include the Gram-positive \textit{L. monocytogenes} and Gram-negative \textit{S. typhimurium}, whereby early electron microscope studies have demonstrated the presence of these bacterial species inside cells (Yokoyama \textit{et al.}, 1987; Armstrong and Sword, 1966).

Many studies have implicated macrophages and neutrophils as immune cells important in the elimination of intracellular bacterium (Conlan and North, 1991). This is achieved through engulfment of pathogens and anti-microbial functions, such as the production of reactive oxygen species and enzymes and phagosome acidification (McRipley and SBarra, 1967; Rathman \textit{et al.}, 1996). NK cells also offer effective protection against intracellular bacterial infection, recognising infected cells due to down-regulation of MHC class I molecules as proposed by the “missing self” hypothesis (Ljunggren and Kärre, 1990; Kim \textit{et al.}, 2005). This leads to cytotoxic activity and cytokine production (\textit{e.g.} IFN-\(\gamma\)) to kill infect cells containing intracellular bacteria (Nakae \textit{et al.}, 1990). It should also be pointed out that other receptors exist on the surface of NK cells such as CD137 to also respond pathogens to lead to effector functions (Lee \textit{et al.}, 2005).

If cells of innate immunity are unable to clear infection, T cells are activated as part of adaptive immunity to boost effector function (Czuprynski \textit{et al.}, 1985). CD8\(^+\) T cells have similar effector phenotype as described for NK cells, such as cytolytic activity and cytokine production (Harty \textit{et al.}, 1992; Harty and Bevan, 1992; Roberts \textit{et al.}, 1993) and also require MHC class I molecules to present processed intracellular bacterial antigens for activation (Pamer, 1994; Darji \textit{et al.}, 1997; Szalay \textit{et al.}, 1994).
The role of MHC class I dependent pathways including CD8 activation in *S. pyogenes* infections has received little attention due to the classical view that this Gram-positive bacterium is an extracellular pathogen. This has implied that antigens might be presented through MHC class II presentation to CD4⁺ T cells. There is however, a body of experimental evidence that *S. pyogenes* can infect and survive within cells, such as neutrophils and macrophages (Staali et al., 2003; Thulin et al., 2006). This suggests that processed streptococcal antigens may MHC class I biosynthetic pathway for presentation to CD8⁺ T cells. The aim of this chapter was to clarify whether MHC class I molecules (and hence CD8⁺ T and/or NK cells) are involved in host defense against *S. pyogenes* infection.

β₂m knockout mice, characterised by the lack of MHC class I molecule surface expression (Koller et al., 1990) were infected with a lethal dose of WT *S. pyogenes* and no difference in survival was found between infected WT and β₂m knockout mice (Figure 4.1). However, bacterial counts taken after 24h infection in C57BL/6 and β₂m knockout mice indicated a trend of increased bacterial spread in knockout mice (Figure 4.2) and correlating with elevated serum IL-17A levels (Figure 4.3). However, serum IL-6, often used in protocols as a surrogate marker for severity/lethality, was reduced in β₂m knockout animals (figure 4.3).

qRT-PCR analysis of draining lymph nodes revealed overall increased gene expression of all immune transcripts studied (Figure 4.4-5), suggestive of a global change in gene expression in the absence of class I expression during *S. pyogenes* infection. My data certainly indicate that MHC class I expression may play a role during the early stages of GAS infection. This role could be for CD8⁺ T cell mediated killing of infected cells. In infected β₂m knockout mice, severely decreased absolute numbers of CD8⁺ T cells were found in both the draining lymph node and the spleen (Figure 4.7).

Twenty-four hours into an acute infection seems too early for a role of CTL killing of intracellularly infected host cells. A hypothesis that would link a set of changes, whereby immune transcripts associated with virtually all T cell subsets are enhanced would be decreased class I expression somehow enhanced the ability of the bacterial
superantigens to induce a cytokine storm. As I have discussed elsewhere in this thesis, this would also be compatible with the observed increase in bacterial spread. From the experiments I have reported here, it is impossible to be certain in what way an MHC class I-dependent responses might normally be curtailing this effect. An obvious candidate would be a CD8\(^+\) regulatory population that normally dampens the effect. In another example of bacterial infection (albeit using a capsular polysaccharide not expressed by GAS), a CD8\(^+\)CD28\(^-\) regulatory T lymphocyte population was induced by TCR cross-linking (Mertens et al., 2009). It should also be noted that there is some literature stating staphylococcal superantigens such as SEB (Häffner et al., 1996) and SEA (Baskar et al., 1996) may be able to interact with MHC class I molecules to lead to the activation of T cells, but whether is also true for streptococcal superantigens is unknown.

The findings in CD8 depleted mice to some extent reiterated those in the \(\beta_2\)m knockout mice, most clearly with respect to the increased bacterial spread. The fact that the phenotype in CD8-depleted mice was subtler than in the \(\beta_2\)m knockouts may be compatible with a view that part of the knockout phenotype was attributable to altered function of another cell type, such as NK cells. Ex vivo cytokine flow cytometric analyses of lymphoid tissues from infected mice depleted of CD8\(^+\) T cells revealed a more dominant role for IFN-\(\gamma\) in the draining lymph node and IL-17A in the spleen (Figure 4.16-17). An increase percentage of Foxp3\(^+\) and CD4\(^+\) T cells within the lymphocyte population were making IFN-\(\gamma\) in the draining lymph node in the absence of CD8\(^+\) T cells upon infection with GAS (Figure 4.16). The same cell populations had switched to making IL-17A in the spleen (Figure 4.17). Whether these changes in cytokine profiles are due to compensatory mechanisms due to the loss of CD8\(^+\) T cells or whether these cell types are normally under the control of CD8\(^+\) T\(_{\text{regs}}\) is unknown. It has been suggested that during viral infection, CD8\(^+\) T\(_{\text{regs}}\) can be induced and can suppress the proliferation of viral-specific T cells (Nigam et al., 2010). More research is required whether this is also the case in my model of bacterial infection.

From the findings stated above, it would suggest that CD8\(^+\) T cells are protective during acute \(S.\ pyogenes\) infection. The importance of CD8\(^+\) T cells during
intracellular bacterial infection has been highlighted in many publications. *L. monocytogenes* is a typical intracellular bacterium and CD8\(^+\) T cells capable of secreting IFN-\(\gamma\) have been found to be induced as early as 16h post infection in mice (Bou Ghanem *et al.*, 2011). Literature exists correlating GAS and autophagy, demonstrating that the immune system has evolved a system to eliminate intracellular *S. pyogenes* (Nakagawa *et al.*, 2010; Yamaguchi *et al.*, 2010; Yoshimori and Amano, 2009). While the process of autophagy was not examined in my model of acute sepsis, this could lead itself to further work. Work has also been carried out on *S. pneumoniae*, another related Gram-positive bacterium has demonstrated that mice lacking CD8\(^+\) T cells also exhibit increased bacterial spread and decreased survival compared to WT controls (Weber *et al.*, 2011). This is very similar to results obtained from my own studies.

Figures 4.9 and 4.16 show, in the context of the \(\beta_2m\) deficient mice and CD8 depletion respectively, that an enhanced population of Foxp3\(^+\) IFN-\(\gamma\)\(^+\) cells in the spleen was detected (Figure 4.9). This convergence of data seems to argue that these cells are normally constrained by CD8 immunity. During the time that I was doing this work, reports began to appear about other settings in which these cells are found. It has now been shown for Foxp3\(^+\) cells to make pro-inflammatory cytokines such as IL-17A and IFN-\(\gamma\) during inflammatory conditions (Espoito *et al.*, 2010). These findings are also mirrored in humans, whereby Foxp3\(^+\) T\(_{\text{regs}}\) can produce IFN-\(\gamma\) in the presence of IL-12 (Dominguez-Villar *et al.*, 2011).

CD8\(^+\) T\(_{\text{regs}}\) are less well defined compared to CD4\(^+\) T\(_{\text{regs}}\) (Joosten and Ottenhoff, 2008). Mayer and colleagues (Mayer *et al.*, 2011) have identified a population of CD8\(^+\) T\(_{\text{regs}}\), similar to conventional CD4\(^+\) T\(_{\text{regs}}\) in terms of expression of key markers such as CD25, GITR and CTLA-4 but lacked suppressive capacity. Whether CD8\(^+\) regulatory T cells may normally suppress the production of IL-17A and IFN-\(\gamma\) from Foxp3\(^+\) or \(\gamma\delta\)\(^+\) cells during acute *S. pyogenes* as suggested in this model require further investigation.

The role of MHC class I dependent antigen processing and presentation during sepsis has previously been explored. Sherwood and colleagues (Sherwood *et al.*, 2003) used
a CLP model of poly-microbial sepsis in β2m knockout mice. They however, found this strain of mice were more resistant to mortality and attributed their increased survival to the loss of pathogenic CD8+ T cells and NK cells. This is the opposite of my findings in *S. pyogenes* induced sepsis, where β2m knockout mice have a worst outcome during the early stages of infection. This is reminiscent of data from another disease model of *K. pneumoniae* infection where the authors observed increased bacterial burden in infected β2m knockout mice (Cogen and Moore, 2009). However, this protection was not due to CD8+ T cells or NKT cells as shown by depletion studies (Cogen and Moore, 2009). In contrast to this paper, NK cells may be pathogenic in LPS-induced shock in β2m knockout mice (Emoto et al., 2001). With this in mind, it was necessary to investigate the individual roles of CD8+ T and NK cells during acute *S. pyogenes* infection.

The role of NK cells was also studied in my model of acute sepsis. Given the lack of available tools to deplete NK cells, anti-NK1.1 antibody clone PK136 was chosen for my depletion studies (Figure 4.18). Other groups have use anti-asialo GM1 to deplete NK cells but it should be noted that a subset of monocytes and macrophages also express Asialo-GM1, therefore this antibody was deemed unsuitable for specific NK cell depletion.

*In vivo* treatment with anti-NK1.1 antibody prior to infection with *S. pyogenes* resulted in increased weight loss (Figure 4.19), systemic bacterial burden as shown in the blood and liver (Figure 4.20) and increased serum IL-6 (Figure 4.21) compared to infected isotype treated control mice. Serum levels of IL-17A and IFN-γ were lower in infected PK136 treated mice compared to infected isotype treated controls (Figure 4.21). This demonstrated that NK cells are protective during acute *S. pyogenes* infection. I have subsequently been breeding mice lacking the E4bp4 transcription factor implicated as crucial for NK cell development as published by Brady and colleagues (Gascoyne et al., 2009) to determine in a knockout model whether I can confirm a clear role of NK cells.

More in depth analysis of IL-17A and IFN-γ cytokine production by different cell types by flow cytometry showed IL-17A responses in the draining lymph node were
severely impaired upon PK136 treatment prior to infection with GAS (Figure 4.23). All studied cell types within the lymphocyte population exhibited an overall decreased in IL-17A production (Figure 4.23), suggestive that this cytokine may be protective during \textit{S. pyogenes} infection, previously suggested and discussed in Chapter 3.

From these depletion studies, I would summarise that diminished NK cell mediated responses appeared to be more detrimental to host immunity than the loss of CD8$^+$ T cell mediated immunity. Without NK cells, it would appear that IL-17A responses by other cell types are not mounted and this was reflected by the decreased levels of IL-17A in serum of infected PK136 treated mice (Figure 4.21) and also more significant bacterial burden across more sampled organs was detected in infected PK136 treated mice compared to infected CD8$^+$ T cell depleted mice (Figure 4.20).

As NK cells form part of innate immunity, perhaps this cell type is more crucial in mounting early responses against \textit{S. pyogenes} infection. This would act to contain the infection and allow time for CD8$^+$ T cells to be activated and expand before contributing to the immune response during the later stages of infection.

Very little is known regarding the role of NK cells during \textit{S. pyogenes} infection. It has been demonstrated that NK cells are pathogenic during an intravenous model of \textit{S. pyogenes} infection with depletion mediated by anti-asialo GM1 treatment (Goldmann \textit{et al.}, 2005a) or in a CLP model of poly-microbial sepsis (Barkhausen \textit{et al.}, 2008). These findings are also very similar to an \textit{E. coli} infection mouse model (Badgwell \textit{et al.}, 2002). This is in contrast to my findings, however, there is evidence in other infectious diseases models to suggest that NK cells may have a protective role. For example, NK cells are required for mounting an effective IFN-γ response against \textit{L. monocytogenes} infection (Shegarfi \textit{et al.}, 2010). A mechanism whereby NK cells enhance bacterial clearance through interactions with macrophages in a CLP model of poly-microbial sepsis has also been proposed (Godshall \textit{et al.}, 2003) and NK cell dysfunction may be an indicator of poorer survival outcome (Hirsh \textit{et al.}, 2004b).
In conclusion, my results would suggest that an MHC class I dependent effector mechanism (or regulatory effect) is required during the early stages of *S. pyogenes* infection and that CD8$^+$ T cell and NK cells are protective during GAS infection. This offers an exciting caveat for studies of this oft-presumed extracellular pathogen.
Chapter 5

5  Creating superantigen knockout streptococcal isolates

5.1  Introduction

Pathogens have adapted to evade and subvert the resultant immune response. A number of microbial virulence factors fall into this category. *S. pyogenes* is an example of a bacterium, which expresses a wide variety of virulence factors to escape and modulate the host immune system post infection. These virulence factors include surface expressed M proteins and secreted superantigens. Superantigens are unlike conventional antigens as they bind to MHC class II molecules in a region away from the peptide-binding groove and to the TCR variable chain of the T cell, and do not require antigen processing (White *et al.*, 1989; Dellabona *et al.*, 1990).

Most research has focused on the role of superantigens during invasive streptococcal disease as studies have demonstrated these virulence factors can induce pro-inflammatory mechanisms and drive specific T cell expansion (Sriskandan and Altmann, 2008). While the presence of stimulation by superantigens is not inextricably linked to the ability of GAS to cause sepsis, there is supporting evidence connecting clinical cases of bacterial sepsis and toxic shock syndrome with the presence of secreted superantigens (Proft *et al.*, 2003).

Studies investigating the magnitude of cytokine responses upon stimulation of human PBMCs with streptococcal superantigens have revealed SMEZ as a potent inducer of both T_H1 and T_H2 cytokine production, whereas SPEA representing the least potent (Proft *et al.*, 2007). From this study, great variation between healthy donors was found, with some individuals biased towards T_H1 responses and others favouring T_H2 cytokine production (Proft *et al.*, 2007). Despite evidence suggesting SPEA as a weak
stimulating superantigen, it is highly associated with invasive disease (Tyler et al., 1992; Yu and Ferretti, 1989; Hauser et al., 1991). This would suggest different superantigens have diverse effects on the immune system; with some leading to potent effects, whereas some not. Further investigation is needed to dissect the precise relationship between immune responses and clinical relevant superantigens.

Due to the lack of availability to date of streptococcal derived, immunodominant peptides or antigens, it is currently not possible to study the direct antigenic effects on the immune system; this is a source of ongoing research in the lab. The only tool at our disposal to study streptococcal antigenic immune responses is whole, heat-killed streptococcal preparations. However, co-culture with such a stimulus only offers a broad approach to investigating ensuing immune responses as this crude preparation contains numerous streptococcal membrane bound proteins and secreted virulence factors, such as superantigens. Thus, care is required to decipher results attained from stimulation experiments with heat-killed bacterial cultures.

As it was not viable at present to study GAS associated antigenic-specific effects on the immune system, it was conceived that investigation of responses to infection in the presence or absence superantigenic effects on immunity might be possible. Sriskandan and colleagues (Sriskandan et al., 1999) have previously created two S. pyogenes isolates lacking either SPEA (M1 serotype mutant) or SMEZ (M89 serotype mutant) superantigens (Sriskandan et al., 2001). These knockout mutants were used for in vivo infection models and showed clear differences in terms of virulence and survival compared to WT isolates. This provides supporting evidence that superantigens are an important virulence factor secreted by S. pyogenes, although whether or to what extent this involved modulation of host immune responses is unknown.

All previous data presented in Chapter 3 and 4 have utilised a M1T1 serotype WT S. pyogenes isolate called H305 (NCTC8198) (Sriskandan et al., 1999). This is a clinical isolate extracted from a patient with scarlet fever, which developed into necrotising fasciitis. It has been useful in murine models of acute sepsis due to its invasive nature and full complement of secreted superantigens, such as SPEA and SMEZ. The results attained from infection experiments in Chapter 3 and 4 have not addressed the
superantigenic nature of the isolate and that the observed immune responses, such as increased cytokine production, may be due to the presence of superantigen virulence factors.

To investigate the effect of superantigens during acute GAS infection in my murine model of infection, it was deemed necessary to create additional M1 serotype streptococcal mutants with known superantigens genetically knocked out. These knockout isolates would be a useful tool to study the relative effects of the different superantigens upon infection \textit{in vivo}, reflecting a more natural experimental model, contrasting to infection models whereby purified recombinant superantigens were administrated \textit{in vivo}.

In this chapter, I have focused on the creation and characterisation of a panel of GAS superantigen knockout mutants. These streptococcal mutants would be used for later \textit{in vivo} infection experiments in order to allow me to investigate whether there are any specific interaction between superantigens and immune cells, in particular T_{reg}.
5.2 Creation of streptococcal *smez* superantigen knockout isolate by insertional duplication mutagenesis

To create a panel of superantigen knockout isolates to study superantigenic effects during infection, different mutagenic approaches were required. *S. pyogenes* WT strain, H305 secretes SMEZ and SPEA, two potent superantigens previously linked to the pathology of septic shock and potent inducers of host pro-inflammatory cytokines (Sriskandan *et al.*, 2001; Unnikrishnan *et al.*, 2002).

![Schematic of insertional duplication mutagenesis](image)

**Figure 5.1: Schematic of insertional duplication mutagenesis.**

Insertion of pGhost*smez* plasmid into streptococcal WT genomic DNA would disrupt functional *smez* transcription to create a *smez* KO mutant. Primers used for verifying insertion and correct orientation of pGhost*smez* plasmid are indicated. Positions of enzyme restriction sites for Southern blotting are also shown above.

Mutagenesis to knock out superantigens in *S. pyogenes* had been previously been employed successfully. A self-replicating plasmid called pGhost*smez* was made and used to genetically knock out *smez* in a M89 serotype *S. pyogenes* isolate (H293)
(Unnikrishnan et al., 2002). This plasmid was modified to contain three copies of the first 300bp of smez (smez’) to allow for homologous recombination with WT genomic DNA after bacterial transformation. At 30°C, after electroporation, pGhostsmez would self-replicate within S. pyogenes. However, temperature switching to 37°C would induce the plasmid to insert into the bacterial genome, recombining via smez’ and unroll into the middle of the WT gene to interrupt functional smez transcription and hence disrupt SMEZ expression in a process called insertional duplication mutagenesis (Figure 5.1).

In order to create a smezΔ M1 serotype isolate for functional studies into the relationship between superantigens and T_reg, the same mutagenesis strategy employed to knock out smez in H293 was used in H305 (M1 serotype WT isolate). pGhostsmez was extracted from E. coli (H376) and digested with EcoRI restriction enzyme to ensure it was the correct plasmid (see Methods 2.5.16). Restriction digest produced the expected 300bp fragment representing the three copies of smez’ cloned into the pGhost shuttle vector (8Kb) (see Methods 2.5.16). pGhostsmez was electroporated into H305 and four clones were produced after antibiotic selection with erythromycin as described in Chapter 2.

