INVESTIGATION OF THE ABBERENT IMMUNE RESPONSE IN BEHÇET SYNDROME

Nicola Louise Ambrose

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National Heart and Lung Institute

Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Behçet Syndrome (BS) is a multisystem disorder characterized by mucocutaneous lesions (oral ulcers, genital ulcers, and skin pustules), arthritis and intraocular inflammation. The pathogenesis of BS is poorly understood but it is considered a complex polygenetic syndrome, with environmental triggers. A Th1 skew has been reported. Recent genome wide association studies have confirmed the association with HLA B51, as well as identifying novel single nucleotide polymorphisms including IL-10, IL23/12R, STAT4, ERAP1 and CPLX1. **Aims:** This project aimed to dissect abnormal cutaneous inflammatory response in BS to gain an understanding as to which pathways are responsible for the pathergy response. Secondary aims were to dissect any observations in more detail *in vitro*. In Chapter 3, the hypothesis that the abnormal cutaneous inflammatory response to "non-specific" stimulation in BS is associated with a failure of deactivation of macrophages during the resolution phase of inflammation was explored using human *in vivo* models of inflammation (MSU injections and skin blisters). In Chapter 4, the hypothesis that the abnormal cutaneous inflammatory response may involve neuropeptides was explored. We applied capsaicin topically to BS and HC, and measured the resultant change in dermal blood flow using laser doppler imaging to evaluate non-invasively if neuropeptides such as CGRP may play a role in the abnormal cutaneous inflammatory response in BS. Chapter 5 summarizes key *in vitro* experiments undertaken alongside *in vivo* experiments. The IFN-γ / chemokine pathways were explored in detail in CD14+ monocyte populations in BS, HC and disease control subjects. **Results:** CXCL10 was identified as a chemokine of interest using the Cantharidin skin blister model of inflammation. We did not find statistically significant differences in neuropeptide responses between BS and HC, although there was a trend towards less CGRP in the BS cohort. *In vitro* work focused on peripheral blood derived monocytes stimulated with IFN-γ. BS monocytes produced significantly more CXCL10 protein versus healthy control (HC) monocytes from two hours onwards, despite equivalent quantities of mRNA, revealing a significantly increased protein:mRNA ratio. This increased protein:mRNA ratio was found to be a specific response. Other IFN-γ induced

chemokines examined did not show this response profile (CXCL9,CXCL11, CCL2); monocytes stimulated with other cytokines (e.g. TNF-α) did not show this specific response; and the increased CXCL10 protein mRNA ratio was not observed in either Rheumatoid Arthritis (RA) or Systemic Lupus Erythematosus (SLE) patients. Sucrose density gradients, to segregate cell lysate mRNA into free RNA or polysome-associated RNA, did not further differentiate HCs from BS, with equal quantities of mRNA in BS and HC samples being found in association with polysomes, possibly implicating microRNAs as translation control factors of interest. **Conclusions**: We have discovered that BS monocytes have dysfunctional post-transcriptional regulation of CXCL-10 mRNA, resulting in overexpression of CXCL-10 protein upon IFN-γ stimulation. This may contribute to the exaggerated inflammatory responses that characterises BS and could also be diagnostically useful.

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Contents

INVESTIGATION OF THE ABBERENT IMMUNE RESPONSE IN BEHÇET SYNDROME 1
Declaration ... 2
Copyright Declaration ... 3
Abstract ... 4
Acknowledgements ... 6
Contents ... 8
List of tables ... 13
List of figures ... 14
Abbreviations ... 18
1. Introduction ... 22
1.1 Epidemiology and prevalence ... 24
1.2 Clinical manifestations ... 25
1.2.1 Orogenital ulceration ... 25
1.2.2 Pathergy ... 26
1.2.3 Skin lesions ... 27
1.2.4 Ocular inflammation ... 27
1.2.5 Neurological system ... 28
1.2.6 Musculoskeletal system ... 29
1.2.7 Gastrointestinal manifestations ... 29
1.2.8 Cardiovascular system ... 30
1.2.9 Genitourinary system ... 31
1.2.10 Constitutional symptoms ... 31
1.3 Reaching the diagnosis ... 31
1.4 The pathergy test and monosodium urate crystal test ... 32
1.5 Prognosis ... 33
1.6 Inflammation and genetics ... 33
1.6.1 Genetics and BS ... 35
1.6.2 Innate immune dysfunction ... 36
1.6.3 The inflammasome / IL-1β pathway ... 36
1.6.4 Toll-like receptors and cytokines ... 37
1.6.5 Matrix metalloproteinases ... 38
1.6.6 Natural killer cells and receptors ... 38
1.6.7 γδ T cells ... 39
1.6.8 Adaptive Immunity ... 41