![Figure 5.2: Insertion of pGhostsmez into WT H305.](image)

PCR using genomic DNA extracted from H305 (WT) and smez KO clones (1-4) were amplified for the presence for ribosomal RNA (A): aphl kanamycin resistance cassette (B) and intact WT smez (C). Samples were run on a 2% agarose gel with Hyperladder II DNA markers (Bioline Ltd, UK). All samples were positive for rRNA (750bp). Only smez KO clones (1-4) contained aphl (700bp) and only H305 contained intact WT smez (800bp).
To confirm insertion of pGhostsmez into H305 after electroporation, PCR of extracted genomic DNA from the four potential smez KO clones was carried out. Amplification of ribosomal RNA DNA was performed by PCR with rRNA(F) and rRNA(R) primers to verify genomic DNA was extracted from the four smez KO clones as a positive control. All four KO clones and H305 produced the expected 750bp fragment confirming there was genomic DNA (Figure 5.2A). Next, the kanamycin resistance cassette (aphl) located in pGhostsmez, permitting antibiotic selection was detected with primers Aphl(F) and Aphl(R) in the smez KO clones (700bp); however, this was absent in H305 WT genomic DNA (Figure 5.2B). Only WT genomic DNA from H305 contained the intact copy of smez (800bp) as shown by PCR with smezP1 and smezR600 primers, whereas the four KO clones did not as insertion of the plasmid into the middle of the smez gene produced a fragment too big to be amplified under standard PCR conditions (Figure 5.2C). Overall, it was concluded that the four smez KO clones produced after electroporation did contain pGhostsmez.

It should be noted that homologous recombination can occur between WT smez and any one of the three copies of smez’ triplets in pGhostsmez containing clones with differing plasmid-derived fragments in the middle of WT smez. pGhostsmez can also unroll into WT smez in either direction, conferring two orientations of the plasmid in the bacterial genome. To determine the orientation of pGhostsmez in the bacterial genome of the four smez KO clones and whether homologous recombination had occurred in the predicted manner via the first smez’ triplet as shown in Figure 5.1, PCR analysis was carried out to amplify the DNA fragment from the smez promoter (smezP1 primer) to the kanamycin resistance cassette (Aphl(R) primer), which was predicted to be only seen in smez KO clones. KO clones 2 and 3 produced the expected size band of 2.5Kb, indicative of recombination occurring via the first smez’ (Figure 5.3A). Interestingly, KO clones 1 and 4 did not produce the expected PCR band size, suggesting that mutagenesis had occurred in a manner not previously predicted to disrupt intact WT smez as shown in Figure 5.2C.
Figure 5.3: Orientation of pGhostsmez in smez KO clones.

(A) Genomic DNA from H305 and smez KO clones (1-4) were amplified using smezP1 and aphI(R) primers. PCR products were run on a 1% agarose gel with Hyperladder I DNA markers (Bioline Ltd, UK). Smez KO clones 2 and 3 showed expected size bands (2.5Kb) corresponding to homologous recombination occurring with the first smez’ triplet leading to full length integration of pGhostsmez plasmid into host genome.

(B) Southern blotting with smez probe was carried out using XbaI digested genomic DNA from H305 (WT) and smez KO clones (1-4). H305 contained WT 3.5Kb smez fragment, whereas this was replaced with two fragments (6 and 7Kb) in smez KO clone 2, indicating that pGhostsmez had inserted in the predicted manner as shown in Figure 5.1.

The orientation of pGhostsmez was also determined by Southern blotting. Genomic DNA from the four smez KO clones was digested with XbaI restriction enzyme and fragments were probed for the presence of smez. Only KO clone 2 produced the expected cut fragments of 6Kb and 7Kb containing smez (Figure 5.3B) demonstrating insertional duplication mutagenesis had occurred in the predicted manner shown in Figure 5.1 to knock out smez. Overall, by PCR analysis and Southern blotting, smez
in KO clone 2 was knocked out and this clone was added to the *S. pyogenes* isolate collection and renamed as H623 (*smez*<sup>−/−</sup>).

5.3 Complementing *smez* knockout phenotype in H623

To confirm that *smez* had been knocked out in H623, the *smez*<sup>−/−</sup> genotype was complemented with a previously described pDL278smez plasmid encoding *smez* gene (Russell and Sriskandan, 2008) to determine if the WT SMEZ phenotype could be restored in the knockout mutant.

pDL278 was modified to contain the WT *smez* gene along with its endogenous promoter region to create pDL278smez. Thus, bacterial transformation with this plasmid would lead to SMEZ expression. pDL278smez was extracted from *E. coli* (H431) and digested with BamHI restriction enzyme to ensure correct plasmid extraction (See Methods 2.5.16). pDL278, the naked shuttle vector was also digested with BamHI (See Methods 2.5.16) as a control digest. Both plasmids produced linearised fragments upon digestion (6Kb) and pDL278smez also produced the expected *smez* fragments (900bp) (See Methods 2.5.16). pDL278smez was electroporated into H623 to produce the SMEZ complemented isolate, designated H626 (*smez*<sup>−/+</sup>).

5.4 Phenotypic analysis of H623 and H626

As the genotype of H623 had been demonstrated to be *smez*<sup>−/−</sup>, the phenotype of this mutant was next determined to ensure there was no SMEZ protein production. This was compared with the complemented mutant, H626 to confirm that the knocked down phenotype was attributed to the *smez*<sup>−/−</sup> genotype.
Figure 5.4: Comparison of mitogenic activities of H305, H623 and H626.

10-fold dilutions of cell-free supernatants from H305 (WT), H326 (spea⁻), H361 (spea⁻), H623 (smez⁻) and H626 (smez⁻⁺) were co-cultured with splenocytes from HLA-DQ8.Aβ⁰ mice (n=6 females). ³H-thymidine incorporation into cells (in triplicates) in the last 8h of a 5 day incubation was measured and ΔCPM calculated by subtracting background proliferation of un-stimulated cells from stimulated cells counts. Data represents mean values ± SEM. Kruskal-Wallis statistical was used and any significant difference are indicated: * p≤0.05, **p≤0.01, ***p≤0.0001.

Cell-free supernatants were produced from H623 and H626 to determine their mitogenic activities, since secreted superantigens in supernatant can be used to induce T cell proliferation (Cunningham, 2000). A 10-fold dilution series of bacterial supernatants was co-cultured in a proliferation assay with murine HLA-DQ8.Aβ⁰ splenocytes, established to be sensitive to the effects of streptococcal superantigens (Faulkner et al., 2007). Disrupting smez in H623 resulted in significantly less mitogenic stimulation of HLA-DQ8.Aβ⁰ splenocytes compared with H305 WT bacterial supernatant in a dose-dependent manner (Figure 5.4). This suggested that no SMEZ protein was present in the supernatant. Complementing H623 with pDL278smez in H626 restored the T cell proliferative response to near WT levels (Figure 5.4). These observations were also true when WT and smez⁻⁺ bacterial supernatant was co-cultured with human PBMCs but will need to be confirmed with more samples (Figure 5.5).
10-fold dilutions of cell-free supernatants from H305 (WT) and H623 (smez−/) were co-cultured with human PBMCs (n=2 healthy controls) in a preliminary study to confirm murine splenocyte proliferation assays. \(^{3}\)H-thymidine incorporation into cells (in triplicates) in the last 8h of a 3-day incubation was measured and ΔCPM calculated by subtracting background proliferation of un-stimulated cells from stimulated cells counts. Data represent mean values ± SEM. Kruskal-Wallis statistical was used and any significant difference is indicated: * p≤0.05.

M1 serotype \textit{S. pyogenes} H326 (spea−/) and H361 (spea−/) isolates, previously made by Sriskandan and colleagues (Sriskandan \textit{et al.}, 1999; Unnikrishnan \textit{et al.}, 2001) were included in the proliferation assay to compare with the phenotype of H623. Depletion of SPEA protein production significantly reduced the mitogenic ability of H326 to induce splenocyte proliferation, correlating with published data (Unnikrishnan \textit{et al.}, 2001) and this knockout phenotype was restored in the complemented mutant, H361. Interestingly, knocking out SMEZ resulted in slightly less mitogenic effect compared to H326 (Figure 5.4) and could be accounted for by \textit{S. pyogenes} secreting less SMEZ than SPEA.

In summary, it was concluded that smez had been knocked out in H623 and complemented in H626 as shown by PCR and proliferation assay and these mutants
would be useful for later in vitro and in vivo infection experiments to study the effects of superantigens on T\textsubscript{reg} and T\textsubscript{eff} function.

5.5 Creation of spea\textsuperscript{-}/smez\textsuperscript{-} double knockout S. pyogenes mutant

From further examination of the data from the proliferation assay of H623 (Figure 5.4), it was observed that there was still some remaining mitogenic effect on splenocytes on addition of supernatant from this knockout mutant. This could be attributed to the presence of other, weaker, superantigens secreted by H623, such as SPEA, SPEF, SPEG and SPEJ (Sriskandan et al., 1999; Nooh et al., 2006). To expand the functional studies with the created knockout mutants, it was resolved that knocking out both smez and spea would be useful to study the contribution of both these superantigens towards the pathogenesis of sepsis.

Using the same technique of insertional duplication mutagenesis with pGhostsmez, smez was knocked out in H326 (spea\textsuperscript{-}) to create 6 potential double knockout (spea\textsuperscript{-}/smez\textsuperscript{-}) clones (DKO 1-6). PCR analysis for rRNA, aphI kanamycin resistance cassette and intact smez was carried out confirming the insertion and orientation of pGhostsmez in H326 as earlier described in Section 5.2 with the same predicted sized fragments (Figure 5.6A-C). DKO 2, 3 and 5 produced PCR products (2.5Kb) to indicate homologous recombination has occurred via the first smez’ triplet and genomic WT smez, whereas DKO 1, 4 and 6 produced fragments (300bp smaller) suggestive that recombination had occurred via the second smez’ triplet (Figure 5.6D). The double knockout clones were also checked by PCR to ensure they were still spea\textsuperscript{-} by using primers for the spea promoter (S1) and the aphIII kanamycin cassette from pUCMUT2.2 plasmid (A1) (Sriskandan et al., 1999). All the double knockout clones produced the same 1.8Kb sized fragment along with H326 (Figure 5.6E). This PCR product was absent in H305 (WT) and H623 (smez\textsuperscript{-}), verifying spea was still knocked out in the double knockout mutants and the insertional duplication mutagenesis had not affected the original spea\textsuperscript{-} genotype.
Figure 5.6: Insertion and orientation of pGhostsmez into H326 (spea−−)

Genomic DNA from H305, H623, H326 and double knockout (DKO) clones (1-6) were amplified for the presence of rRNA; 750bp (A); aphI kanamycin resistance cassette; 700bp (B), intact smez; 800bp (C). The orientation of pGhostsmez was checked using smezP1 and aphI(R) primers: 2.5Kb product (D) as previously shown in Figure 5.3. The presence of pUCMUT2.2, the plasmid used to replace WT spea was amplified using S1 and A1 primers (1.8Kb product) to show spea was still knocked out in DKO clones (E).

5.6 Phenotypic analysis of spea−−smez−− double knockout S. pyogenes mutant

As PCR analysis confirmed that insertional duplication mutagenesis had occurred in all the double knockout mutants to disrupt smez and spea expression, double knockout clone 2 was picked for the isolate collection as homologous recombination via the first smez' triplet had occurred in the same manner as in H623. This clone was renamed H658 (spea−−smez−−) and was complemented with pDL278smezspea (see Methods 2.5.23) to produce H786 (spea−−smez−−). The phenotype of these mutants was next examined.
Figure 5.7: Comparison of mitogenic activities of H305, H658 and H786.

Cell-free supernatants (1:10 dilution) were co-cultured with splenocytes from HLA-DQ8.Aβ0 mice (n=3 females) in a proliferation assay. ³H-thymidine incorporation into cells (in triplicates) in the last 8h of a 5-day incubation period was measured and ΔCPM calculated by subtracting background proliferation of un-stimulated cells from stimulated cells. Data represents mean values ± SEM. Kruskal-Wallis statistical was used and any significant difference are indicated: * p≤0.05, **p≤0.01, ***p≤0.0001.

Cell-free supernatants from all the M1 superantigen mutants were prepared and the mitogenic effect of the secreted superantigens in the supernatant was tested in a proliferation assay as described in Section 5.4. Proliferation of splenocytes from HLA-DQ8.Aβ0 mice was greatly reduced when co-cultured with supernatants from H658 compared to H326 and was significantly decreased compared to wild-type H305. This knockout phenotype was restored in H786 to near WT levels (Figure 5.7).

With the creation of numerous superantigen knockout and complemented streptococcal isolates by insertional duplication mutagenesis, the mutants for in vivo infection had to be chosen. As the complemented strains were produced by electroporation of modified pDL278 plasmid, requiring antibiotics to maintain the presence of the plasmid within the host bacterium, these isolates were not deemed suitable for in vivo infection. Antibiotics administration would be necessary alongside the bacterial inoculum to maintain the presence of the complementing plasmid within
the streptococcal isolate and this would skew the immune response observed in the host. With this decision, only four GAS isolates (Table 5.1) were chosen for later in vitro and ex vivo phenotypic analysis due to their stable genomic integrated superantigen knockout genotype.

Table 5.1: Superantigen knockout isolates for in vivo infection

The streptococcal isolates proposed for later in vivo murine infection experiments and their superantigen genotypes are shown, along with the form of mutagenesis used to generate the mutants.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Superantigen genotype</th>
<th>Mutagenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>H305 (WT)</td>
<td>spea⁺ sme⁺</td>
<td>N/A</td>
</tr>
<tr>
<td>H326</td>
<td>spea⁺ sme⁺</td>
<td>Allelic replacement</td>
</tr>
<tr>
<td>H623</td>
<td>spea⁺ sme⁻</td>
<td>Insertional duplication</td>
</tr>
<tr>
<td>H658</td>
<td>spea⁻ sme⁻</td>
<td>Insertional duplication</td>
</tr>
</tbody>
</table>

5.7 Phenotypic characterisation of superantigen knockout isolates

With the creation of the panel of streptococcal superantigen knockout isolates for later in vivo infection experiments (Table 5.1), further in vitro phenotypic analysis was performed to ensure mutagenesis had not affected other essential aspects of the mutants. The growth rate of each streptococcal isolate was measured in vitro in liquid culture (Figure 5.8). Overnight cultures of each of the isolates were inoculated into fresh broth and optical density at 600nm was followed every hour for 8h as an indirect method of assessing bacterial growth. There was no difference in the rate of growth of the four GAS isolates in broth (Figure 5.8), indicating the mutagenesis process had not affected the rate of proliferation and growth of these isolates in culture.
Growth rates of the streptococcal superantigen knockout mutants (H305, H326, H623 and H658) in Todd-Hewitt Broth were measured optically at 600nm in three independent experiments. Linear regression statistical testing was carried out and there was no significant difference between growth rates of each of the streptococcal superantigen knockout mutants.

(B) Heparinised human blood (n=4 healthy control donors, in triplicate) or heparinised HLA-DQ8.Aβ0 blood (n=3 females, in triplicate) (B) was cultured with exponential phase streptococcal superantigen KO mutants (H305, H326, H623 and H658) for 3h at 37°C. Bacterial counts (CFU) were determined and multiplication factor calculated by dividing infected blood CFU by inoculum CFU. 1-way ANOVA statistical test was carried out and no significant difference were found.
The rate of growth of GAS superantigen knockout isolates *ex vivo* was assessed by a pilot opsonophagocytosis assay (Figure 5.9). Exponential phase streptococcal mutants were cultured with human whole blood (Figure 5.9A) or HLA-DQ8.Aβ⁰ murine blood (Figure 5.9B) for 3h before the multiplication factor from the original inoculation dose was calculated. Initial analysis showed no significant difference in the rate of growth of the four GAS isolates in either human or mouse blood (Figure 5.9), confirming the mutagenesis had not affected the rate of growth of the isolates in whole blood. Interestingly, it would appear that the multiplication effect of H326 and H623 in heparinised human blood could be divided into two groups of donors, those who phagocytose *S. pyogenes* well and those who did not. As the HLA-class II genotype of the healthy donors used for this experiment was not known, the pattern of opsonophagocytosis cannot be correlated to immune responses based on HLA-class II preferential binding of superantigens expressed by the four streptococcal isolates.

**Figure 5.10: Initial analysis of *ex vivo* mitogenic activities of superantigen knockout isolates.**

Sera (1:20) from streptococcal infected (H305, H326, H623 & H658) or PBS immunised (PBS) mice (n=2) were co-cultured with human PBMCs (n=5 healthy control donors) in a pilot proliferation assay. ³H-thymidine incorporation into cells (in triplicates) in the last 8h of a 3-day incubation period was measured and ΔCPM calculated by subtracting background proliferation of un-stimulated cells from stimulated cells. ConA was used a positive control for proliferative responses and serum from PBS immunised mice determined the background signal. Data represents mean values ± SEM. Kruskal-Wallis statistical test was used and no significant difference were found.
The *ex vivo* mitogenic phenotype of the streptococcal isolate was studied to verify the superantigen knockout phenotype was retained during infection in a mouse model. HLA-DQ8. Aβ^0^ mice were infected with the panel of superantigen knockout isolates for 24h and serum collected to stimulate human PBMCs in a proliferation assay (Figure 5.10). The murine serum would contain the secreted superantigens from the streptococcal mutants. There was no significant difference between the different superantigen knockout infected murine sera used to stimulate human PBMCs. This could have been due to the extremely low dilution and number of samples of serum used. However, the pattern of mitogenic stimulation was similar to that observed *in vitro* with HLA-DQ8. Aβ^0^ splenocytes (Figure 5.7) and human PBMCs (Figure 5.5). The serum from H305 infected mice induced the most proliferation of PBMCs and then followed by H326, H623 and H658 infected serum induced the least amount of proliferation.

Overall, it was concluded that process of mutagenesis to create the panel of four streptococcal superantigen knockout isolates had not affected the growth rate of the mutants, either in broth or whole blood and they retained their superantigenic identities during infection in a mouse model.
5.8 Discussion

Overall, due to the lack of availability of bacterial reagents to study streptococcal antigenic responses, I sought out in this chapter to create a new panel of genetically stable streptococcal superantigen knockout isolates to dissect out the different superantigenic responses during acute GAS infection.

As discussed in Chapter 1, superantigens are an important class of virulence factors secreted by both streptococcal and staphylococcal clinical isolates and are tentatively linked to the pathogenesis of bacterial sepsis. In STSS, there is an approximate correlation with number and or potency of superantigens (Proft et al., 2003), and this in turn has been supposed to facilitate pathogenesis through an ill-described process utilising the so-called “cytokine storm”.

I have created a number of streptococcal superantigen knockout isolates based on a M1 serotype WT S. pyogenes clinical isolate, formerly cultured from a patient with scarlet fever. By using insertional duplication mutagenesis, smez was knocked out of H305 to create H623, a new M1 serotype isolate. This had previously never been done before as published work was based on an M89 S. pyogenes isolate (Sriskandan et al., 1999).

By PCR analysis, it was confirmed this knockout mutant did not contain intact smez gene (Figure 5.2) and proliferation assay performed with supernatant from this mutant co-cultured with HLA-DQ8.Aβ0 splenocytes showed a significantly decreased mitogenic effect on target cells (Figure 5.4). This knockout phenotype was reinforced by complementation studies with H626 to show that upon addition of pDL278smez, a plasmid carrying the smez gene and endogenous promoter, the knockout phenotype could be restored back to near WT levels in a proliferation assay (Figure 5.4).

These results were reminiscent of the smez−/− phenotype shown in H377, the M89 serotype knockout isolate mutated with pGhostsmez (Unnikrishnan et al., 2002), whereby this isolate was shown to have reduced mitogenic activity in human PBMCs and HLA-DQ8 splenocytes. In this paper from our lab, the authors studied the
cytokine profile of PBMCs co-cultured with H377 cell-free supernatant by ELISA and found the presence of SMEZ could account for a time-dependent release of pro-inflammatory cytokines, such as IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\) and IL-8 (Unnikrishnan et al., 2002). They also confirmed there was a specific V\(\beta\)11\(^+\) T cell expansion post WT \(S.\) \textit{pyogenes} (H293) stimulation of murine splenocytes and this was absence upon smez knockout mutant (H377) co-culture (Unnikrishnan et al., 2002). These phenotypic traits were not addressed in my characterisation experiments and could be future work to truly validate the smez knockout phenotype of H623.

A spea\(^{+}\)smez\(^{-}\) double superantigen knockout mutant (H658) was created based on an existing spea\(^{+}\) M1 \(S.\) \textit{pyogenes} isolate (H326). Knocking out both smez and spea in the mutant appeared to greatly reduce its ability to induce proliferative responses on HLA-DQ8.A\(\beta\)^0 splenocytes compared to both H305 (WT) and even H326 (spea\(^{+}\)) (Figure 5.7). This knockout phenotype was returned to WT levels by the additional electroporation with pDL278smezspea plasmid to create H786 (Figure 5.7). This work thus, documents the first double superantigen knockout mutant made based on H305 WT \(S.\) \textit{pyogenes} isolate.

With the deliberate exclusion of complemented isolates for future \textit{in vivo} infection experiments due to their unstable superantigen expression profile, the four remaining \(S.\) \textit{pyogenes} isolates were further characterised to determine whether other aspects of their phenotype were affected. The growth rate of H305, H326, H623 and H658 were found to be no different if grown in liquid broth or whole blood, suggesting that the lack of superantigens does not affect their growth (Figure 5.8 and Figure 5.9). Characterisation of H623 and H658 had never been conducted until now, although H326 had previously been found to have no difference in growth rate in broth or murine whole blood compared to H305 (Unnikrishnan et al., 2001). H377, the M89 smez knockout mutant was also shown to have no difference in growth rate in broth or murine whole blood compared to H293 WT isolate, indicative that the insertion of pGhostsmez was unlikely to affect the growth of the bacterium (Unnikrishnan et al., 2002).
The superantigenic phenotype of the four selected *S. pyogenes* mutants was retained *in vivo* in the serum of infected mice, conferring a stable expression pattern of superantigens (Figure 5.10). This reassures that, during *in vivo* infection experiments, the subsequent immune response would be due to the presence or absence of the given superantigens secreted by the various knockout mutants.

Few papers have been published regarding genetic superantigen knockout streptococcal mutants. The field has been dominated by experiments concentrating on the use of purified recombinant superantigens administrated *in vitro or in vivo*. Superantigens from *S. equi*, a Group C Streptococci and causative agent of strangles in horses were co-cultured *in vitro* with equine PBMCs to induce T cell proliferation and IFN-γ production (Paillot *et al.*, 2010). Recombinant SPEA has also been inoculated into HLA transgenic mice to study the resultant immune response (Welcher *et al.*, 2002; Rajagopalan *et al.*, 2006). Some papers have published work pertaining to isolates with certain point mutations within a particular superantigen, but these experiments have been limited to structural analysis of interactions between superantigens and TCR or MHC class II molecules (Kline and Collins, 1996).

In summary, the use of these four *S. pyogenes* superantigen knockout mutants described in this chapter would be hugely beneficial in understanding the relative contributions of different superantigens during acute sepsis within the context of an *in vivo* infection model. This will be discussed in Chapter 6.
Chapter 6

6 Characterisation of regulatory T cells during acute Streptococcus pyogenes infection

6.1 Introduction

The conventional view of the immune response considers innate immunity to be recruited and activated first during infection, acting fast within minutes to hours to eliminate and prevent the spread of pathogens. This is generally followed by the functionally linked but slower adaptive immunity, providing support to the innate response with terminally differentiated effector cells and offering long-lasting protection in the form of memory cells and, in the case of B cells, affinity maturation (Kamat and Henney, 1976; Nossal et al., 1965; Nossal and Mäkelä, 1962; Vischer et al., 1967).

Innate immunity offers a broad specificity against pathogens using germline-encoded Pattern Recognition Receptors (PRRs) that recognise common conserved pathogenic features or Pathogen-Associated Molecular Patterns (PAMPs) (Uematsu and Akira, 2006; Beutler, 2009; Kawai and Akira, 2010). With this in mind, innate immunity acts in a non-specific manner, which is in contrast to adaptive immunity, which utilises antigen specific receptors generated by V(D)J recombinase (Yancopoulos et al., 1986).

PRRs expressed on innate cell types, such as scavenger receptors on macrophages are key in pathogen recognition and mediate up-take and killing microorganisms as part of the normal immune response. To date, there are several families of PRRs, the most commonly studied being Toll-Like Receptors (TLRs), expressed on most innate immune cells, such as DCs and macrophages (Takeuchi and Akira, 2010).
Recognition of microbial ligands by innate cell types initiates a chain of events, culminating in the activation of adaptive immunity in the form of B or T cells (Hou et al., 2008).

Adaptive immunity is slower acting than innate immunity, as it requires activation and priming, usually by innate components such as DCs before lymphocyte clonal expansion, which can take days before being able to efficiently aid the host response (Kamat and Henney, 1976). It should also be noted that members of adaptive immunity express PRRs (Hornung et al., 2002). Both T and B cells themselves have been shown to express a wide array of TLRs, both on their surface and also in intracellular compartments, suggestive that microbial products can directly activate these subsets of cells, without the need of innate cell mediated signals (Zarember and Godowski, 2002).

A particular subset of CD4\(^+\) T cells have been highly researched in the past few years due to their immuno-suppressive function, termed regulatory T cells (T\(_{\text{regs}}\)). A role for T\(_{\text{regs}}\) has been implicated during several different infectious models with differing outcomes (Mittrücker and Kaufmann, 2004; Belkaid, 2007) and previously discussed in Chapter 3.

The induction of T\(_{\text{regs}}\) during the later stages of infection has been deemed as beneficial to the host in order to limit tissue damage caused by the activated immune system. Depletion of T\(_{\text{regs}}\) during murine malaria infection with Plasmodium yoelii protected mice from death, suggesting that suppression of effector cells was protective in this system (Hisaeda et al., 2004). However, there is evidence that T\(_{\text{regs}}\) may also contribute to delaying the initial immune response to control the spread of pathogens during infection. Pathogen-specific T\(_{\text{regs}}\) have also been identified during M. tuberculosis infection and have been shown to limit the appearance of IFN-\(\gamma\) producing effector T cell subsets associated with protective immunity (Shafiani et al., 2010).

To date, very little research has been carried out on the function or kinetics of T\(_{\text{regs}}\) induction during S. pyogenes infection. Costalonga and colleagues (Costalonga et al., 2008) had reported that S. pyogenes in nasal-associated lymphoid tissue (NALT)
induced Th1 subsets, although Treg induction was not found in this experimental model. Previous work has predominantly focused on streptococcal superantigens and Treg induced tolerance (Papiernik, 2001). However, these studies were mainly based on staphylococcal superantigens.

Repeated exposure of superantigens, such as SEA and SEB from S. aureus can induce Tregs characterised by expression of CTLA-4 and IL-10 production with suppressive capacity (Noël et al., 2001). Similar experiments have demonstrated that superantigen induced Tregs, dissimilar from nTreg commonly described in humans and mice, as they do not express CD25 (Feunou et al., 2003). CD4⁺CD25⁺ T cells can be stimulated to gain suppressive function post repeated exposure to SEA in models of tolerance (Grundström et al., 2003). It should also be pointed out that bacteria are not the only source of this virulence factor as virus can also produce superantigens (Punkosdy et al., 2011).

The precise kinetics of induced Tregs during infection has been investigated in many different models. During the early stages of mucosal infection with HSV, both activated effector and regulatory T cells are recruited to the site of infection (Lund et al., 2008). Tregs with suppressive function are seen to proliferate in draining lymph nodes and at the site of infection in the first four days post inoculation (Lund et al., 2008). Depletion of Tregs in Foxp3.DTR transgenic mice infected with HSV increases viral load in the mucosa and central nervous system and mice rapidly succumb to infection (Lund et al., 2008), indicating that Tregs are beneficial in this model.

Tregs subset of cells express TLRs, either on their surface or in internal compartments. For example, CD4⁺CD25⁺ cells have been shown to express TLR4 and can be activated via LPS stimulation (Caramalho et al., 2003) and enhance their proliferation and survival (Gelman et al., 2004; Liu and Zhao, 2007). Tregs also express TLR2 although their function is still debated (Sutmuller et al., 2006).

The natural ligands for TLR2 are Lipoteichoic Acid (LTA) or bacterial lipoproteins. tlr2⁻/⁻ mice exhibit decreased peripheral CD4⁺CD25⁺ Treg cells compared to WT littermates as shown by flow cytometry, leading to the conclusion that this particular TLR is involved in Treg cell population expansion (Crellin et al., 2005; Netea et al.,
This has been reinforced by work in which purified CD4^+CD25^+ T_{regs} co-cultured with PAM3Cys, a TLR2 agonist were found to have enhanced proliferative abilities and hence increased T_{reg} numbers (Tang et al., 2008). The addition of PAM3Cys also increased CD25 expression (Tang et al., 2008) and the MyD88 dependent pathway was also required as myd88^{-/-} mice exhibited decreased CD4^+CD25^+ T_{regs} similar to the tlr2^{-/-} mice (Sutmuller et al., 2006). The precise mechanism by which TLR2 activates T_{regs} is still unclear. Whether TLR2-mediated activation of T_{regs} plays a role during *S. pyogenes* infection warrants further investigation.

In this chapter, I will characterise the kinetics of T_{regs} during acute *S. pyogenes* infection and investigate whether secreted streptococcal superantigens have any influence on T_{reg} induction. This will be achieved through *in vivo* infection in our HLA-DQ transgenic mouse model with streptococcal superantigen knockout isolates created and described in Chapter 5. I will also briefly examine whether TLR2 has a part to play in the induction of T_{regs} during GAS infection and correlate this to the presence or absence of streptococcal superantigens.
6.2 Kinetics of T\textsubscript{regs} during chronic *S. pyogenes* infection

![Figure 6.1: Representative staining of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} T cells.](image)

HLA-DQ8.Aβ\textsuperscript{0} females were infected with 9x10\textsuperscript{6} CFU H305 WT *S. pyogenes* i.m at t=0h. At defined time points, groups of mice (n=4 at t=0h and 48h, n=5 at t=24h and 36h) were culled and DLNs (A) and spleens (B) were harvested for flow cytometry analysis of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells. Representative dot-plots gated on CD4\textsuperscript{+} cells show Foxp3 expression (y-axis) and CD25 expression (x-axis). Percentages in quadrants are based on gated CD4\textsuperscript{+} cells.

To characterise the kinetics of T\textsubscript{regs} during acute *S. pyogenes* infection, HLA-DQ transgenic mice were utilised in this study, having formerly been demonstrated to be sensitive to GAS infection, in particular to the presence of secreted streptococcal superantigens (Faulkner *et al.*, 2007). Before carrying out a time course of infection, draining inguinal lymph nodes and spleens were harvested 24h post infection for flow cytometric analysis of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells (Figure 6.1). From this infection protocol, I established that it was possible to detect the presence of
CD4⁺CD25⁺Foxp3⁺ Treg cells in this model by flow cytometry (Figure 6.1). HLA-DQ8.Apβ0 mice were next infected with 9x10⁶ CFU H305, a scarlet fever clinical S. pyogenes isolate via intra-muscular route in a longer chronic infection protocol (Figure 6.2).

**Figure 6.2: Detection of Foxp3⁺ cells during chronic S. pyogenes infection.**

HLA-DQ8. Apβ0 females were infected with 9x10⁶ CFU H305 i.m at t=0h. At defined time points, groups of mice (n=4 at t=0h and 48h, n=5 at t=24h and 36h) were culled and DLNs (A) and spleens (B) were harvested for flow cytometry analysis of CD4⁺CD25⁺Foxp3⁺ cells. Representative histograms show Foxp3 expression (black line) of CD4⁺CD25⁺ T cells compared to Fluorescence Minus-One (FMO) controls (shaded histogram). Percentages of Foxp3⁺ cells shown by the bracket are of gated CD4⁺CD25⁺ cells.

Having validating the choice of readout for the detection of Tregs during GAS infection, draining inguinal lymph nodes and spleens were harvested at various time points post infection with 9x10⁶ CFU H305 S. pyogenes before CD4⁺CD25⁺Foxp3⁺ Treg cells were stained and measured by flow cytometry (Figure 6.2).
Figure 6.3: Kinetics of T\textsubscript{reg} during chronic S. pyogenes infection.

HLA-DQ8.A\textsuperscript{0} females were infected with 9x10\textsuperscript{6} CFU H305 WT S. pyogenes i.m at t=0h. At defined time points, groups of mice (n=4 at t=0h and 48h, n=5 at t=24h and 36h) were culled and DLNs (A) and spleens (B) harvested for flow cytometry analysis of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells. Mean and SEM of percentage of CD25\textsuperscript{+}Foxp3\textsuperscript{+} of CD4\textsuperscript{+} T cells (top), percentage of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} of total cells (middle) and Foxp3 expression per cell (bottom) are shown. Kruskal-Wallis statistical test was carried out and any significant differences are indicated: *p<0.05.

In the draining lymph node, the expression of Foxp3 remained constant within the CD4\textsuperscript{+}CD25\textsuperscript{+} T cell population during the time course of S. pyogenes infection. In the spleen, there was a decrease in expression of Foxp3 per cell (MFI) from CD4\textsuperscript{+}CD25\textsuperscript{+} population, with a significant drop at 36h post infection in relation to uninfected t=0h controls (Figure 6.3). The percentage of CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells within the CD4\textsuperscript{+} T cell population appeared to increase in the draining lymph node, whereas this population was marginally contracted in the spleen compared to un-infected controls (Figure 6.3).
It was interesting to observe changes in the kinetics of $T_{\text{regs}}$ within a relative short amount of time (24-36h) post GAS infection, as adaptive immune responses are generally characterised in textbook terms as appearing several days post the initial infection (Murphy et al., 2007). With this in mind, it was decided the disease phenotype would be examined within a shorter time course of an acute $S. \text{pyogenes}$ infection to try to correlate this with early changes in the kinetics of $T_{\text{regs}}$.

6.3 Increase bacterial burden during acute $S. \text{pyogenes}$ model of infection

As relative quick changes in the kinetics of Foxp3$^+$ $T_{\text{regs}}$ were observed during $S. \text{pyogenes}$ infection, a shorter time course of GAS infection was established with a higher dose of H305 WT $S. \text{pyogenes}$ to induce acute disease and sepsis in the HLA transgenic mouse model. Disease pathology along with changes in $T_{\text{regs}}$ were investigated at 4, 8, 12 and 24h post infection. H305 bacterial overnight cultures for $\text{in vivo}$ infection were resuspended in PBS to give a dose of approximately $10^8$ CFU per 50µl. However, due to experimental constraints, it was not viable to achieve the same dose for each experiment, as it was not possible to quantify the precise CFU of the inoculum before infection. Serial dilution of the inoculum was the only method in order to quantify the dose, however, this would only give the CFU dose the next day after infection.

As the infection progressed, the mice had increased percentage weight loss (Figure 6.4A), indicative that systemic infection was induced within 24h post infection. In contrast, mice injected with PBS appeared to recover and put on weight from 12 to 24h post inoculation. There was increased bacterial burden in the sampled thigh, blood, spleen and liver tissue over time (Figure 6.4B), correlating with the increased percentage body weight loss. No $S. \text{pyogenes}$ was detected in sham treated mice (data not shown).
Figure 6.4: Pathogenic responses during acute *S. pyogenes* infection in HLA-DQ8.Aβ⁰ transgenic model.

A. Percentage total weight loss during acute WT *S. pyogenes* infection. HLA-DQ8.Aβ⁰ females were infected with 4x10⁸ CFU H305 WT *S. pyogenes* or PBS (sham) i.m at t=0h. The start weight of each mouse was recorded. At defined time points, groups of mice (n=7 per infected time point and n=4 per sham time point) were culled and the end weight recorded. The percentage total weight loss was calculated and the mean and SEM are represented.

B. Bacterial counts during acute WT *S. pyogenes* infection. HLA-DQ8.Aβ⁰ females were infected with 1x10⁸ – 10⁹ CFU H305 WT *S. pyogenes* or PBS (sham) i.m at t=0h as part of three independent experiments. At defined time points, groups of mice were culled and bacterial counts were determined (n=17 per infected time point for blood, spleen and liver and n=14 per infected time point for thigh tissue). No bacterial growth was detected from any sampled organs of sham-infected mouse (data not shown).
HLA-DQ8.Aβ0 females were infected with 4x10⁸ CFU H305 WT *S. pyogenes* or PBS (sham) i.m at t=0h. At various time points, groups of mice were culled (n=6 per infected time point and n=4 per sham infected time point) and serum collected for cytokine analysis. Serum IL-6 and IL-17A concentrations were measured by ELISA, mean and SEM represented. No significant differences were found using 1-way ANOVA statistical analysis. Experiment was carried out with Dr. R. Ingram.

Cytokine analysis of IL-6 and IL-17A by ELISA was performed on serum collected from the short time course of infection. IL-6 levels were below levels of detection in sham-treated mice, correlating with lack of induced disease (Figure 6.5). However, in *S. pyogenes* infected mice, there was a steady increase in IL-6 in the serum, peaking at 8h post infection before declining (Figure 6.5). IL-17A levels in the serum displayed a different pattern of production. Interestingly, IL-17A was detected in sham-treated mice, despite the absence of microbial infection (Figure 6.5). This is reminiscent of immune-surveillance mechanisms mediated by innate cell types such as γδ T cells (Hayday, 2009). *S. pyogenes* infected mice had slightly higher levels of serum IL-17A at 4, 8 and 24h post infection (Figure 6.5).
6.4 Changes in gene expression during acute WT *S. pyogenes* infection

**Figure 6.6:** qRT-PCR analysis of transcription factors expression during acute *S. pyogenes* infection.

HLA-DQ8.Aβ0 females were infected with 1x10^6 - 10^9 CFU H305 WT *S. pyogenes* infected at t=0h as part of 2 independent experiments or PBS (sham). At various time points, groups of mice were culled (n=10 per infected time point and n=5 per sham time point) and DLN (A) and spleens (B) harvested for qRT-PCR analysis of transcription factor gene expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
qRT-PCR analysis was carried out on draining lymph nodes and spleen from *S. pyogenes* infected mice from the short time course of acute infection. Fold changes in gene expression were calculated relative to time matched sham controls. Changes in the expression of transcription factors genes as markers for defined T helper cell subsets were investigated to see if infection induced augmentation of gene expression.

In the draining lymph node, *foxp3* gene expression was significantly increased above time-matched sham controls, indicative of elevated T<sub>regs</sub> appearing within 4h of infection (Figure 6.6A). This also correlated with increased with *gitr* expression, another possible marker for T<sub>regs</sub> (McHugh *et al*., 2002) (Figure 6.6A). *t-bet*, *gata-3* and *rorγt* gene expressions were also studied as master regulators for T<sub>H1</sub>, T<sub>H2</sub> and T<sub>H17</sub> cells subsets respectively. During acute GAS infection, *t-bet*, *gata-3* and *rorγt* gene expression was increased over time in the draining lymph nodes (Figure 6.6A), suggestive of T<sub>H1</sub>, T<sub>H2</sub> and T<sub>H17</sub> differentiation.