1.6.9 Chemokines 45
1.6.10 Dampening inflammation 46
1.6.11 The role of neuropeptides in inflammation 48
1.6.12 Environmental Triggers 49
1.7 Summary 51
1.8 Project Aims overview 51
2. Materials and Methods 53
2.1 53
2.2 Study subjects and study design 53
2.2.1 Ethics 53
2.2.2 Subject evaluation 53
2.2.3 Study design 54
2.2.4 Statistical methodology 54
2.3 Monosodium urate crystals (MSU) 54
2.3.1 Protocol for MSU crystal preparation 54
2.3.2 Endotoxin testing 55
2.3.3 MSU administration and size measurement 55
2.4 Skin blister models 56
2.4.1 Suction blisters 56
2.4.2 Cantharidin blisters 56
2.4.3 Blister fluid processing 57
2.5 Monocytes isolation and culture 58
2.5.1 Peripheral venous blood sampling 58
2.5.2 CD14+ monocyte positive selection 58
2.6 Flow cytometry 59
2.6.1 Flow cytometry theory 59
2.6.2 Fluorescence minus one 59
2.6.3 Antibodies for flow cytometry 60
2.6.4 Flow cytometry protocol 61
2.7 Neuropeptide investigation 61
2.7.1 Thermal threshold testing (TTT) 61
2.7.2 Capsaicin mode of action 61
2.7.3 Real-time blood flow imaging system: Laser-doppler 62
2.8 Protein analysis 63
2.8.1 ELISA 63
2.8.2 Multiplex bead analysis – Cytokine analysis of blister fluid 64
2.9 RNA extraction and cDNA generation 65
2.9.1 RNeasy protein extraction method 65
2.9.2 Trizol RNA extraction 65

2.9.3 Trizol LS RNA extraction 66
2.9.4 RNA purification and concentration analysis 66
2.9.5 Quantitative real-time PCR 67
2.10 Sucrose-density-gradient polysome experiment 68
2.10.1 Sucrose-density-gradient polysome experiment theory 68
2.10.2 Establishing the sucrose density gradient 69
2.10.3 Cell lysis and ultracentrifugation 69
2.10.4 Sucrose density gradient polysome experiment 70
2.10.5 Green Fluorescent Protein (GFP) spike 70
3. Examining the exaggerated cutaneous inflammatory response using a skin blister model of inflammation 72
3.1.1 Abnormal inflammatory responses in BS 72
3.1.2 Intradermal (id) injection of monosodium urate (MSU) crystals 73
3.1.3 Immunopathology of BS 74
3.1.4 Inflammatory responses to MSU crystals *in vitro* 74
3.1.5 Suction blister model of skin inflammation 75
3.1.6 Monocyte and macrophage biology 76
3.1.7 CD163 as a marker of deactivated macrophages 78
3.2 Hypothesis 78
3.3 Aims 78
3.3.1 Research aims in detail 78
3.4 Results 79
3.4.1 Assessment of a suction blister model to study cutaneous inflammation 79
3.4.2 Establishment of a 3 day protocol for cantharidin skin blisters. 80
3.4.3 Demographics of the BS and HC cohorts 81
3.4.4 Monosodium urate injections 83
3.4.5 Naked eye assessment of cantharidin skin blisters 83
3.4.6 Cantharidin volume and cell count 85
3.4.7 Flow cytometry 89
3.4.8 Blister fluid cytokine and chemokine concentrations 93
3.5 Discussion 97
3.5.1 Skin blister models of inflammation 97
3.5.2 Macrophage activation states 97
3.5.3 Other cells and cytokines in early and late blisters 97
3.6 Concluding remarks 98
4. Investigation of the role of neuropeptides in BS 99
4.1 Introduction 99
4.1.1 The neuro-immune system 99
4.1.2 Neuropeptides 100