In the spleen, *foxp3* gene expression was decreased below time-matched sham controls, indicative of perhaps declining T<sub>reg</sub> levels (or at least, Foxp3 transcription) in this organ (Figure 6.6B). This however, did not match with *gitr* expression (Figure 6.6B). It should be noted that GITR is not a T<sub>reg</sub> specific marker and is also expressed on activated T cells (Nocentini and Riccardi, 2009). *t-bet* and *gata-3* gene expression was up-regulated (Figure 6.6B), suggestive that T<sub>H1</sub> and T<sub>H2</sub> T cell subsets may be differentiating in the spleen. There was however, no difference in *rorγt* gene expression compared to sham-treated controls (Figure 6.6B). This suggested that there were no changes in T<sub>H17</sub> polarisation or activation in the spleen.

qRT-PCR analysis of fold changes in cytokine gene expression during the short time course of GAS infection was next studied. Draining lymph nodes and spleens were harvested from sham and *S. pyogenes* infected mice at various time point post infection with 10<sup>8</sup> CFU H305.

*tgf-β* gene expression was up-regulated in *S. pyogenes* infected draining lymph nodes, whereas, there was no significant difference in *il-10* gene expression in the lymph
node in *S. pyogenes* infected mice compared to PBS treated mice. Acute phase cytokines genes such as *il-6* and *inf-alpha* expression were up-regulated in infected mice, correlating with serum IL-6 levels (Figure 6.5) and reflective of systemic disease onset. Despite the increase in expression of *t-bet* and *ror-gamma* in the draining lymph node (Figure 6.6A), the gene expression of the hallmark cytokines of T\(_H^1\) and T\(_H^17\) cells; IFN-\(\gamma\) and IL-17A respectively did not reflect this. The level of transcription of these cytokines was significantly down-regulated compared to sham-treated controls (Figure 6.7). There was no change in gene expression pattern of *il-4* transcription. *il-9* expression was significantly down-regulated over the time course of acute infection (Figure 6.7), despite evidence of a trend of up-regulation of *gata-3*, particularly at 12h post infection (Figure 6.6A). Although *il-9* was significantly up-regulated upon infection compared to sham controls at 4 and 8h, the increased expression of the master regulator of T\(_H^2\) cells does not correlate with increased expression of the respective hallmark cytokines, *il-4* and *il-9* as shown by qRT-PCR analysis.
Figure 6.7: qRT-PCR analysis of cytokine expression in the lymph node during acute *S. pyogenes* infection.

HLA-DQ8. Ap0 females infected with 1x10^8 - 10^9 CFU H305 WT *S. pyogenes* infected at t=0h as part of 2 independent experiments or PBS (sham). At various time points, groups of mice were culled (n=10 per infected time point and n=5 per sham infected time point) and DLN harvested for qRT-PCR analysis of cytokine gene expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Figure 6.8: qRT-PCR analysis of cytokine expression in the spleen during acute *S. pyogenes* infection.

HLA-DQ8.Aβ0 females infected with 1x10^8 - 10^9 CFU H305 WT *S. pyogenes* infected at t=0h as part of 2 independent experiments or PBS (sham). At various time points, groups of mice were culled (n=10 per infected time point and n=5 per sham infected time point) and spleens harvested for qRT-PCR analysis of cytokine gene expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Although the gene expression of *tgf-β* in the draining lymph node was significantly up-regulated, the expression of this cytokine in the spleen was significantly down-regulated (Figure 6.8). *il-10* was significantly up-regulated in the spleen (Figure 6.8), suggestive this anti-inflammatory cytokine, associated with T<sub>reg</sub> function may be playing a dominant role in the spleen. *il-6* was up-regulated in the spleen at all time points similar to the draining lymph node. *tnf-α* was only significantly up-regulated at later time points of the infection course (Figure 6.8). In contrast to the draining lymph node, *ifn-γ* and *il-17a* gene expression was elevated compared to sham-treated controls (Figure 6.8). Increased *ifn-γ* correlated with increased *t-bet* expression in the spleen, however, the pattern of *il-17a* expression did not match decreased rorγt transcription. This suggested that other cell types, apart from T<sub>H</sub>17 cells may be contributing to the increased expression of *il-17a*, such as NK cells. *il-4* and *il-9* expression in the spleen did not differ from sham-treated controls and during the later stages of GAS infection decreased (Figure 6.8). This suggests T<sub>H</sub>2 cytokines may not play a significant part in the spleen during acute *S. pyogenes* infection, although *il-9* gene expression was significantly increased 4 and 8h post infection in the draining lymph node.

![Figure 6.9: qRT-PCR analysis of mpo expression during acute *S. pyogenes* infection.](image)

HLA-DQ8.Aβ<sup>0</sup> females infected with 1x10<sup>8</sup> - 10<sup>9</sup> CFU H305 WT *S. pyogenes* infected at t=0h as part of 2 independent experiments or PBS (sham). At various time points, groups of mice were culled (n=10 per infected time point and n=5 per sham infected time point) and DLN (A) spleens (B) harvested for qRT-PCR analysis of *mpo* expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
qRT-PCR analysis was performed for changes in innate immune markers. *mpo* was studied as a marker for neutrophil activation (Schmekel *et al.*, 1990). Gene expression of this gene was found to be down-regulated both in the draining lymph node and spleen (Figure 6.9), suggesting that neutrophils are not activated during acute *S. pyogenes* infection.

In summary, qRT-PCR analysis revealed induction of *foxp3* expression in the draining lymph node, possibly due to up-regulation of *tgf-β*. This was contrasted in the spleen, where *foxp3* transcription was down-regulated. Gene markers for pro-inflammatory cytokines, such as *il-6*, *tnf-α*, *ifn-γ* and *il-17a* were significantly elevated in the spleen over time, reflective of the response to developing systemic infection.

### 6.5 Kinetics of T<sub>regs</sub> during acute WT *S. pyogenes* infection

![Figure 6.10: Representative staining of T<sub>regs</sub> during acute *S. pyogenes* infection.](image)

HLA-DQ8.AB<sup>0</sup> females were infected with 4x10<sup>8</sup> CFU H305 WT *S. pyogenes* infected at t=0h. 24h post infection DLN were harvested for flow cytometric analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells. Representative staining based on gated CD4<sup>+</sup> T cells expressing CD25 and Foxp3: isotype control (left) and Foxp3 stained (right) from DLN. Percentage of Foxp3<sup>+</sup> of CD4<sup>+</sup>CD25<sup>+</sup> T cells is shown in the gate. Experiment carried out with Dr. R. Ingram.
As qRT-PCR analysis for the acute time course of infection experiment suggested a maintained elevation in foxp3 expression in the draining inguinal lymph node and a progressive fall in the spleen, the kinetics of Tregs were followed directly by flow cytometry. A short time course of infection with H305 S. pyogenes was carried out in the HLA-DQ8.Aβ0 murine model. Draining lymph nodes and spleen were harvested for flow cytometric analysis for CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells (Figure 6.10).

**Figure 6.11: Flow cytometric analysis of Tregs during acute WT S. pyogenes infection.**

HLA-DQ8.Aβ0 females were infected with 4x10<sup>8</sup> CFU H305 WT S. pyogenes infected at t=0h or PBS (sham). At various time points, groups of mice were culled (n=7 per infected time point and n=6 per sham infected time point) and DLN (A) and spleens (B) harvested for flow cytometric analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells. Mean and SEM of absolute numbers (left), percentage of total cells (middle) and Foxp3 MFI (right) of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells are represented. Data is normalised to time-matched sham controls. 1-way ANOVA statistical test was carried out on absolute numbers and Foxp3 MFI data and Kruskal-Wallis statistical test was carried out on percentage of total cells data. Any significance differences are indicated: *p<0.05, ***p<0.001. Experiment carried out with Dr. R. Ingram.

From flow cytometric analysis of Tregs during a short time course of acute GAS infection, increased expression of Foxp3 per cell in terms of MFI (Figure 6.11A) was observed in the draining lymph node. However, there were only moderate changes in the absolute numbers of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells (Figure 6.11A) and percentage of CD25<sup>+</sup>Foxp3<sup>+</sup> of CD4<sup>+</sup> T cells (Figure 6.11A) over time. The increase in Foxp3 MFI
in Tregs over the time course of GAS infection, mirrors previous qRT-PCR data of increased foxp3 gene expression in the draining lymph node with significant upregulation observed from 8 to 24h (Figure 6.6A).

In the spleen, there was a decrease in the absolute number of CD4+CD25+Foxp3+ cells from 4 to 24h (Figure 6.11B). This was reflected in the gradual fall in the percentage of CD25+Foxp3+ of CD4+ T cells (Figure 6.11B). Interestingly, there was a slight increase in Foxp3 MFI over time in the spleen (Figure 6.11B), indicating that although there are less Tregs in the spleen, they are expressing more Foxp3 per cell. The Foxp3 MFI flow cytometric data does not agree with qRT-PCR analysis of infected spleen samples, whereby gene expression of foxp3 was significantly increased from 4 to 8h post infection and then decreased (Figure 6.6B), but would generally correlate with overall decrease in the absolute numbers of Tregs in the spleen over the 24h of GAS infection.

Overall, changes in Tregs induction and absolute numbers were detected during acute S. pyogenes infection by qRT-PCR and flow cytometric analysis. Systemic bacterial burden increased with time post infection, matched with amplified IL-6 levels in serum. foxp3 gene expression increased in the draining inguinal lymph node, whereas fell in the spleen and correlated with changes in elevated Foxp3 MFI in the draining lymph node and decrease in absolute numbers of Tregs in the spleen.
6.6 Streptococcal superantigens contributes to disease pathology

Figure 6.12: Pathogenic responses during superantigen knockout *S. pyogenes* mutant infection.

HLA-DQ8.Aβ0 females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or 2-4x10⁸ CFU H305, 3-7x10⁸ CFU H326, 3-7x10⁸ CFU H623, 2-6x10⁸ CFU H658 at t=0h as part of 2 independent experiments for 24h. (A) Percentage total weight loss was calculated and mean and SEM represented. Kruskal-Wallis statistical test was carried out and any significance differences are indicated: *p<0.05, **p<0.01. (B) Bacterial counts for thigh, blood, spleen and liver were calculated and mean and SEM represented. Kruskal-Wallis statistical test was carried out and any significant differences are indicated: *p<0.05, **p<0.01. Sham infected mice did not show any bacterial growth from sampled organs (data not shown).
S. pyogenes secretes superantigens as virulence factors; this has been presumed to operate through subversion of appropriate immunity, though there is a paucity of described mechanism (Proft and Fraser, 2003). It has been documented that streptococcal superantigens are in most cases correlated with invasive diseases, such as bacterial sepsis and necrotising fasciitis (Sriskandan and Altmann, 2008). In order to link changes in disease pathology to T_{reg} kinetics to the presence or absence of streptococcal superantigens, bacterial mutants were created with known (and phenotypically strongest) superantigens knocked out as described in Chapter 5.

HLA-DQ8.Aβ^{0} mice exhibiting heightened sensitivity to streptococcal superantigens [due to the high affinity binding between HLA-DQ and the streptococcal superantigens (Llewelyn et al., 2004)] were utilised in acute infection with a panel of superantigen knockout isolates (Table 5.1). Previous experiments had suggested significant decrease in the absolute numbers of T_{regs} in the spleen and increase in Foxp3 expression in the draining lymph nodes 24h post infection with WT H305 infection. HLA-DQ8.Aβ^{0} transgenic mice were infected via intra-muscular route with the superantigen knockout isolates for 24h before bacterial counts, serum cytokine ELISA, qRT-PCR and flow cytometric analysis were carried out.

Knocking out spea superantigen gene in either WT (H326) or a smez^{-/} mutant (H658) S. pyogenes resulted in significantly increase percentage weight loss compared to sham-treated mice (Figure 6.12A). The lack of smez superantigen gene in WT S. pyogenes (H623) lead to a significant reduction in percentage weight loss upon infection compared to H326 (spea^{-/}) (Figure 6.12A), indicative of less severe systemic infection.

Bacterial counts were carried out to assess the spread of infection with the various superantigen knockout isolates. Sham-treated mice did not exhibit any growth of β-haemolytic bacteria (data not shown). H658 infection lead to significantly decreased bacterial counts in the thigh compared to WT S. pyogenes (Figure 6.12B), suggesting the lack of SMEZ and SPEA may contribute to this reduction in CFU. In the blood, it was observed H623 infected mice had the highest amount of recovered bacteria, even
higher than H326 (Figure 6.12B). This was also true in the spleen and liver (Figure 6.12B).

The removal of SMEZ in the WT strain (H623) resulted in increased bacterial burden compared to WT infection. My data would also suggest that the lack of SPEA in H326 and H658 would lead to less severe systemic infection, particularly in the blood. However, it was interesting to note, H326 had been shown to induce bacteraemia compared to H305 WT infection but this was carried out in CD1 outbred mice (Sriskandan et al., 1999). The use of HLA transgenic mice (thus conferring superantigen sensitivity in contrast to mouse H-2A, which bind poorly) may explain the difference in pathology induced by H326 infection.

![Figure 6.13: Cytokine responses during superantigen knockout S. pyogenes mutant infection.](image)

HLA-DQ8. Aβ0 females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or superantigen knockout *S. pyogenes* isolate (2-4x10^8 CFU H305, 3-7x10^8 CFU H326, 3-7x10^8 CFU H623, 2-6x10^8 CFU H658) at t=0h as part of 2 independent experiments for 24h. Serum was collected and concentrations of IL-6, IL-17A and IFN-γ were determined by ELISA and mean and SEM represented. 1-way ANOVA statistical test was carried out and any significant differences indicated: *p<0.05, **p<0.01, ***p<0.001.

Infection with the superantigen knockout mutants resulted in elevated levels of IL-6, with no observed significant difference between the various isolates. The increase in recovered bacteria systemically post H623 infection correlated with a significant increase of IL-17A in the serum compared to sham-treated mice (Figure 6.13). WT *S. pyogenes* (H305) and H623 infected mice appeared to have increased concentration of IFN-γ in the blood (Figure 6.13). It would appear that the lack of SPEA in H325 lead to decreased levels of serum IL-17A and IFN-γ (Figure 6.13), suggestive that
this streptococcal superantigen may be a potent mediator of the observed “cytokine storm” in septic patients. The reverse can be said for H623, whereby upon removal of this superantigen lead to increased production of pro-inflammatory cytokines (Figure 6.13).

In conclusion, the presence of streptococcal superantigens such as SMEZ and SPEA modulate the disease pathology, particularly at the site of infection. This is demonstrated by the reduction in recovered bacteria in the thigh. However, the lack of SMEZ in H623 resulted in increased bacteraemia and systemic release of IL-17A, indicating different superantigens may have different effects on the immune response.

6.7 Streptococcal superantigens play a role in specific gene expression during in vivo infection

As differences in disease pathology were observed upon infection with the superantigen knockout mutants, the gene expression pattern during in vivo infection was next examined to see if the presence or absence of superantigens influenced the transcriptional profile. HLA-DQ8.Aβ0 mice were infected with 10^8 CFU of each of the various streptococcal superantigen knockout isolates and draining lymph nodes and spleens were harvested for qRT-PCR analysis.

qRT-PCR analysis of draining inguinal lymph node cDNA showed a reduction in foxp3 expression in the absence of smeZ transcription during H623 and H658 infection and this was not significantly different from sham-treated mice (Figure 6.14A). However, gitr and ctla4 expression did not match the reduction seen for foxp3 transcription, reflective that these two markers are not restricted to Tregs (Figure 6.14A). t-bet and roryt expression was up-regulated during infection with no differences between all the different GAS knockout isolates (Figure 6.14A). There was no change in gata3 gene expression upon infection with the S. pyogenes superantigen knockout mutants (Figure 6.14A). This suggests that in the draining
lymph node, only foxp3 gene expression was primarily affected by the absence of SMEZ superantigen.

Figure 6.14: qRT-PCR analysis of transcription factors expression during acute superantigen knockout S. pyogenes infection.

HLA-DQ8.Aβ0 females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or 2-4x10^8 CFU H305, 3-7x10^8 CFU H326, 3-7x10^8 CFU H623, 2-6x10^8 CFU H658 at t=0h as part of 2 independent experiments for 24h. DLN (A) and spleen (B) were harvested for qRT-PCR analysis of transcription factor gene expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
In the spleen, foxp3 expression was unchanged during in vivo infection with the different superantigen knockout isolates (Figure 6.14B), which contradicts the transcriptional profile observed in the draining lymph node (Figure 6.14A). gitr expression followed a similar pattern of expression to foxp3 and was significantly down-regulated in spea knockout mutants compared to WT H305 (Figure 6.14B). ctl4 expression was significantly up-regulated in all strains of S. pyogenes compared to sham-treated mice with no difference between the different isolates (Figure 6.14B). t-bet and rorb expression was down-regulated in H658 compared to WT H305 in the spleen, indicating that SPEA may be required to induce transcriptional changes in these genes (Figure 6.14B). gata3 expression was unchanged in the spleen, similarly to the draining lymph node (Figure 6.14B).
Figure 6.15: qRT-PCR analysis of cytokine expression in the DLN during acute superantigen knockout *S. pyogenes* infection.

HLA-DQ8.AB^0^ females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or superantigen knockout *S. pyogenes* isolate (2-4x10^8^ CFU H305, 3-7x10^8^ CFU H326, 3-7x10^8^ CFU H623, 2-6x10^8^ CFU H658) at t=0h as part of 2 independent experiments for 24h. DLN were harvested for qRT-PCR analysis of cytokine gene expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
In the inguinal draining lymph nodes, most of the transcripts measured were up-regulated apart from *il-17a* and *il-9* (Figure 6.15). *il-4* transcription was unchanged upon infection with all streptococcal isolates in the draining lymph node (Figure 6.15). There was no significance between found between H305 WT isolates and the streptococcal knockout isolates (Figure 6.15). Increased transcription of pro-inflammatory cytokines such as *il-6* and *tnf-α* were reflective of systemic infection (Figure 6.15) and correlated with high IL-6 production in the serum of *S. pyogenes* infected mice (Figure 6.13). *ifn-γ* was only significantly up-regulated in H623 compared to sham infected mice, suggestive that the removal of SMEZ may contribute to this effect (Figure 6.15) and matched the production of serum IFN-γ (Figure 6.13).

In the spleen, all transcripts apart from *tgf-β* and *tnf-α* were up-regulated upon infection with the GAS isolates (Figure 6.16). The only significant difference between H305 WT infection and the superantigen knockout mutants was found with H326 infection. The lack of SPEA in this isolates resulted in decreased *il-10* and *il-6* gene expression (Figure 6.16), correlating with a modest decrease in serum IL-6 (Figure 6.13) and reduced bacterial burden (Figure 6.12B).
Figure 6.16: qRT-PCR analysis of cytokine expression in the spleen during acute superantigen knockout *S. pyogenes* infection.

HLA-DQ8.Aβ0 females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or superantigen knockout *S. pyogenes* isolate (2-4x10^8 CFU H305, 3-7x10^8 CFU H326, 3-7x10^8 CFU H623, 2-6x10^8 CFU H658) at t=0h as part of 2 independent experiments for 24h. Spleens were harvested for qRT-PCR analysis of cytokine gene expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Figure 6.17: qRT-PCR analysis of mpo expression during acute superantigen knockout S. pyogenes infection.