4.1.3 Methods of studying neuropeptides 104
4.2 Hypothesis and aims 105
4.2.1 Rationale for investigating capsaicin induced blood flow in BS 105
4.3 Results 106
4.3.1 Pilot study of 5 BS and 5 HC to evaluate the usefulness of thermal threshold testing 106
4.3.2 Establishing a protocol for laser blood flow measurement 107
4.3.3 Perimed Laser Doppler Imager versus Moor Laser Speckle Photometry Imager 108
4.3.4 Establishing a final protocol for the use of capsaicin 109
4.3.5 Interpretation of results 110
4.3.6 Study cohort 112
4.3.7 Change in blood flow within 'Region of interest 1' (ROI1) 113
4.3.8 Change in blood flow within 'Region of interest 2' (ROI2) 114
4.3.9 Flare response assessment using total pixel counts 116
4.4 Discussion 117
4.4.1 BS capsaicin flare responses appeared to be smaller than that of HC 118
4.4.2 Study limitations 118
4.4.3 Reasons why it may be plausible that BS subjects secrete less CGRP than HCs 119
4.5 Future work 119
5. Exploring the IFN-γ–Chemokine pathway in BS *in vitro* 121
5.1 Context 121
5.2 Background 121
5.2.1 Interferons 121
5.2.2 Chemokines 127
5.2.3 BS and IFN-γ pathways 130
5.2.4 mRNA Translation: from gene to protein 130
5.2.5 miRNAs with IFN-γ and CXCL10 138
5.2.6 Polysome analysis 138
5.2.7 BS and miRNA work to date 139
5.2.8 The choice of SLE as a disease control 139
5.2.9 The choice of RA as a disease control 139
5.3 Hypothesis 140
5.4 Aims of *in vitro* work 140
5.5 Methods 141
5.6 Results 141
5.6.1 Demographics 141
5.6.2 Pilot data 146
5.6.3 Comparison of CXCL10 protein response in BS and HCs 148

5.6.4 Assessing the specificity of observed differences in CXCL10 protein and mRNA 152

5.6.5 Correlation of observations to clinical features such as MSU response, disease activity and medications 159

5.6.6 Polysome profiling to assess post-transcriptional regulation of IFN-γ - CXCL-10 164

5.7 Discussion 169

5.7.1 Clinical relevance of the observation 173

5.7.2 Correlation of elevated CXCL10 protein *in vitro* to low CXCL10 observed in skin blisters 174

5.8 Future work and potential clinical usefulness of this observation 175

6. Concluding Remarks 176

7. REFERENCES 179

List of tables

Table 1-1. Overlapping features of autoinflammatory conditions and BS 24

Table 1-2. Differential diagnosis of recurrent oral ulcerations 26

Table 1-3. The International Study Group Criteria for diagnosis of BS 32

Table 1-4 A summary of some common genetic tests 35

Table 1-5. Summary of innate immunity genetic markers identified in BS 40

Table 1-6. Summary of some HLA genetic markers identified in BS 43

Table 1-7. T helper cell populations 45

Table 1-8. Summary of T helper and chemokine markers identified in BS..... 46

Table 1-9. Summary of anti-inflammatory markers identified in BS 48

Table 2-1. Antibodies used for flow cytometry experiments..... 60

Table 2-2. Oligonucleotides used for qRT PCR 68

Table 2-3. Equipment used for sucrose density gradient experiment 70

Table 3-1. Suction blister volumes and cell counts in 6 healthy controls..... 79

Table 3-2. Demographics of BS and HC subjects recruited for cantharidin skin blister study... 82

Table 5-1 CXCL9, CXCL10 and CXCL11 stimulus preference and promoter..... 129

Table 5-3. Demographics of cohort 2 HC and BS subjects recruited for *in vitro* experiments. 143

Table 5-4. Demographics of cohort 3 HC, BS, RA and SLE subjects recruited for *in vitro* experiments. 144

Table 5-5. Demographics of pooled cohort of HC and BS subjects recruited for *in vitro* experiments. 145

List of figures

Figure 2-2 A cantharidin elicited skin blister. 57

Figure 3-1. The final protocol to establish 24 hour and 48 hour cantharidin skin blisters, and an ID MSU injection test. 81

Figure 3-2. Cantharidin skin blister application. 81

Figure 3-3. The maximum diameter of erythema in HC and BS subjects 24 and 48 hours after the intradermal injection of MSU crystals. 83

Figure 3-4. The typical appearance of cantharidin skin blisters in HC (left) and BS (right) subjects. 84

Figure 3-5. MSU responses classified as 'strong' or 'weak' depending on the size of erythema at 48 hours. 84

Figure 3-6. The correlation between having a large MSU response and development of erythema around the cantharidin skin blister. 84

Figure 3-8. The cell count (cells x 10^4 / mL) of 24 and 48 hour blisters in HC and BS. 86

Figure 3-9. Cantharidin blister total cell counts (x10^4) in HC and BS at 24 and 48 hours. 87

Figure 3-10. The percentage of neutrophils present in cantharidin skin blisters. 88

Figure 3-11. The percentage of macrophages present in cantharidin skin blisters. 88

Figure 3-12. The percentage of lymphocytes; eosinophils and 'other' cells present in cantharidin skin blisters. 89

Figure 3-13. A sample of gating of flow cytometry sample prepared from peripheral blood derived mononuclear cells. 90

Figure 3-14. CD163 expression by flow cytometry of peripheral blood derived monocytes (PB), 24 hour blister cells and 48 hour blister cells. 91

Figure 3-15. HLA DR Expression by flow cytometry of peripheral blood derived monocytes (PB), 24 hour blister cells and 48 hour blister cells. 92

Figure 3-16. The percentage of CD3+ cells that co-stained for CD4 or CD8..... 93

Figure 3-17. The Th1 skew associated cytokines present in blister fluid at 24 and 48 hours..... 94

Figure 3-18 The Th2 skew associated cytokines present in blister fluid at 24 and 48 hours..... 95

Figure 3-19 The Th17/9 skew associated cytokines in blister fluid at 24 and 48 hours. 96

Figure 3-20. The acute phase response associated proteins present in the blister fluid at 24 and 48 hours..... 96

Figure 4-1. The results of thermal threshold testing in 5 BS and 5 HCs. 107

Figure 4-2. The preliminary dose finding experiment showing doppler flux values in HC and BS subjects following the application of either 300µg (top row) or 1000µg (bottom row) of capsaicin..... 108

Figure 4-3. A typical image obtained by the Perimed Imager. 109

Figure 4-4. A typical image obtained by the Moor Imager..... 109

Figure 4-5. A sample of Moor images following the application of capsaicin into the rubber O ring. 111

Figure 4-6. A sample of Moor images following the application of capsaicin into the rubber O ring. 111

Figure 4-7. The mean values of HC and BS for % increase in flux over time within ROI1. ... 114

Figure 4-8. The percentage increase in mean flux from baseline within ROI1 was calculated for each subject at each time point allowing an area under the curve (AUC) value to be calculated. 114

Figure 4-9. The mean values of HC and BS for % increase in flux over time within ROI2. ... 115

Figure 4-10. The percentage increase in mean flux from baseline within ROI2 was calculated for each subject at each time point allowing an area under the curve (AUC) value to be calculated. 115

Figure 4-11. The mean total pixel counts over time in HC and BS cohorts. 116

Figure 4-12. The total pixel counts over time were plotted and an area under the curve (AUC) was calculated for each subject. 116

Figure 4-13. The mean total pixel counts over time in HC and BS cohorts. 117

Figure 4-14. The total pixel counts over time were plotted and an area under the curve (AUC) was calculated for each subject, using a threshold of 500 flux units. 117

Figure 5-1 Proposed model for the control of mechanisms of mRNA translation of ISGs. 125

Figure 5-2. Members of the chemokine superfamily. 128

Figure 5-4. Serum protein levels of IFN-γ and CXCL10 in samples from HC (n=69), BS (n= 72) and SLE (n=65). 147

Figure 5-5. CXCL10 protein concentration. CD14+ monocytes from HC and BS subjects. .. 148

Figure 5-6. CXCL10 protein concentration. CD14+ monocytes from HC and BS subjects. ... 149

Figure 5-7. CXCL10 mRNA expression. CD14+ monocytes from HC and BS subjects. 150

Figure 5-8. The relationship of CXCL10 protein to mRNA. CD14+ monocytes from HC and BS subjects..... 151

Figure 5-9. CXCL10 protein:mRNA ratio. CD14+ monocytes from HC and BS subjects. 152

Figure 5-10. CXCL9, CXCL11 and CCL2 protein concentration. CD14+ monocytes from HC and BS subjects. 153

Figure 5-11. CXCL9 mRNA and protein:mRNA ratio. CD14+ monocytes from HC and BS subjects..... 154

Figure 5-12. CXCL10 evaluation in Disease controls. CD14+ monocytes from HC, BS, RA and SLE subjects..... 156