HLA-DQ8.β0 females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or superantigen knockout S. pyogenes isolate (2-4x10⁸ CFU H305, 3-7x10⁸ CFU H326, 3-7x10⁸ CFU H623, 2-6x10⁸ CFU H658) at t=0h as part of 2 independent experiments for 24h. DLN and spleen were harvested for qRT-PCR analysis of mpo expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.

qRT-PCR analysis of innate markers such as MPO was determined to examine whether the presence or absence of superantigens had an impact on neutrophil activation. In the lymph node, mpo expression was found to be down-regulated upon infection with S. pyogenes compared to sham-treated controls, with a significantly increase in H623 compared to H305 (Figure 6.17). In the periphery, there was an increase in mpo expression in the spleen during H326 infection, inducing high level of expression compared to PBS-treated mice (Figure 6.17).

Overall, it was deduced that the lack of SMEZ [often considered the most potent of the superantigens (Unnikrishnan et al., 2002)] in H623 and H658 was associated with reduced expression of foxp3 in the draining lymph node, possibly via tgf-β induction and that SPEA may be responsible for systemic induction of t-bet. This suggests that particular superantigens may contribute to the differentiation of different T cell subsets, influencing cytokine production. For example, ifn-γ expression was up-regulated in H623 infected lymph nodes, perhaps due to the lack of Treg differentiation induced by SMEZ.
6.8 Kinetics of T\textsubscript{regs} during streptococcal superantigen knockout isolates infection

To confirm whether SMEZ superantigen was responsible for Foxp3 induction as suggested by qRT-PCR analysis, direct detection of T\textsubscript{regs} was performed in HLA-DQ8.AB\textsuperscript{0} mice infected with 10\textsuperscript{8} CFU of each of the various streptococcal knockout isolates. Draining lymph nodes and spleens were harvested to assay the kinetics of T\textsubscript{regs} during \textit{in vivo} infection by flow cytometry.

In the draining lymph nodes, it was interesting to note that infection with H623 (smez\textsuperscript{−/−}) resulted in a significantly decreased expression of Foxp3 per cell (MFI) compared to sham controls (Figure 6.18A). This mirrored the down-regulation of \textit{foxp3} expression in the lymph node of H623 infected mice (Figure 6.14A). Despite a fall in Foxp3 expression per cell, there was a significantly increase in the percentage of CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{+} cells of the total cell population upon infection with H623 (Figure 6.18A). In the spleen, there was a consistent reduction in Foxp3 MFI expression upon WT \textit{S. pyogenes} infection (Figure 6.18B). H326 infection resulted in a slight increase in Foxp3 MFI (Figure 6.18B). Overall, in the spleen, there was no change in the percentage of CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{+} cells of the total cell population (Figure 6.18B).
Figure 6.18: Flow cytometric analysis of T<sub>reg</sub> during acute superantigen knockout <i>S. pyogenes</i> infection.

HLA-DQ8.Ab<sup>0</sup> females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or 5x10<sup>8</sup> CFU H305, 7x10<sup>8</sup> CFU H326, 8x10<sup>8</sup> CFU H623, 6x10<sup>8</sup> CFU H658 at t=0h for 24h. DLN (A) and spleens (B) were harvested for flow cytometric analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells. Mean and SEM of Foxp3 MFI (left) and percentage of total cells (right) of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells are represented. 1-way ANOVA statistical test was carried out on absolute numbers and Foxp3 MFI data and Kruskal-Wallis statistical test was carried out on percentage of total cells and percentage of lymphocytes data. Any significance differences are indicated: *p<0.05, **p<0.01, ***p<0.001.

In summary, analysis of T<sub>reg</sub> kinetics during superantigen-dependent infection by flow cytometry suggests the presence of SMEZ was responsible for Foxp3 MFI expression in T<sub>reg</sub> in the draining lymph node, however it did not affect the expansion in absolute numbers or percentages of this subset during infection. Very little effect of the presence or absence of streptococcal superantigens was seen in the spleen.
6.9 Bacterial profile upon depletion of T\(_{regs}\) in the absence of streptococcal superantigens

From the experiments so far, it would appear the absence of certain superantigens such as SMEZ resulted in down-regulation of foxp3 gene expression (Figure 6.14A) and decrease in Foxp3 expression in CD4\(^+\)CD25\(^+\)Foxp3\(^+\) cells in the draining lymph nodes (Figure 6.18A). This was accompanied by high levels of recovered bacteria in the blood (Figure 6.12B). This was suggestive that this streptococcal superantigen may have a role in T\(_{reg}\) induction, correlating with systemic disease upon H623 infection. I decided to investigate the disease profile upon specific depletion of T\(_{regs}\) in superantigen-mediated infection. This was achieved by crossing HLA-DQ8.A\(\beta^0\) mice with Foxp3.DTR mice to create a new transgenic model (HLA.DQ8.A\(\beta^0\).DTR) to study the role of T\(_{regs}\) during GAS infection.

HLA.DQ8.A\(\beta^0\).DTR mice were divided into 2 main treatment groups, those receiving doses of PBS to maintain their population of Foxp3\(^+\) cells and those receiving doses of Diphtheria Toxin (DT) to deplete this population. Each of these treatment groups was infected with the panel of four superantigen knockout isolates (n=8 for DT treated mice and n=2 for PBS treated group). Sham infected mice (n=2) were also included in this study for comparison to infected mice.
Figure 6.19: Bacterial counts from superantigen knockout *S. pyogenes* infection in HLA-DQ8.Aβ0.DTR transgenic model.

HLA-DQ8.Aβ0.DTR females were treated with DT or PBS i.p (n=8 per DT per isolate group and n=2 per PBS per isolate group) every day for 2 days prior to infection with PBS (sham, n=2) or superantigen knockout *S. pyogenes* isolate (2x10^9 CFU H305, 2x10^9 CFU H326, 9x10^8 CFU H623, 4x10^8 CFU H658) for 24h. Bacterial counts for thigh, blood, spleen and liver were calculated and mean and SEM represented. Kruskal-Wallis statistical test was carried out and any significant differences are indicated: *p<0.05. Sham infected mice did not show any bacterial growth from sampled organs (data not shown).

Specific depletion of Foxp3^+^ cells by the administration of DT resulted in increased levels of recovered superantigen knockout mutant bacteria in the thigh compared to infected mice containing Foxp3^+^ cells (Figure 6.19). The only exception to this was during H658 infection, where no difference was found between mice with or without T_{reg}S (Figure 6.19). In the blood, spleen and liver, there were only subtle differences in the bacterial counts between the different superantigen knockout isolates either in DT or PBS treated groups (Figure 6.19). No bacterium was recovered from any tissues from sham-infected mice (data not shown).
Figure 6.20: Cytokine responses from superantigen knockout *S. pyogenes* infection in HLA-DQ8.Aβ8.DTR transgenic model.

HLA-DQ8.Aβ8.DTR females were treated with DT or PBS i.p (n=8 per DT per isolate group and n=2 per PBS per isolate group) for 2 days prior to infection with PBS (sham, n=2) or superantigen knockout isolate (2x10⁹ CFU H305, 2x10⁹ CFU H326, 9x10⁸ CFU H623, 4x10⁸ CFU H658) for 24h. Serum was collected and concentrations of IL-6, IL-17A and IFN-γ determined by ELISA and mean and SEM represented. 1-way ANOVA statistical test was carried out and no significant differences were found.

Cytokine ELISA analysis of serum harvested from *S. pyogenes* superantigen knockout isolates infected DT or PBS treatment groups revealed that IL-6 was marginally elevated in the knockout mutant infected mice compared to H305 treated groups (Figure 6.20). There was no difference between DT and PBS treated groups. There appeared to have slightly higher levels of IL-17A and IFN-γ in the blood of the infected DT groups compared to PBS treated groups (Figure 6.20). This was in line with the concept of depleting Tregs removes control over effector cell populations and hence cytokine production.

It would appear that depletion of Foxp3⁺ cell during infection had the greatest effect at the site of local infection, with more bacteria detected in the thigh. This effect was dependent on the presence of superantigens such as SPEA and SMEZ. Systemically, no significant differences were observed upon the removal of Foxp3⁺ cells or superantigens. Thus the presence of Foxp3⁺ cells was associated with worse bacterial clearance at the site of acute infection and survival of GAS was enhanced by the presence of SMEZ and SPEA.
6.10 Flow cytometric analysis of cytokine producing cells upon removal of \( \text{T}_{\text{regs}} \)

Analysis of IFN-\( \gamma \) and IL-17A producing cell types during superantigen knockout infection was carried out to better define the source of the modest elevation seen in serum cytokines. Groups of HLA.DQ8.A\( \beta^0 \).DTR mice were treated with DT or PBS to remove or maintain Foxp3\(^+\) cells before infection with the four streptococcal superantigen knockout isolates or PBS (sham) for 24h. Inguinal lymph nodes and spleens were harvested for flow cytometric analysis of IFN-\( \gamma \) and IL-17A producing cell populations.

**Figure 6.21:** Cell types in DLN from superantigen knockout \( S. \text{pyogenes} \) infection in HLA-DQ8.A\( \beta^0 \).DTR transgenic model.

HLA-DQ8.A\( \beta^0 \).DTR females were treated with DT or PBS i.p (n=8 per DT per isolate group and n=2 per sham per isolate group) every day for 2 days prior to infection with either PBS (sham, n=2) or \( 2 \times 10^7 \) CFU H305, \( 2 \times 10^9 \) CFU H326, \( 9 \times 10^8 \) CFU H623, \( 4 \times 10^8 \) CFU H658 for 24h. In the last 4h of infection, BFA was administrated i.p. DLN were harvested for \textit{ex vivo} cytokine flow cytometry analysis. Absolute number of cell types (top) and percentage of cytokine producing cells (bottom) from lymphocyte gate in the draining lymph node were calculated and mean and SEM represented. Kruskal-Wallis statistical test was carried out and no significant difference was found.
In the inguinal draining lymph nodes, there was generally increased absolute numbers in all cell populations upon GAS infection compared to sham-infected controls. Depletion of Foxp3+ cells was not associated with significant differences in the absolute numbers of any cell populations, nor in the percentage of IFN-γ+ and IL-17A+ cells in the lymphocyte gate (Figure 6.21).

![Figure 6.22](image)

**Figure 6.22:** IL-17A and IFN-γ production in DLN from superantigen knockout *S. pyogenes* infection in HLA-DQ8.Aβ0.DTR transgenic model.

HLA-DQ8.Aβ0.DTR females were treated with DT or PBS i.p (n=8 per DT per isolate group and n=2 per sham per isolate group) for 2 days prior to infection with PBS (sham, n=2) or superantigen knockout isolate (2x10⁹ CFU H305, 2x10⁹ CFU H326, 9x10⁸ CFU H623, 4x10⁸ CFU H658) at t= for 24h. In the last 4h of infection, BFA was administrated i.p. DLNs were harvested for *ex vivo* cytokine flow cytometry analysis. Percentage of lymphocytes (top) and absolute number (bottom) of IL-17A (A) and IFN-γ (B) producing cells in the draining lymph node calculated and mean and SEM represented. Kruskal-Wallis statistical test was carried out and no significant difference was found.

The main source of IL-17A during *S. pyogenes* infection in this transgenic model surprisingly appeared to be CD8+ T cells (Figure 6.22A), reminiscent of the findings on GAS infection discussed in Chapter 4. Upon depletion of Foxp3+ cells, there were higher absolute numbers of CD8+ T cells and NK cells compared to sham-infected controls. IFN-γ responses seemed to be more dominant than IL-17A responses with
more cells producing this cytokine. CD8$^+$, CD4$^+$ T cells and NK cells were the main source of IFN-γ (Figure 6.22B) with CD8$^+$ T cells dominating. Upon the depletion of Foxp3$^+$ cells in the DT treated group, there were higher absolute numbers of CD8$^+$ T cells and NK cells producing IFN-γ compared to sham-infected controls, little difference being seen when mice were infected with the various superantigen knockout isolates (Figure 6.22B).

![Graph](image1)

**Figure 6.23:** Cell types in the spleen from superantigen knockout *S. pyogenes* infection in HLA-DQ8.Aβ0.DTR transgenic model.

HLA-DQ8.Aβ0.DTR females were treated with DT or PBS i.p (n=8 per DT per isolate group and n=2 per sham per isolate group) for 2 days prior to infection with PBS (sham, n=2) or 2x10^7 CFU H305, 2x10^7 CFU H326, 9x10^8 CFU H623, 4x10^8 CFU H658 for 24h. In the last 4h of infection, BFA was administrated i.p. Spleens were harvested for ex vivo cytokine flow cytometry analysis. Absolute number of cell types (top) and percentage of cytokine producing cells from lymphocyte gate (bottom) in the spleen were calculated and mean and SEM represented. Kruskal-Wallis statistical test was carried out and no significant difference was found.
In the spleen, there was a general reduction in the absolute number of all cell populations in infected mice upon the administration of DT compared to sham controls (Figure 6.23). There was however, an increase in the percentage of IL-17A and IFN-γ producing cells within the total population upon infection compared to sham controls with little difference between mice containing or depleted of Foxp3\(^+\) cells (Figure 6.23).

**Figure 6.24: IL-17A and IFN-γ production in the spleen from superantigen knockout S. pyogenes infection in HLA-DQ8.Aβ\(^0\).DTR transgenic model.**

HLA-DQ8.Aβ\(^0\).DTR females were treated with DT or PBS i.p (n=8 per DT per isolate group and n=2 per sham per isolate group) for 2 days prior to infection with PBS (sham, n=2) or superantigen knockout isolate (2x10\(^9\) CFU H305, 2x10\(^9\) CFU H326, 9x10\(^8\) CFU H623, 4x10\(^8\) CFU H658) for 24h. In the last 4h of infection, BFA was administrated i.p. Spleens were harvested for ex vivo cytokine flow cytometry analysis. Percentage of lymphocytes (top) and absolute number (bottom) of IL-17A (A) and IFN-γ (B) producing cells in the spleen were calculated and mean and SEM represented. Kruskal-Wallis statistical test was carried out and no significant difference was found.

NK cells were found to be main source of IL-17A in the spleen with a trend to increased absolute numbers or responding cells upon the removal of Foxp3\(^+\) cells (Figure 6.24A). This was also true for CD8\(^+\) T cells producing IL-17A (Figure
There were equal number of CD4$^+$, γδ$^+$ and NKT cells upon Foxp3$^+$ removal compared to mice treated with PBS (Figure 6.24A). As with the inguinal lymph node, the IFN-γ responses in the spleen were greater than IL-17A responses. CD4$^+$, CD8$^+$ cells and NK cells were all found to produce this cytokine (Figure 6.24B). Interestingly, H658 infected mice with their Foxp3$^+$ population intact appeared to contain large absolute numbers of NK producing IFN-γ, although this does not reach significance (Figure 6.24B).

As little difference was found in the production of IL-17A and IFN-γ upon the removal of Foxp3$^+$ cells during in vivo infection in the absence of superantigens, multi-cytokine analysis was performed to determine whether any other cytokines were affected by the removal of Foxp3$^+$ cells. Splenocytes from Foxp3.DTR mice, treated with PBS or DT were stimulated with cell-free supernatant from the four superantigen knockout isolates for 24h before supernatant collected for CBA analysis.

![Figure 6.25: Differential cytokine production upon removal of Foxp3$^+$ cells.](image)

Foxp3.DTR mice (n=5 mice per treatment group) were given 100µl PBS or DT (12.5µg/ml) for 2 days before spleens were harvested. 10$^6$ splenocytes were stimulated with cell-free supernatants from superantigen knockout isolate (H305, H326, H623 and H658), PMA/Ionomycin (positive control) and media only (negative control) for 24h. Supernatant was collected and CBA was carried out to measure the concentration of IFN-γ, TNF, IL-17A, IL-4, IL-9 and IL-10. Mean and SEM of concentrations minus background from media stimulated cells are represented and 1-way ANOVA statistical analysis was carried out and any significant differences indicated: *p<0.05.
PMA/Ionomycin stimulation of the splenocytes was carried out to serve as a positive control for cytokine production. Upon Foxp3+ cell removal, IFN-γ production was generally elevated compared to PBS treated splenocytes, in particular upon stimulation with cell-free supernatant from H623 (Figure 6.25). There was no difference in the production of TNF, IL-17A and IL-4 between PBS and DT-treated splenocytes (Figure 6.25). However, stimulation of DT-treated splenocytes with supernatant from H658 resulted in decreased production of IL-17A and IL-4, although not significant (Figure 6.25). IL-9 production was not stimulated by any of the *S. pyogenes* supernatants, with the exception of H326 in PBS-treated splenocytes (Figure 6.25). IL-10 production was not detected upon H305 supernatant stimulation in PBS-treated splenocytes (Figure 6.25).

Overall, the removal of Foxp3+ cell mediated control during infection with the panel of four streptococcal superantigen knockout isolates resulted in only a modest changes in systemic disease pathology. Although Foxp3+ cells may somehow aid bacterial clearance at the site of infection as shown by increased bacterial counts in infected DT-treated mice (Figure 6.19), this appears to be only partially dependent on superantigens (Figure 6.19). There was only a marginal increase in the production of IL-17A and IFN-γ in the serum of infected mice (Figure 6.20), mirrored by the subtle changes in the cytokine production as shown *ex vivo* (Figure 6.21-24) and *in vitro* (Figure 6.25). This collection of data would suggest that T<sub>regs</sub> do not play a significant role during systemic infection but may be beneficial at the site of the infection via cytokine-independent mechanisms.

### 6.11 TLR2 expression on T<sub>regs</sub> during acute *S. pyogenes* infection

There is evidence for a possible role for TLR2 in the activation of T<sub>regs</sub> during microbial infection (Sutmuller *et al.*, 2006). Whether this class of innate receptors is also involved in my model of *S. pyogenes* infection is unclear but it may explain how the rapid adaptive immune response observed during acute GAS infection. Flow
cytometry was performed to confirm that T\textsubscript{regs} expressed TLR2 and whether GAS influenced protein expression.

HLA-DQ8.A\textbeta\textsuperscript{0} blood was collected and cultured \textit{in vitro} with exponential phase H305 and H293 \textit{S. pyogenes} isolates for 4h before flow cytometric analysis of TLR2 expression on Foxp3\textsuperscript{+} cells was carried out in a pilot study. H305 stimulation increased expression of intracellular TLR2 in Foxp3\textsuperscript{+} cells compared to un-stimulated and H293 (M89 serotype GAS, lacking SMEZ) stimulated blood (Figure 6.26). This suggested there might be a specific interaction between \textit{S. pyogenes} and TLR2, in particular in T\textsubscript{regs}.

![Figure 6.26: Representative TLR2 expression of Foxp3\textsuperscript{+} cells in un-stimulated and GAS infected blood.](image)

Pooled HLA-DQ8.A\textbeta\textsuperscript{0} whole blood was preliminarily infected with H305 or H293 \textit{in vitro} for 4h and intracellular TLR2 stained in gated Foxp3\textsuperscript{+} cells. (Red line: isotype matched control; green line: TLR2 expressed in un-stimulated blood; blue line: H305 infected blood; brown line: H293 infected blood).

qRT-PCR analysis of HLA-DQ8.A\textbeta\textsuperscript{0} draining inguinal lymph nodes and spleens during acute WT \textit{S. pyogenes} infection revealed significantly elevated expression of \textit{tlr2} in the lymph node (Figure 6.27). The increase of \textit{tlr2} gene expression was also matched by the up-regulation of \textit{foxp3} expression over the same time course of
infection (Figure 6.6A), suggesting that tlr2 expression may be linked to WT S. pyogenes infection, perhaps in Tregs.

![Figure 6.27: qRT-PCR analysis of tlr2 expression during acute S. pyogenes infection.](image)

**Figure 6.27: qRT-PCR analysis of tlr2 expression during acute S. pyogenes infection.**

HLA-DQ8.Aβ0 females were infected with 1x10^8 - 10^9 CFU H305 S. pyogenes at t=0h as part of 2 independent experiments or PBS (sham). At various time points, mice were culled (n=10 per infected time point and n=5 per sham infected time point) and DLN or spleens harvested for qRT-PCR analysis of tlr2 expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.

![Figure 6.28: qRT-PCR analysis of tlr2 expression during acute superantigen knockout infection.](image)

**Figure 6.28: qRT-PCR analysis of tlr2 expression during acute superantigen knockout infection.**

HLA-DQ8.Aβ0 females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or superantigen knockout isolate (2-4x10^6 CFU H305, 3-7x10^6 CFU H326, 3-7x10^6 CFU H623, 2-6x10^6 CFU H658) as part of 2 independent experiments for 24h. DLNs and spleen were harvested for qRT-PCR analysis of tlr2 gene expression. Infected samples were normalised to tissue specific reference genes and sham treated samples. Fold change in expression was determined by REST analysis and mean and SEM represented. Any significant differences are indicated: *p<0.05, **p<0.01, ***p<0.001.
Previous evidence has suggested that streptococcal superantigens may contribute to increased expression of TLR2 on monocytes (Hopkins et al., 2008). Whether this interaction can also be extrapolated to T\textsubscript{regs} in my model of acute sepsis was next examined. HLA-DQ8.\textalpha\beta0 mice were infected with 10\textsuperscript{8} CFU of the streptococcal superantigen knockout isolates and the expression of tlr2 was studied by qRT-PCR. No differences were found in the expression of tlr2 in either the draining lymph node or spleen 24h post infection between the four various superantigen knockout isolates (Figure 6.28). However, the loss of SPEA in H326 and H658 up-regulated tlr2 expression in the spleen above sham-treated levels. This suggests SPEA may inhibit tlr2 gene expression in the spleen.

![Flow cytometric analysis of surface TLR2 expression during acute superantigen knockout S. pyogenes infection.](image)

Figure 6.29: Flow cytometric analysis of surface TLR2 expression during acute superantigen knockout S. pyogenes infection.

HLA-DQ8.\textalpha\beta0 females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or superantigen knockout S. pyogenes isolate (5x10\textsuperscript{8} CFU H305, 7x10\textsuperscript{8} CFU H326, 8x10\textsuperscript{8} CFU H623, 6x10\textsuperscript{8} CFU H658) for 24h. DLN (A) and spleens (B) were harvested for flow cytometric analysis of TLR2 expression on CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells. Mean and SEM of surface TLR2 MFI (left) and percentage of lymphocytes (right) of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+}TLR2\textsuperscript{+} cells are represented. 1-way ANOVA statistical test was carried out on absolute numbers and Foxp3 MFI data and Kruskal-Wallis statistical test was carried out on percentage of total cells and percentage of lymphocytes data. Any significant differences are indicated: **\textit{p}<0.01.
Flow cytometric analysis of surface TLR2 expressed on T\textsubscript{regs} was performed on the draining lymph node and spleen in the various superantigen knockout isolate infected mice. There was a significantly increased percentage of TLR2\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells in the lymphocyte population upon infection in the absence of SMEZ (H623) compared to sham-treated controls in the draining lymph node (Figure 6.29A). The expression of surface TLR2 on T\textsubscript{regs} in the spleen revealed a different picture compared to the draining lymph node. H658 infected mice showed elevated surface TLR2 MFI expression on CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells relative to H305 infected mice (Figure 6.29B). This is indicative that the absence of superantigens, in particular SPEA and SMEZ, contributed to the increased expression of surface protein TLR2 on T\textsubscript{regs}.

![Flow cytometric analysis of surface TLR2 expression](image)

**Figure 6.30:** Flow cytometric analysis of intracellular TLR2 expression during acute superantigen knockout \textit{S. pyogenes} infection.

HLA-DQ8.Aβ\textsuperscript{0} females (n=8 per isolate and n=3 per sham group) were infected with PBS (sham) or 5x10\textsuperscript{5} CFU H305, 7x10\textsuperscript{5} CFU H326, 8x10\textsuperscript{5} CFU H623, 6x10\textsuperscript{5} CFU H658 for 24h. DLN (A) and spleens (B) were harvested for flow cytometric analysis of TLR2 expression on CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells. Mean and SEM of surface TLR2 MFI (left), percentage of lymphocytes (right) of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+}TLR2\textsuperscript{+} cells are represented. 1-way ANOVA statistical test was carried out on absolute numbers and Foxp3 MFI data and Kruskal-Wallis statistical test was carried out on percentage of total cells and percentage of lymphocytes data. Any significant differences are indicated: *p<0.05, **p<0.01.
The expression of intracellular TLR2 on T\textsubscript{regs} was also assayed by flow cytometry. The percentage of TLR2\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells of lymphocytes was increased in H623 infected draining lymph node compared to sham-infected controls (Figure 30.B), similar to the surface expression of TLR2 (Figure 6.29A). This was indicative that the presence of SMEZ may inhibit the intracellular expression of TLR2 on T\textsubscript{regs} in the lymph node and spleen. In the spleen, there was a decrease in intracellular TLR2 MFI on CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} upon GAS infection, except for H326, which has the opposite effect of increased intracellular TLR2 expression in T\textsubscript{regs} (Figure 6.30B). This suggests that SPEA inhibits intracellular TLR2 expression in T\textsubscript{regs}. There was also increased percentage of TLR2\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells of the lymphocyte gate in the absence of SPEA and SMEZ (H658 infection) (Figure 6.30B). Overall, there appeared to be some evidence that streptococcal superantigens may be linked to TLR2 transcription and protein expression. The absence of SMEZ streptococcal superantigen in H623 during \textit{in vivo} infection resulted in an increase in the percentage of surface and intracellular TLR2\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} cells of lymphocytes. This was evidence that this particular superantigen may negatively influence TLR2 expression of T\textsubscript{regs}, but whether this is directly connected to \textit{foxp3} expression observed in Figure 6.14A and hence T\textsubscript{reg} induction, is unknown.

6.12 The role of TLR2 during acute WT \textit{S. pyogenes} infection

As TLR2 may have a role during T\textsubscript{reg} function and development as shown by other infectious disease models, this was also explored in my acute \textit{S. pyogenes} infection model. To achieve this, TLR2\textsuperscript{−/−} mice were utilise to determine the precise role of this innate receptor during GAS infection.
Figure 6.31: Percentage survival of *S. pyogenes* infected TLR2\(^{-/-}\) mice.

C57BL/6 (n=8) and TLR2\(^{-/-}\) (n=8) females were infected with \(6 \times 10^8\) CFU H305 WT *S. pyogenes* i.m at t=0h. Mice were monitored for percentage total weight loss or signs of septic shock and culled at defined end-points.

WT C57BL/6 and TLR2\(^{-/-}\) mice were infected with a lethal dose of H305 WT *S. pyogenes*. There appeared to be no difference in terms of survival between the two strains of mice infected (Figure 6.31). This implies that TLR2 has little role during lethal systemic infection.

This was also mirrored by little difference in bacterial counts recovered from WT and TLR2\(^{-/-}\) mice infected with \(10^9\) CFU H305 for 24h (Figure 6.32). There was a trend of lower bacterial counts in the blood of TLR2\(^{-/-}\) infected mice, indicative of perhaps decreased systemic bacterial burden. Serum IL-6 and IL-17A levels were also measured with no significant difference found, although serum IFN-\(\gamma\) was statistical higher in infected TLR2\(^{-/-}\) mice compared to WT mice (Figure 6.33).
Figure 6.32: Bacterial counts from *S. pyogenes* infected TLR2\(^{-/-}\) transgenic mice.

C57BL/6 (n=8) and TLR2\(^{-/-}\) (n=7) females were infected with 1x10^9 CFU H305 WT *S. pyogenes* i.m at t=0h for 24h. Bacterial counts were calculated for thigh, blood, spleen and liver and mean and SEM represented. Kruskal-Wallis statistical test was carried out and no significant differences found.

Figure 6.33: Cytokine responses from *S. pyogenes* infected TLR2\(^{-/-}\) transgenic mice.

C57BL/6 (n=8) and TLR2\(^{-/-}\) (n=7) females were infected with 1x10^9 CFU H305 WT *S. pyogenes* via i.m for 24h. Serum was collected and concentrations of IL-6, IL-17A and IFN-γ measured by ELISA. Mean and SEM are represented and 1-way ANOVA statistical test was carried out and any significant differences indicated: *p<0.05.

In summary, it appears that TLR2 signaling plays little role during acute *S. pyogenes* infection as demonstrated by the lack of significant difference in survival or bacterial counts recovered in WT and TLR2\(^{-/-}\) transgenic mice.
6.13 Discussion

The role of T\textsubscript{regs} during acute \textit{S. pyogenes} infection has not previously been characterised in detail. Furthermore, the precise role of immune responses induced by superantigens has not been fully characterised, though it is always assumed to be an immune subversion strategy. From the experiments performed and described in this chapter, it was clear that the adaptive immune response was initiated quicker than originally perceived, within hours of WT GAS infection. Increased bacterial burden over time (Figure 6.4B) correlated with high levels of serum IL-6 (Figure 6.5). \textit{foxp3} gene expression was increased in the draining lymph node and down-regulated in the spleen (Figure 6.6). This pattern of gene expression was also matched in the protein production as measured by flow cytometry in the draining lymph node and spleen (Figure 6.11).

The change in numbers of Foxp3\textsuperscript{+} T\textsubscript{regs} in the draining lymph node and spleen could be explained by the migration of T\textsubscript{regs} from the periphery to the lymph node at the site of infection. Tissue-specific migration can be achieved through the expression of particular receptors. For example, the integrin \(\alpha_4\beta_7\) and the chemokine receptor CCR9 are expressed by T\textsubscript{regs} to allow homing to the intestines and associated lymphoid tissues in response to inflammation (Menning \textit{et al.}, 2010). Other chemokines such as CCR2, 5 and 7 allow T\textsubscript{regs} to migrate to draining lymph nodes (Zhang \textit{et al.}, 2009a). Chemokine receptor and integrin expression on Foxp3\textsuperscript{+} T\textsubscript{regs} during acute GAS infection could be further investigated as another parameter to determine if this subset of cells is being recruited to the site of infection to dampen down immune responses.

Elevation of T\textsubscript{reg} absolute numbers and increased bacterial burden over time suggested that T\textsubscript{regs} may be contributing to pathogenesis in this \textit{in vivo} infection model. This is in line with data reported in Chapter 3, whereby \textit{S. pyogenes} infected Foxp3.DTR transgenic mice depleted of Foxp3\textsuperscript{+} cells have decreased bacterial burden, particularly in the blood (Figure 3.15). This observation however, can only be
confirmed if the suppressive phenotype of the increased number of T\textsubscript{regs} detected is fully characterised.

The observed change in T\textsubscript{reg} kinetics can be related to the presence of streptococcal superantigens. Models correlating T\textsubscript{regs} with superantigens produced as part of bacterial infection have not been previously described. The HLA-transgenic model utilised in this chapter provided me with a useful tool to study the effects on T\textsubscript{regs} induction by superantigens, normally secreted \textit{in vivo} by \textit{S. pyogenes}.

SPEA enhanced T\textsubscript{H1} associated transcription factor as demonstrated by the down-regulation of \textit{t-bet} during H326 (spea\textsuperscript{+/-}) infection (Figure 6.14B). SMEZ induced both Foxp3 gene (Figure 6.14A) and protein expression (Figure 6.18A). Knocking out these superantigens correlated with increased bacterial counts, particular in the blood in H623 infected mice (Figure 6.12B) and decreased bacterial burden in H326 infected mice (Figure 6.12B). This was paradoxical data, as it would suggest that the induction of Foxp3\textsuperscript{+} T\textsubscript{reg} cells, not observed during smez\textsuperscript{+/-} infection, may be beneficial during infection. The working hypothesis thus suggests that potent superantigen activation of T\textsubscript{reg}, is beneficial to bacterial pathogenesis and spread. This may be either because of some beneficial effect of the T cell cytokine storm, or more contentiously, because \textit{S. pyogenes} may sometimes be able to spread as an intracellular pathogen (LaPenta \textit{et al.}, 1994; Osterlund and Engstrand, 1997; Thulin \textit{et al.}, 2006; Staali \textit{et al.}, 2003). Removing the T\textsubscript{regs} from the activated mix thus simply allows the phenotype to be enhanced.

Results also suggested that T\textsubscript{H1} cells induced by SPEA are pathogenic as spea\textsuperscript{+/-} mice had lower levels of \textit{t-bet} expression and bacterial burden (Figure 6.12B). This was in contrast to data presented in Chapter 3 proposing T\textsubscript{regs} as pathogenic during acute \textit{S. pyogenes} infection. The different murine models used could explain this difference. Foxp3.DTR mice are not sensitive to superantigens and the heightened responses observed in HLA transgenic mice might be more prone to Foxp3\textsuperscript{+} mediated control induced by superantigens.

Depletion of Foxp3\textsuperscript{+} T\textsubscript{regs} during superantigen-mediated infection of HLA-DQ8.Ap\textsuperscript{0}.DTR mice reduced any specific effects of superantigens on T\textsubscript{regs} as no
significant difference was found in the systemic bacterial counts after infection with the superantigen knockout isolates (Figure 6.19). A difference in recovered bacterial numbers, dependent on superantigens was observed in the thigh upon depletion of Foxp3\(^+\) cells (Figure 6.19), suggesting that superantigens interacting on T\(_{\text{regs}}\) at the site of infection are beneficial. This supports the general conclusion that there was a previously unknown interaction between streptococcal superantigens and T\(_{\text{regs}}\) in this model.

*Ex vivo* intracellular staining of various cell populations during infection with the superantigen knockout *S. pyogenes* isolates upon Foxp3\(^+\) cell depletion did not reveal any strong changes in cytokine production (Figure 6.22 & 6.24). Only modest elevations in IFN-\(\gamma\) production in CD8\(^+\) and NK cells were observed upon the removal of T\(_{\text{reg}}\) (Figure 6.22). The importance of these cell types during acute streptococcal infection has already been highlighted in Chapter 4. This suggests that if superantigens such as SMEZ are inducing T\(_{\text{regs}}\) to alter the pathogenesis, it is not via release of T\(_{\text{reg}}\) mediated control over effector cell production of cytokines.

Previous work from other groups has demonstrated an interaction between superantigens and T\(_{\text{reg}}\) induction (Papiernik, 2001; Taylor and Llewelyn, 2010), reinforcing results in this chapter showing superantigens, such as SMEZ can induce T\(_{\text{regs}}\). Most work has concentrated on purified superantigen commonly secreted by *S. aureus*, such as SEA and SEB. Repeated exposure of superantigens induce a state of tolerance with modulated Th1 cytokines release (e.g. IFN-\(\gamma\) and IL-2) and was associated with induction of regulatory CD4\(^+\)CD25\(^+\) T cells secreting IL-10 (Noël *et al.*, 2001; Pontoux *et al.*, 2002; Feunou *et al.*, 2003; Grundström *et al.*, 2003). This regulatory T cell was confirmed to be Foxp3\(^+\) T\(_{\text{regs}}\) due to induced Foxp3 expression (Schartner *et al.*, 2008). The addition of TGF-\(\beta\) before re-stimulation of CD4\(^+\) T cells with SEB enhanced this cell population’s capacity to produce this cytokine and was correlated with suppressive activity over B cell mediated antibody production (Zheng *et al.*, 2002).

Not all superantigens have been associated with T\(_{\text{reg}}\) induction. SEB has been shown to inhibit T\(_{\text{regs}}\) isolated from human PBMCs in an APC-dependent manner (Cardona
et al., 2006). This was achieved through GITR-L expression on monocytes induced by SEB exposure and was followed by T_{reg} proliferation and loss of suppressive function (Cardona et al., 2006). This could be extrapolated to explain why SPEA secreted by \textit{S. pyogenes} showed a slight trend of inhibiting \textit{foxp3} gene expression and stimulated induction of T_{H1} cells, as characterised by increased \textit{t-bet} gene expression (Figure 6.14).

Superantigens driven responses are not just limited to T_{reg} and other cells of adaptive immunity. Innate immunity is also influenced by the presence of superantigens during infection. Tanriver and colleagues (Tanriver et al., 2009) showed that endogenous superantigens encoded in the Mouse Mammary Tumour Virus (MMTV) genome, randomly integrated into the mouse genome prevented the migration of NK cells and matured DCs. This was reminiscent of qRT-PCR analysis showing that upon the absence of SMEZ compared to WT infection, there was significant up-regulation of \textit{mpo}, an activation marker of neutrophils (Figure 6.17). Perhaps, this particular superantigen linked to \textit{foxp3} expression could be also be preventing the recruitment and activation of innate cell population, such as neutrophils.

Microbial products are key in initiating the immune response during infection with most research focused on TLRs. T_{reg} have been shown to express TLRs, for example TLR2 (Figure 6.26). It has also been suggested that TLR2 signaling can also stimulate T_{reg} proliferation (Chen et al., 2009a). Much interest has been generated in this specific PRR, generally associated with increased signal transduction during Gram-positive infection with some groups concentrating on TLR2 expression of T_{reg} during fungal infection (Sutmuller et al., 2006). Superantigens can induce TLR2 expression on monocytes (Hopkins et al., 2008), however whether TLR2 expression on T_{reg} is involved during GAS infection was partially addressed in this chapter and requires further work.

TLR2 gene expression was induced during a short time course of \textit{S. pyogenes} infection and matched with the pattern of up-regulation in \textit{foxp3} expression (Figure 6:27). This increase in \textit{tlr2} expression weakly correlated with the presence or absence of streptococcal superantigens as \textit{in vivo} infection with superantigen knockout isolates did not change \textit{tlr2} expression in the draining lymph node (Figure 6.28).
However, upon infection in the spleen with smeZ<sup>−/−</sup> (H623) a slight down-regulation in tlr2 expression was detected and was compared with sham controls (Figure 6.28). This weakly suggested that SMEZ superantigen might be involved in the induction of tlr2 gene expression in the spleen.

Superantigens also affected TLR2 expression on the surface and in intracellular compartments of T<sub>regs</sub> during in vivo infection (Figure 6.29 & 6.30). The percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells of lymphocytes expressing surface TLR2 was significantly elevated during H623 infection in the draining lymph node (Figure 6.29A) and the expression of surface TLR2 per CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in the spleen was increased in H658 infected mice compared to WT H305 controls (Figure 6.29B). However, absolute numbers of T<sub>regs</sub> expressing intracellular TLR2 was reduced during H623 infection, correlating with qRT-PCR data (Figure 6.30B). Flow cytometric analysis of T<sub>regs</sub> in the draining lymph node showed that SMEZ might in fact be inhibitive resulting in decreased surface TLR2 protein expression and percentage of T<sub>reg</sub> of lymphocytes to express surface TLR2.

Correlating increased bacterial burden seen in H623 infected mice with decreased foxp3 expression and increased percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells expressing surface TLR2 would suggest a possible switch in T<sub>reg</sub> phenotype in the absence of SMEZ. Previous work has documented that TLR2 controls the expansion and function of T<sub>regs</sub> (Sutmuller et al., 2006). As the phenotype of the T<sub>regs</sub> during this model of acute sepsis is unknown, we cannot state whether TLR2 is suppressing the function of T<sub>regs</sub> as proposed by Oberg and colleagues (Oberg et al., 2010) or stimulating T<sub>H1</sub> responses (Patel et al., 2005).