Figure 5-13. LPS response in BS, HC and Disease Controls. CD14+ monocytes. 158

Figure 5-14. Comparison of *in vitro* IFN-γ – CXCL10 response with the *in vivo* MSU response. 160

Figure 5-15. Comparison of CXCL10 (a) protein and (b) mRNA levels to medications that BS subjects were concurrently taking. 161

Figure 5-16. Correlation of CXCL10 (a) protein and (b) protein:mRNA with BS disease activity (DAS). 162

Figure 5-17. Comparison of CXCL10 (a) protein and (b) protein:mRNA with clinical subgroups of BS subjects. 164

Figure 5-18. A representative voltage tracing of polysome factions within a sucrose density gradient passing though under laser over time. 165

Figure 5-19. The percentage occupancy of CXCL10 mRNA. 166

Figure 5-20. The proportion of the mRNA present within the free RNA portion or the polysomal portion of each sample (represented as a percentage of the whole). 168

Abbreviations

ACR	American College of Rheumatology
ACTH	Adrenocorticotropic hormone
AGO	Argonaute
ARE	Adenine and uridine rich element
AUC	Area under the curve
BFI	Blood flow imaging
BS	Behçet Syndrome
CCL2	Chemokine (C-C motif) ligand 2, MCP-1
CCL20	Chemokine (C-C motif) ligand 20, MIP-3α
CD	Crohn's disease
CGRP	Calcitonin gene related peptide
CPLX1	Complexin 1
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	cycle threshold
CXCL10	C-X-C motif chemokine 10, IP-10
CXCL11	Chemokine (C-X-C motif) ligand 11, I-TAC
CXCL9	Chemokine (C-X-C motif) ligand 9, MIG
CXCR3	Chemokine (C-X-CR motif) ligand 3, MIG
DAS	Disease activity score
DBF	Dermal blood flow
DICE	Differentiation control element
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis

EGF	Epidermal growth factor
eIF	Eukaryotic translation initiation factor
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
EULAR	The European League against Rheumatism
FACS	Flow cytometry and cell sorting
FMF	Familial Mediterranean fever
FMO	Fluorescence minus one
GFP	Green fluorescent protein
GWAS	Genome wide association study
HC	Healthy control subject
HIDS	Hyperimmunoglobulinemia D with Periodic Fever Syndrome
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPA	The hypothalamic-pituitary-adrenal axis
HSP	Heat shock protein
IBD	Inflammatory bowel disease
ICAM1	Intercellular adhesion molecule 1
IFN-γ	Interferon – gamma
IL	Interleukin
ISG criteria	International study group criteria
ISRE	IFN stimulated response element
ISRE	Internal ribosome entry sequence
JAK	Janus Kinase
LPS	Lipopolysaccharide
MAGIC	Mouth and genital ulcers with inflamed cartilage syndrome

MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
miRNA	microRNA
MMP	Metalloproteinase
MSU	Monosodium urate crystal
MTX	Methotrexate
nBS	neuro-Behçet Syndrome
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NK	Natural Killer
NOMID	Neonatal onset multisystem inflammatory disease
OURF	Upstream open reading frame
PABP	PolyA binding protein
PAPA	Pyogenic arthritis, pyoderma gangrenosum and acne syndrome
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositide 3-kinases
PU	Unit of flux
qRT-PCR	Quantitative real time reverse transcription PCR
QST	Quantitative sensory testing
R	Receptor
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species

SAPHO	Synovitis, acne, pustulosis, hyperostosis and osteitis. CRMO
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
SNP	Single-nucleotide polymorphism
SP	Substance-P
SpA	Spondyloarthritis
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF-α	Tumor necrosis factor – alpha
TRAPS	TNF receptor associated periodic syndrome
Treg	Regulatory T cell
TRPV1	Transient receptor potential cation channel subfamily V member 1
UTR	Untranslated region
VCAM1	Vascular cell adhesion protein 1