Whether there is an interaction between superantigens and TLR2 expression still remains inconclusive and more research required. Other streptococcal virulence factors may be driving TLR2 signaling upon GAS infection. For example, the role of M proteins has previously been identified to act via TLR2 on human monocytes to drive an inflammatory response (Pålhamn et al., 2006). This particular virulence factor has not been addressed in this study.
The precise role of TLR2 during *S. pyogenes* infection was further investigated through the use of *TLR2*−/− knockout mice. However, upon either lethal or sub-lethal infection with WT *S. pyogenes*, there was no significant difference in survival or bacterial burden (Figure 6.31 & 6.32). This was indicative that TLR2 and subsequent downstream signaling were dispensable during GAS infection. There was a slight drop in the number of recovered bacteria from the blood of infected TLR2−/− mice, suggesting these mice were less bacteraemic than WT mice (Figure 6.32). An increase in serum IFN-γ was also detected in TLR2−/− mice (Figure 3.33). Perhaps, pro-inflammatory responses from innate immune cell types may be activated independent of TLR2 signaling, similar to work published by Gratz and colleagues (Gratz *et al.*, 2008).

Overall, the kinetics of Tregs during acute *S. pyogenes* infection was revealed and correlated to the presence of key streptococcal superantigens for the first time. SMEZ was found to be responsible for enhanced Foxp3 gene and protein expression, inducing Tregs, which limited bacterial burden. This suggests that in this model of acute sepsis, Tregs are beneficial. The precise mechanism of signal transduction leading to *foxp3* up-regulation is unknown. SMEZ was yet again found to induce *tlr2* gene expression in the spleen and correlated with low absolute numbers of Tregs expressing this intracellular receptor in the absence of this superantigen. However, these effects were small as TLR2 overall was found to be redundant in the survival post lethal *S. pyogenes* infection.
Chapter 7

7 Conclusions and general discussion

In this thesis, I initially characterised the relative contribution of adaptive and innate immune responses during acute \textit{S. pyogenes} infection. I present evidence indicative of a potential role for T\textsubscript{regs} in exacerbation of pathology during acute bacterial sepsis and interactions with specific streptococcal virulence factors. In the following section, I will summarise my results and the implications of these findings within the context of previous published data regarding immunity mounted during Gram-positive infection.

7.1 Summary of the role of T\textsubscript{regs} during acute streptococcal infection

\textit{S. pyogenes} is the causative agent of divergent diseases, from non-invasive (impetigo and “strep throat”) to severely invasive (necrotising fasciitis and bacterial sepsis) (Cunningham, 2000). High mortality is associated with bacterial sepsis and remains a large burden on the nation health service. This Gram-positive human pathogen has a large array of virulence factors to evade and modulate the immune response (Cunningham, 2000). Most studies conducted on T cell mediated immunity during bacterial systemic infection have utilised the poly-microbial CLP animal model of experimental sepsis (Sherwood \textit{et al.}, 2004; Chung \textit{et al.}, 2006; Enoh \textit{et al.}, 2008; Enoh \textit{et al.}, 2007). However, very little work has been performed regarding the direct intimate interaction of this bacterium with T cells during \textit{in vivo} acute infection.

From the initial studies of αβ and γδ T cells during acute \textit{S. pyogenes} infection as discussed in Chapter 3, it appears that T cell-mediated immunity is detrimental to survival outcome after GAS infection via an intra-muscular route of challenge. On further dissection of this observation, regulatory αβ\textsuperscript{+} T cells, classically defined as
T\(_{\text{regs}}\) were found to be pathogenic, as induced depletion of this subset in Foxp3.DTR mice limited dissemination of systemic infection. Unleashing of T\(_{\text{reg}}\)-mediated control of \(\gamma\delta^+\) T cells lead to increased IL-17A production in the spleen and was supported by a trend to increased, systemic bacterial burden in IL-17A\(^{-/-}\) and TCR \(\delta^+\) mice; however this observation will need to be further confirmed.

Upon more detailed investigation of the kinetics of T\(_{\text{regs}}\) during S. pyogenes in Chapter 6, increased Foxp3 gene and protein expression in peripheral lymphoid organ correlated with increased bacterial burden over a short time course of GAS infection in HLA-DQ8.A\(\beta^0\) transgenic mice. This further supports the conclusion that the presence of T\(_{\text{regs}}\) is detrimental during S. pyogenes infection. Exploiting the sensitivity of this HLA transgenic model to streptococcal superantigens, it was determined that SMEZ superantigen expression was necessary to induce foxp3 gene expression. Depletion of Foxp3\(^+\) cells in HLA-DQ8.A\(\beta^0\).DTR mice resulted in the loss of superantigen driven systemic pathology. Thus, this is evidence that specific streptococcal virulence factors can modulate T\(_{\text{reg}}\)-mediated immune responses.

Overall, fast kinetics of T\(_{\text{regs}}\) in terms of foxp3 gene expression was observed within hours of acute bacterial infection, indicative that this branch of adaptive immunity initiates immune regulation faster than might have been thought. This induction was partially attributed to secreted potent streptococcal superantigens rapidly promoting a state of immune suppression.

7.2 The hypothesised role of T\(_{\text{regs}}\) during acute GAS infection

Much scientific progress has been made since the original proposal of “suppressor T cells” in the 1970s (Gershon, 1975). T\(_{\text{regs}}\) have been characterised as suppressors of both adaptive and innate immune responses (Sakaguchi, 2004) and research has been conducted regarding this cell type within the context of various infectious pathogens with differing survival outcomes shown (Belkaid, 2007). From the published work to
date, it is becoming more and more clear, a fine balance between \( T_{\text{reg}} \)-mediated suppression and enhanced effector function exists in order to protect the body from collateral damage caused by potent anti-microbial responses without impeding efficient eradication of harmful pathogens from the host (Belkaid and Rouse, 2005; Suvas and Rouse, 2006).

During infection, it may be beneficial for pathogens to induce, recruit and maintain the survival of \( T_{\text{reg}} \), so as to promote their own persistence within the body. \( T_{\text{reg}} \) induction can be achieved by influencing the cytokine milieu to favour conversion of naïve CD\(^{+}\) T cells into \( T_{\text{reg}} \) through the production of IL-10 or TGF-\( \beta \) from other cell types, such as monocytes (Walther \textit{et al.}, 2005; Suvas \textit{et al.}, 2003). The resultant effect of \( T_{\text{reg}} \) induction during infectious diseases has been extensively studied, however, different pathogens can invoke different immunological responses, which will be discussed in the following section.

A protective effect of \( T_{\text{reg}} \) during infection has been demonstrated in murine models with various pathogenic microorganisms. For example, \( T_{\text{reg}} \) delay the recruitment of DCs, NK and T cells into draining lymph nodes of mice upon infection with Herpes Simplex virus (HSV) to limit immunopathology (Lund \textit{et al.}, 2008). Parasitic infection with African trypanosomes can lead to potent TH\(^{1} \) immune responses, however, n\( T_{\text{reg}} \) producing IL-10 can expand in the spleen and liver of infected C57BL/6 mice to constrain immune pathology exacerbated by CD\(^{+}\) IFN-\( \gamma \) T cells (Guilliams \textit{et al.}, 2007). \( T_{\text{reg}} \) were also found to suppress inflammation during \textit{Pneumocystis carinii} infection (McKinley \textit{et al.}, 2006), suggestive that this cell type may ameliorate disease. It has also been proposed that \( T_{\text{reg}} \) can activate immune cells as well as suppress to indirectly counter infection. This has been demonstrated during \textit{Candida albicans} infection in mice, whereby \( T_{\text{reg}} \) can up-regulate the expression of TH\(^{17} \)-associated cytokine genes such as \textit{il-17a}, \textit{il-17f} and \textit{il-22} in CD\(^{4} \) T cells to aid fungal clearance (Pandiyan \textit{et al.}, 2011).

The profound importance of \( T_{\text{reg}} \) in suppressing over-activated immune responses and inflammation during infection is also seen in the context of the gastro-intestinal tract and autoimmunity. The link between \( T_{\text{reg}} \) and a pathological intestinal immune
responses was initially demonstrated in human patients with IPEX syndrome: autoimmunity arises from the lack of Foxp3+ cells often encompassing severe intestinal inflammation (Gambineri et al., 2003). As the gut is home to many different commensal bacteria and a major source of exogenous antigens, it is easy to appreciate the beneficial role of Tregs in dampening down immune response and preventing inflammation under normal conditions as an example of oral tolerance (Barnes and Powrie, 2009). Effector cells such as γδ T cells, normally under the control of Tregs have been implicated as pathogenic with the ability to induce inflammation in the intestines to lead to colitis (Park et al., 2010). The number of Tregs can be regulated within the gut to also maintain tolerance. The administration of anti-RANK-L monoclonal antibody in vivo augmented Treg-mediated suppression of colitogenic CD4+ T cells to suggest that the RANK/RANK-L signaling pathway between dendritic and Treg cells may be an important suppressive mechanism by which Tregs can prevent DCs from presenting antigen to naïve CD4+ T cells (Tomita et al., 2009). This overall highlights the importance of Treg-mediated suppression of activated effector cells to control intestinal inflammation.

The beneficial effects of Treg-mediated suppression have not been found across all types of infections. Detrimental effects of the presence and function of Tregs have been proposed for plasmodium infection in mice, whereby depletion of CD4+CD25+ cells protected mice against lethal infection with Plasmodium yoelii (Hisaeda et al., 2004). Delayed immune responses were also observed in mice infected with M. tuberculosis due to suppression by Tregs, allowing expansion of mycobacterium in the lung (Shafiani et al., 2010). A similar observation was also made during Friend viral infection, whereby, Treg-mediated suppression of CD8+ T cells lead to inefficient viral clearance (Myers et al., 2009). nTregs are also induced during Helicobacter pylori infection in the gut to allow bacterial colonisation and persistence by the induction of tolerance and Th17 cell suppression (Kao et al., 2010). Fungi, such as Aspergillus fumigatus can successful evade the immune response through the differentiation of Tregs to allow for establishment of chronic lung infection (Montagnoli et al., 2006), demonstrating that many pathogens exploit immune regulation to their own survival advantage.
Our original premise on initiating this study perhaps veered to the likelihood that, since streptococcal sepsis is often characterised in terms of the key role of an excessive cytokine response in disease outcome, this might be a setting in which T_{regs} would have a protective role to dampen down heightened effector function. Since infection by GAS is generally regarded as a case of superantigen shock from an overactive immune response, along with genetic risk factors (Kotb et al., 2002; Welcher et al., 2002; Llewelyn et al., 2004), this might have been predicted to be a case where T_{regs} are protective. Here in Chapter 3, we found the opposite, that is, T_{regs} appear to be constraining protective immunity as specific removal of Foxp3^{+} cells during the early stages of infection was actually protective in a murine model of acute sepsis, possibly due to the unleashing of suppression over γδ^{+}, CD8^{+} T and NK cells.

Experiments conducted in our HLA transgenic murine model showed that amplified T_{reg} frequency correlated with increased bacterial burden in Chapter 6. Data from this model is in line with studies documenting increased frequencies of circulating T_{regs} in the blood of septic patients (Venet et al., 2004) and also mirrored other animal studies utilising the murine model of poly-microbial sepsis (Wisnoski et al., 2007). Overall, these findings validate the use of our HLA transgenic mice in this study.

As the majority of the experiments conducted in this study were focused on the early stages of infection, the relative contribution of T_{regs} during the later phases of the immune response are still unclear. Ongoing work in the group includes attempting to optimise a long-term infection model, but it has not been trivial to find a point between doses so high that they are lethal before 72h, and lower so that they are cleared by local, innate mechanisms without clear evidence of any adaptive response or pathology. It is not unconceivable that there may be a differential role of T_{regs} as the loss of Foxp3^{+} cells did not affect the overall survival post lethal GAS challenge. This would suggest that either, T_{regs} play little role in the late stages of bacterial sepsis or the host is too overwhelmed by the immune driven damage caused to host tissue in order for T_{regs} to exert any protective immune suppression. This concept has been previously addressed in Foxp3.DTR mice during chronic Salmonella infection, with enhanced T_{reg} activity found during the later stages of infection (Johanns et al., 2010) Depletion of Foxp3^{+} cells at different stages of S. pyogenes infection in our
humanised animal model may provide valuable information in order to clarify this hypothesis.

My findings in the HLA transgenic mouse model, clearly indicate that SMEZ streptococcal superantigen, probably the most potent virulence factor of this bacterium, may act partly through the immune subversion of inducing an inappropriate, acute T\textsubscript{reg} responses. The evolution of these streptococcal virulence factors can greatly enhanced survival within the host. However, it should not be forgotten that virulence factors are non-essential genes compared to the vital genes required for replication and metabolism (Ulrich, 1999). Tight regulation is in place to control the expression of virulence factors to conserve energy utilized by the pathogen (Ulrich, 1999). However, dysregulation of regulatory elements, such as CovRS in \textit{S. pyogenes} can lead to hyper-virulence and associated increase severity of invasive disease (Turner \textit{et al.}, 2009). Mutations in regulatory genes may occur due to immunological pressure presented to the bacterium by the host and may explain the up-surge of invasive M1 serotype strains and the rise in the number of antibiotic resistant strains admitted to intensive care units (Willems \textit{et al.}, 2011).

Bacteriophages are the source of many different DNA elements, such as the genes encoding streptococcal superantigens (Beres \textit{et al.}, 2002). These phages also appear to be present in other bacterial species as well, for example the dairy bacterium \textit{Lactococcus lactis} (Desiere \textit{et al.}, 2011), highlighting the widespread nature of horizontal genetic transfer between bacterial species. The presence of these phages increases the fitness of the bacterium to survive and in some cases act as virulence factors (Desiere \textit{et al.}, 2011). Therefore, \textit{S. pyogenes} isolates have evolved to retain these beneficial phages encoding superantigens to evade and exploit host immune response, such as T\textsubscript{reg} and T\textsubscript{H}1-mediated immunity as discussed in Chapter 6 and ultimately prolong their lifespan within the host.

As \textit{S. pyogenes} is a human pathogen, it is not surprising that streptococcal superantigens have greatest affinity in the context of human TCR V\textbeta/HLA interactions, with approximately 100-1000 times less sensitivity to mouse T cells (Fleisher \textit{et al.}, 1991). T cells from non-human primates (\textit{e.g.} rhesus monkeys) also
exhibit superantigen driven responses such as specific TCR Vβ expansion and pro-inflammatory cytokine production upon stimulation with staphylococcal superantigens: SEA, SEB and SEC (Bawari and Ulrich, 1995). Increased sensitivity of non-primate T cells to superantigens may be due to strong affinity to specific TCR Vβ sequences (Bawari and Ulrich, 1995). This highlights that these pathogens have adapted to subvert specific host species immune cells and emphasises the importance of studying GAS-mediated responses in humanised transgenic models, primates, or human cells directly.

Upon further investigation as discussed in Chapter 6, streptococcal SMEZ superantigen was found to induce Foxp3+ cells in my model of acute sepsis and upon removal of this superantigen, there was decrease in expression of Foxp3 gene and protein and increased bacteraemia (Figure 7.1). One possible mechanism that SMEZ could induce Tregs would be that this superantigen is favouring specific Vβ11+ T cell expansion (Unnikrishnan et al., 2002), particularly amongst Tregs expressing this Vβ allele. As SMEZ is a potent superantigen, it is not unfeasible that other T cell subsets will also be activated, however, qRT-PCR analysis of draining lymph node and spleens from HLA-DQ8.Aβ0 infected with H623 (smez−/−) did not show any significant changes in gene expression of t-bet, gata-3 or rorgt transcription factors for T_h1, T_h2 and T_h17 cells respectively.

While no effect of SMEZ on T cell associated transcription factors apart from foxp3 was observed in my model of acute sepsis, interestingly, SPEA superantigen promoted T_h1 induction via T-bet and lead to increased IFN-γ secretion, which may contribute to the well-documented downstream immunological effects observed during sepsis, known as the “cytokine storm” (Sriskandan and Altmann, 2008) (Figure 7.1). Perhaps, SPEA is inducing specific expansion of Vβ4, 5.1, 2 or 6+ T cells (Sriskandan et al., 2001), leading to activation and differentiation into IFN-γ producing T_h1 cells.

The expansion of Tregs would benefit GAS, due to enhanced suppression of key effector cells, which would normally hinder S. pyogenes colonisation and persistence within the host. From work presented in Chapter 3 and 4, it appears that effector cells
such as NK, CD8$^+$ and $\gamma\delta^+$ T cells may prove beneficial to the host in order to slow disease progression. The production of IL-17A from these cells may be the protective factor. It has been well documented that IL-17A can recruit neutrophils to the site of infection to lead to effective elimination of pathogens (Ye et al., 2001a; Cho et al., 2010). Whether this is also the case in my model of sepsis is unknown, as the precise frequency of neutrophils was not directly assessed. However, preliminary IHC analysis of infected thigh tissue depleted of Foxp3$^+$ cells in Chapter 3 showed decreased levels of MPO staining, a marker for neutrophil activation (Schmekel et al., 1990). This suggests the removal of Foxp3$^+$ cells, paradoxically correlated to decreased neutrophil activation, indicating that neutrophils play a very minor or indeed pathogenic role at the site of infection during acute infection. This requires further investigation.

Other studies have indicated that innate signaling receptors such as TLRs can also influence intestinal homeostasis by activating components of adaptive immunity, in the form of T$_{reg}$. For example, TLR9 is able to recognise un-methylated CpG dinucleotides derived from gut flora commensals to negatively regulate the frequency of T$_{reg}$s in the intestines, suppressing T$_{H1}$ and T$_{H17}$ effector function (Hall et al., 2008). However, a study by Boulard and colleagues have shown that the loss of TLR2 does not affect intestinal inflammation and TLR2$^{-/-}$ mice have normal levels of functional Foxp3$^+$ T$_{reg}$s to suppress effector T cell mediated responses (Boulard et al., 2010). This finding is reminiscent of my results showing the loss of TLR2 during acute S. pyogenes infection had little impact on bacterial burden and resistance to survive lethal challenge, however, the frequency of T$_{reg}$s or transcriptional changes in foxp3 in this model was not assessed, requiring further investigation.

In order for T$_{reg}$ induction to be initiated, S. pyogenes must be directly sensed by immune cells. Investigating the expression of the innate receptor TLR2 on T$_{reg}$s during infection with sme$^{+/-}$ GAS isolate revealed a global decrease in tlr2 gene expression in the spleen and a reduction in the absolute numbers of T$_{reg}$ expressing intracellular TLR2. This would suggest that S. pyogenes and SMEZ could modulate T$_{reg}$-mediated immunity via interactions with TLR2 in this cell population (Figure 7.1). The precise mechanism of signal transduction between TLR2 and Foxp3 is unknown. However, it has previously been shown by Hopkins and colleagues, that
SMEZ can increase the surface expression of TLR2 on monocytes in a MHC class II dependent manner (Hopkins et al., 2008). The requirement of MHC class II for TLR2 signaling and subsequent pro-inflammatory cytokine induction has also been demonstrated by Liu and colleagues (Liu et al., 2011b). Whether these proposed signaling pathways are also true in T\textsubscript{reg} is unclear and more work is required to determine if TLR signaling pathways lead to the observed expansion or differentiation of Foxp3\textsuperscript{+} cells.

Overall, the depletion of Foxp3\textsuperscript{+} mediated suppression does appear to contribute to the unleashing of control over innate and adaptive immune cell types as demonstrated by changes in cytokine production from innate and adaptive cells. However, taken together, during acute GAS infection there is an intimate interaction between host and invading pathogen. \textit{S. pyogenes} has exquisitely evolved to retain bacteriophage-derived superantigens to manipulate immune regulation mechanisms to promote its own endurance within the body at the expense of the ensuing immune response.
A. Onset and progression of invasive streptococcal disease. During the initial infection with *S. pyogenes*, expression of superantigens is induced by the bacterium to evade and modulate the immune response. Secreted SPEA superantigen leads to T_{H1} differentiation and subsequent IFN-γ production, a hallmark cytokine of the so-called “cytokine storm” observed during sepsis. *S. pyogenes* produces SMEZ superantigen to induce Foxp3^+ T_{regs} via TLR2 to suppress effector cells (NK, CD8^+ and γδ^+ T cells) to prevent bacterial clearance. The net effect is the propagation of pathogenic T_{H1} cells and ensuing disease pathology associated with uncontrolled production of pro-inflammatory cytokines and suppression of protective immune cell types to allow *S. pyogenes* to replicate.

B. Bacterial clearance and resolution of infection. If effective host immunity is mounted or the presence of secreted streptococcal superantigens neutralized by antibody responses, T_{H1} cells will diminish in the absence of SPEA and protective effector cells such as NK, CD8^+ and γδ^+ T cells will be unleashed from Foxp3^+ mediated control to lead to direct bacterial elimination by induced apoptosis or phagocytosis or production of cytokines such as IL-17A to enhance resolution of disease.

**Figure 7.1:** Schematic representation of the interaction between streptococcal superantigens and T_{regs}. 

![Diagram](image)
7.3 Future work

This thesis has offered a number of new insights into effector and regulatory cell function during the initial stages of infection, with many intriguing questions raised from the work carried out. From the data presented, streptococcal superantigens appear to play a key role during infection through possible stimulation of T<sub>regs</sub>. To further complement my findings, investigating the unknown phenotype of the T<sub>regs</sub> during the time course of <i>S. pyogenes</i> infection would be crucial to determine whether these cells are exerting enhanced suppressive activity or whether they are dysfunctional. Since completing the studies for this thesis, work of others and myself in the lab has encompassed DNA microarray comparison of effector population from T<sub>reg</sub> depleted or wild-type infected mice. A number of different cell types have been shown here as potentially protective during GAS infection, including NK cells. This work is being further explored through the use of E4bp4 NK cell knockout mice. This approach could be extended to investigate other effector cells types as well. As <i>S. pyogenes</i> is a human pathogen, it would be essential to extend this work to human studies to see if there is a correlation between my finding utilising murine models with data looking directly at human responses.

In summary, future work following this study continuing the characterisation of T<sub>regs</sub> during <i>S. pyogenes</i> infection would include:

- **Detailed phenotypic analysis of T<sub>regs</sub> such as cytokine profile (e.g. IL-10, TGF-β and IL-35), expression of markers associated with induction of apoptosis in target cells (e.g. granzyme A and B).** Chemokine expression could be studied to understand the functional role of increased T<sub>regs</sub> in the draining lymph node post GAS infection. The TCR Vβ repertoire of T<sub>regs</sub> could also be determined to better comprehend superantigen-driven expansion during infection.

- **Investigate further the protective role of NK cells during GAS infection with the use of E4bp4<sup>−/−</sup> mice, which lack NK cells (Gascoyne et al., 2009).** The role of other cell types such as neutrophils, known to produce IL-17A could also be
studied. From my findings, IL-9 appears to be important during *S. pyogenes* infection; it would be interesting to find out whether this cytokine is protective during GAS infection?

- Addressing the nature of antigen-specific T$_{reg}$s with a panel of streptococcal surface proteins (available in our lab through a collaboration with Dr G Grandi, Novartis, Siena) would offer insight into the contribution of specific immune targets in stimulating T cells during infection. This could lead to epitope mapping, followed by vaccine design.

- Ultimately, the role of T$_{reg}$s during *S. pyogenes* infection would be extended to look at human responses utilising PBMCs or *in vitro* cell cultures derived from tonsil samples taken from patients with recurrent GAS-associated tonsillitis.


Awasthi, A. and Kuchroo, V. J. (2009), 'IL-17a directly inhibits Th1 cells and thereby suppresses development of intestinal inflammation', Nature Immunology, 10, 568-570.


Beriou, G., Costantino, C. M., Ashley, C. W., Yang, L., Kuchroo, V. J., Baecher-Allan, C. and Hafler, D. A. (2009), 'IL-17–producing human peripheral regulatory T cells retain suppressive function', *Blood*, 113, 4240-4249.

that secrete helper factor(s) for B-cell proliferation and maturation', *Scandinavian Journal of Immunology*, **28**, 519-27.


TNF receptor-related protein ligand on monocytes', *The Journal of Allergy and Clinical Immunology*, 117, 688-695.


Collart, M. A., Baeuerle, P. and Vassalli, P. (1990), 'Regulation of Tumor Necrosis Factor Alpha Transcription in Macrophages: Involvement of Four κB-Like Motifs and of Constitutive and Inducible Forms of NF-κB', *Molecular and Cellular Biology*, **10**, 1498-06.

Collard, M. A., Baeuerle, P. and Vassalli, P. (1990), 'Regulation of Tumor Necrosis Factor Alpha Transcription in Macrophages: Involvement of Four κB-Like Motifs and of Constitutive and Inducible Forms of NF-κB', *Molecular and Cellular Biology*, **10**, 1498-06.


Costalonga, M., Cleary, P. P., Fischer, L. A. and Zhao, Z. (2008), 'Intranasal bacteria induce Th1 but not Treg or Th2', Mucosal Immunology, 2, 85-95.

Cottalorda, A., Mercier, B. C., Mbikton-Kobo, F. M., Arpin, C., Teoh, D. Y. L., McMichael, A., Marvel, J. and Bonnefoy-Bérard, N. (2009), 'TLR2 engagement on memory CD8+ T cells improves their cytokine-mediated proliferation and IFN-γ secretion in the absence of Ag', European Journal of Immunology, 39, 2673-2681.


Dong, C. (2009), 'Mouse Th17 cells: Current understanding of their generation and regulation', European Journal of Immunology, 39, 640-645.

Eberl, M. and Moser, B. (2009), 'Monocytes and $\gamma\delta$ T cells: close encounters in microbial infection', Trends in Immunology, 30, 562-568.


Eroukhmanoff, L., Oderup, C. and Ivars, F. (2009), 'T-cell tolerance induced by repeated antigen stimulation: selective loss of Foxp3$^+$ conventional CD4 T cells and induction of CD4 T-cell anergy.', European Journal of Immunology, 39, 1078-1087.


Espoito, M., Ruffini, F., Bergami, A., Garzetti, L., Borsellino, G., Battistini, L., Martino, G. and Furlan, R. (2010), 'IL-17$^+$ and IFN-$\gamma$-Secreting Foxp3$^+$ T Cells Infiltrate the Target Tissue in Experimental Autoimmunity', The Journal of Immunology, 185, 7467-73.

by Addition of 22 New M Protein Gene Sequence Types from Clinical Isolates: emm103 to emm124', Clinical Infectious Diseases, 34, 28-38.


Figgdor, C. G., van Kooyk, Y. and Adema, G. J. (2002), 'C-type lectin receptors on dendritic cells and Langerhans cells', Nature Reviews Immunology, 2, 77-84.


Gaffen, S. (2009), 'Structure and signalling in the IL-17 receptor family', *Nature Reviews Immunology*, 9, 556-567.

regulator of T-cell homeostasis', *Current Opinions in Rheumatology*, **15**, 430-35.


Gram, C. (1884), 'Ueber die isolirte Färbung der Schizomycestin in Schnitt-und Trockenpräparaten', Fortschritte der Medicin, 2, 185-89.


Guillaume, L. and Kalil, J. (2010), 'Rheumatic Fever and Rheumatic Heart Disease: Cellular Mechanisms Leading Autoimmune Reactivity and Disease', Journal of Clinical Immunology, 30, 17-23.


Harder, J., Franchi, L., Muñoz-Planillo, R., Park, J., Reimer, T. and Núñez, G. (2009), 'Activation of the Nlrp3 Inflammasome by Streptococcus pyogenes Requires Streptolysin O and NF-kB Activation but Proceeds Independently of TLR Signaling and P2X7 Receptor', The Journal of Immunology, 183, 5823-5829.

Role for Induced Regulatory T Cells in Tolerance Induction in Experimental Colitis', *The Journal of Immunology*, **182**, 3461-3468.


Hilbi, H., Chen, Y., Thirumalai, K. and Zychlinsky, A. (1997), 'The Interleukin 1β-Converting Enzyme, Caspase 1, Is Activated during *Shigella flexneri*-Induced


Huber, S. A. (2009), 'Depletion of γδ T cells increases CD4+FoxP3 (T regulatory) cell response in coxsackievirus B3-induced myocarditis', *Immunology*, **127**, 567-576.


Activation of Tonsillar B Cells after Receptor-Mediated Endocytosis, The Journal of Immunology, 182, 4713-4720.


Joosten, S. A. and Ottenhoff, T. H. M. (2008), 'H Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination', Human Immunology, 69, 760-70.


Kameyama, K., Nemoto, Y., Kanai, T., Shinohara, T., Okamoto, R., Tsuchiya, K., Nakamura, T., Sakamoto, N., Totuka, T., Hibi, T. and Watanabe, M. (2010), 'IL-2 is positively involved in the development of colitogenic CD4+ IL-7Ra^high memory T cells in chronic colitis', European Journal of Immunology, 40, 2423-2436.


Accelerates Neutrophil Recruitment through γδ T-Cell IL-17 Production in a Murine Model of Sepsis', *Infection and Immunity*, 78, 4714-22.

Kastner, L., Dwyer, D. and Qin, F. Q. (2010), 'Synergistic Effect of IL-6 and IL-4 in Driving Fate Revision of Natural Foxp3+ Regulatory T Cells', *The Journal of Immunology*, 185, 5778-86.


Kish, D. D., Gorbachev, A. V. and Farichild, R. L. (2005), 'CD8+ T cells produce IL-2, which is required for CD4+CD25+ T cell regulation of effector CD8+ T cell development for contact hypersensitivity responses', *Journal of Leukocyte Biology*, 78, 725-35.


Liu, F. and Whitton, J. L. (2005), 'Cutting Edge: Re-evaluating the In Vivo Cytokine Responses of CD8+ T Cells During Primary and Secondary Viral Infections', *The Journal of Immunology*, 174, 5936-5940.

Liu, G., Burns, S., Shrestha, S. and Chi, H. (2010a), 'The S1P1-mTOR axis directs the reciprocal differentiation of T_{H1} and T_{reg} cells', *Nature Immunology*, 11, 1047-56.
Liu, G., Ma, H., Qiu, L., Li, L., Cao, Y., Ma, J. and Zhao, Y. (2011a), 'Phenotypic and functional switch of macrophages induced by regulatory CD4\(^+\)CD25\(^+\) T cells in mice', *Immunology and Cell Biology*, 89, 130-42.

Liu, G. and Zhao, Y. (2007), 'Toll-like receptors and immune regulation: their direct and indirect modulation on regulatory CD4\(^+\)CD25\(^+\) T cells', *Immunology*, 122, 149-156.


Llewelyn, M. (2005), 'Human leukocyte antigen class II haplotypes that protect against or predispose to streptococcal toxic shock', *Clinical Infectious Diseases*, 41, S445-448.


Lurie, M. B. (1938), 'Studies on the Mechanism of Immunity in Tuberculosis: The Mobilization of Mononuclear Phagocytes in Normal and Immunized Animals


Maruyama, T., Li, J., Vaque, J. P., Kondel, J. E., Wang, W., Zhang, B., Zhang, P.,
(2011), 'Control of the differentiation of regulatory T cells and Th17 cells by 
the DNA-binding inhibitor Id3', Nature Immunology, 12, 86-95.
Matsumiya, T., Imaizumi, T., Yoshida, H., Satoh, K., Topham, M. K. and Stafforini, 
D. M. (2009), 'The Levels of Retinoic Acid-Inducible Gene I Are Regulated 
by Heat Shock Protein 90-α', The Journal of Immunology, 182, 2717-2725.
Matsuzaki, G. and Umemura, M. (2007), 'Interleukin-17 as an effector molecule of 
in innate and acquired immunity against infections', Microbiology and 
Immunology, 51, 1139-1147.
in Immunology, 12, 991-1045.
'CD8+Foxp3+ T cells share developmental and phenotypic features with 
classical CD4+Foxp3+ regulatory T cells but lack potent suppressive activity', 
European Journal of Immunology, 41, 716-25.
McConnell, K. W., Fox, A. C., Clark, A. T., Chang, N. N., Domínguez, J. A., Farris, 
The Journal of Immunology, 186, 3718-25.
McCormick, J. K., Pragman, A. A., Stolpa, J. C., Leung, D. Y. M. and Schlievert, P. 
M. (2001), 'Functional Characterization of Streptococcal Pyrogenic Exotoxin 
J, a Novel Superantigen', Infection and Immunity, 69, 1381-1388.
McDonald, B., Pittman, K., Menezes, G. B., Hirota, S. A., Slaba, I., Waterhouse, C. 
Signals Guide Neutrophils to Sites of Sterile Inflammation', Science, 330, 
362-66.
McFadden, C., Morgan, R., Rahangdale, S., Green, D., Yamasaki, H., Center, D. and 
Cruikshank, W. (2007), 'Preferential Migration of T Regulatory Cells Induced 
by IL-16', The Journal of Immunology, 179, 6439-6445.
McGeachy, M. J., Chen, Y., Tato, C. M., Laurence, A., Joyce-Shaikh, B., 
Blumenschein, W. B., McClanahan, T. K., O'Shea, J. J. and Cua, D. J. (2009), 
'The interleukin 23 receptor is essential for the terminal differentiation of 
interleukin 17–producing effector T helper cells in vivo.', Nature Immunology, 
10, 314-324.
McHugh, R. S. and Shevach, E. M. (2002), 'Cutting Edge: Depletion of CD4+CD25+ 
Regulatory T Cells Is Necessary, But Not Sufficient, for Induction of Organ-
Specific Autoimmune Disease', The Journal of Immunology, 168, 5979-83.
McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., 
Collins, M. and Byrne, M. C. (2002), 'CD4+CD25+ immunoregulatory T cells: 
gene expression analysis reveals a functional role for the glucocorticoid-
induced TNF receptor', Immunity, 16, 311-323.
(2006), 'Regulatory T Cells Dampen Pulmonary Inflammation and Lung 
Injury in an Animal Model of Pneumocystis Pneumonia', The Journal of 
Immunology, 177, 6215-26.
McRipley, R. J. and SBarra, A. J. (1967), 'Role of the Phagocyte in Host-Parasite 
Interactions. XII. Hydrogen Peroxide-Myeloperoxidase Bactericidal System 


Moon, J. J. and McSorley, S. J. (2009), 'Tracking the Dynamics of Salmonella specific T cell responses', *Current Topics in Microbiology and Immunology*, 334, 179-98.


S. (2002), 'CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung', Nature Medicine, 8, 588-593.


Papathanassoglou, E. D., Giannakopoulou, M. D. and Bozas, E. (2006), 'Genomic variations and susceptibility to sepsis', AACN Advanced Critical Care, 17, 394-422.


Proft, T. and Fraser, J. D. (2003), 'Bacterial superantigens', Clinical Experimental Immunology, 133, 299-306.


Proft, T., Sriskandan, S., Yang, L. and Fraser, J. D. (2003), 'Superantigens and streptococcal toxic shock syndrome', Emerging Infectious Diseases, 9, 1211-1218.


Reardon, C., Wang, A. and McKay, D. M. (2008), 'Transient Local Depletion of Foxp3’ Regulatory T Cells during Recovery from Colitis via Fas/Fas Ligand-Induced Death', The Journal of Immunology, 180, 8316-8326.


Roncarolo, M. G. and Gregori, S. (2008), 'Is FOXP3 a bona fide marker for human regulatory T cells?', European Journal of Immunology, 38, 925-927.


Shibata, K., Yamada, K., Hara, H., Kishihara, K. and Yoshikai, Y. (2007), 'Resident Vδ1 γδ T cells control early infiltration of neutrophils after Escherichia coli infection via IL-17 production.', The Journal of Immunology, 178, 4466-4472.


Tang, Q. and Bluestone, J. A. (2008), 'The Foxp3 regulatory T cell: a jack of all trades, master of regulation', Nature Immunology, 9, 239-244.


Taylor, A. L. and Llewelyn, M. J. (2010), 'Superantigen-Induced Proliferation of Human CD4+CD25 T Cells Is Followed by a Switch to a Functional Regulatory Phenotype', The Journal of Immunology, 185, 6591-98.

Tenorio, E. P., Fernández, J., Olguin, J. E. and Saavedra, R. (2011), 'Depletion with PC61 mAb before Toxoplasma gondii infection eliminates mainly Tregs in BALB/c mice but activated cells in C57BL/6J mice', FEMS Immunology and Medical Microbiology.


Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K. and Spits, H. (2009), 'Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T_{H}^{-17}, T_{H}^{-1} and T_{H}^{-2} cells', Nature Immunology, 10, 864-872.
Trinchieri, G. (1997), 'Cytokines acting on or secreted by macrophages during intracellular infection (IL-10, IL-12, IFN-γ)', Current Opinions in Immunology, 9, 17-23.


Ulrich, R. G. (2008), 'Vaccine based on a ubiquitous cysteinyl protease and streptococcal pyrogenic exotoxin A protects against Streptococcus pyogenes sepsis and toxic shock', Journal of Immune Based Therapies and Vaccines, 6, 8-16.


Unsinger, J., Kazama, H., MCDonough, J. S., Griffith, T. S., Hotchkiss, R. S. and Ferguson, T. A. (2010), 'Sepsis-Induced Apoptosis Leads to Active Suppression of Delayed-Type Hypersensitivity by CD8⁺ Regulatory T Cells through a TRAIL-Dependent Mechanism', The Journal of Immunology, 184, 6766-6772.


Uyttenhove, C., Brombacher, F. and van Snick, J. (2010), 'TGF-β interactions with IL-1 family members trigger IL-4-independent IL-9 production by mouse CD4⁺ T cells', European Journal of Immunology, 40, 2230-2235.

Valmori, D., Raffin, C., Raimbaud, I. and Ayyoub, A. (2010), 'Human RORγ⁺ T cell preferentially differentiate from naïve FOXP3⁺ Treg in the presence of...
lineage-specific polarizing factors', *Proceedings of the National Academy of Sciences of the United States of America*, 107, 19402-07.


Yamazaki, S., Dudziak, D., Heidkamp, G. F., Fiorese, C., Bonito, A. J., Inaba, K., Nussenzweig, M. C. and Steinman, R. M. (2008), 'CD8\(^+\)CD205\(^+\) splenic dendritic cells are specialized to induce FoxP3\(^+\) regulatory T cells', The Journal of Immunology, 181, 6923-6933.


Yoshimori, T. and Amano, A. (2009), 'Group a Streptococcus: a loser in the battle with autophagy', Current Topics in Microbiology and Immunology, 335, 217-26.

Yu, C. and Ferretti, J. J. (1989), 'Molecular Epidemiologic Analysis of the Type A Streptococcal Exotoxin (Erythrogenic Toxin) Gene (speA) in Clinical Streptococcus pyogenes Strains', Infection and Immunity, 57, 3715-19.


Zheng, S. G., Wang, J. and Horwitz, D. A. (2008), 'Cutting Edge: Foxp3+CD4+CD25+ Regulatory T Cells Induced by IL-2 and TGF-β and are Resistant to Th17 Conversion by IL-6', *The Journal of Immunology*, 180, 7112-7115.


Zhuang, Y., Huang, Z., Nishida, J., Brown, M., Zhang, L. and Huang, H. (2009), 'A continuous T-bet expression is required to silence the interleukin-4-producing potential in T helper type 1 cells', *Immunology*, 128, 34-42.