1. Introduction

Hippocrates of Cos (460–377 BC) described an illness with fevers, orogenital ulceration, pustules and eye disease in the 5th Century BC, “but there were also other fevers, as it will be described. Many had their mouths affected with aphthous ulcerations. There were also many defluxions about the genital parts, and ulcerations, boils (phymata), externally and internally about the groins. Watery ophthalmies of a chronic character, with pains; fungous excrescences of the eyelids, externally and internally, called fici, which destroyed the sight of many persons” [1]. From 1700 onwards, several reports were published of cases very similar to Behçet syndrome [2]. In one of these cases, reported in 1930 by the Greek ophthalmologist Benediktos Adamantiades [3], genital ulcers, arthritis and ocular inflammation were brought together as a single disease. In 1937, Hulusi Behçet, a Professor and Director of Dermatology and Syphilology at the University of Istanbul, published details of two patients with recurrent oral ulcers, genital ulcers, and ocular lesions [4]. During the following 3 years he published reports on five further patients in different languages, thereby promoting cultural awareness of the syndrome. Moreover, he added other clinical findings, including acneiform skin lesions, erythema nodosum, and arthralgia, to the so-called triple symptom complex [2]. He was convinced of the autonomy of this multi-system illness and succeeded in drawing the attention of the scientific community to it.

The condition has been given many names, the two most widely accepted being Behçet disease and Behçet syndrome (BS). Given the heterogeneity of clinical manifestations, we prefer the term syndrome rather than disease, thereby avoiding the implication of a single aetiology. BS has a complex polygenetic pre-distribution, with probable environmental triggers resulting in abnormal inflammatory responses [5-8]. Classification of this syndrome as an autoinflammatory or an autoimmune condition, however, has been heavily debated. Autoinflammatory diseases are a group of inherited disorders characterized by episodes of seemingly unprovoked recurrent inflammatory attacks triggered by the innate immune system. Their underlying genetic basis

relates to mutations and/or polymorphisms of genes that encode proteins involved in the regulation of apoptosis, cytokine processing and inflammation [9]. In contrast to classical autoimmune disorders, autoinflammatory diseases show no female predominance, and evidence of antigen-specific T cells or high-titre specific autoantibodies is relatively lacking. In BS, no female preponderance is observed, HLA class II associations have not been demonstrated and no strongly associated autoantibodies have been found. Furthermore, many manifestations of BS overlap with autoinflammatory conditions (Table 1). However, BS does not fit perfectly into the autoinflammatory disease category, as fevers do not tend to be a prominent feature, and onset in children is unusual. If autoinflammatory disease classification is extended to include many polygenetic disorders, including Crohn's disease, gout and Still's disease, it may be reasonable to include BS in this broader grouping [10]. However, it may be best classified as a complex polygenetic syndrome.

The complexity of BS makes dissecting the underlying pathogenesis a challenging prospect. Indeed, BS remains a relatively poorly understood syndrome. Recent genetic studies have shed some light on underlying mechanisms but much work remains to be done. Furthermore, there is a huge unmet clinical need, with no diagnostic test, no biomarkers to monitor disease activity or assess response to treatments and no specific treatments. This chapter reviews the clinical presentation of this syndrome as well as our current understanding of the pathogenesis of BS with emphasis on insights gleamed from recent genetic advances. It also highlights some of the excellent hypothesis-driven cellular research undertaken over the past 5 decades, trying to marry this to recent genetic insights. The evidence for environmental triggers in genetically susceptible individuals is also touched upon. Finally I outline my research aims. Detailed introductions for the three arms of this research project are found in later chapters.

Table 1-1. Overlapping features of autoinflammatory conditions and BS

Overlapping features of autoinflammatory conditions and Behçet Syndrome	
Clinical finding	*Associated autoinflammatory condition*
Aphthous ulcerations	HIDS
Genital ulcerations	HIDS
	PAPA
Acneiform lesions	PAPA
Pathergy	PAPA
Uveitis	Blau Syndrome
	NOMID
	TRAPS
Non deforming arthritis	FMF
	TRAPS
	PAPA
	Blau Syndrome
	Muckle Well's Syndrome
	HIDS
Meningoencephalitis	FMF
	NOMID

Abbreviations: HIDS, Hyperimmunoglobulinemia D with Periodic Fever Syndrome; NOMID, Neonatal Onset Multisystem Inflammatory Disease; PAPA, Pyogenic Sterile Arthritis, Pyogenic Gangrenosum and Acne Syndrome; TRAPS, TNF Receptor Associated Periodic Syndrome.
Table adapted from paper by Ambrose, Haskard[11]

1.1 Epidemiology and prevalence

BS is found throughout the world, with the highest prevalence in countries bordering the Silk Route, an ancient network of interlinking trade routes that connected Asia with the Mediterranean world. BS is linked with HLA B51 and the high frequency of this tissue antigen along the Silk Route indicates that a genetic risk migrated with population movement [12]. Not only is BS more common in countries related to the Silk Route than those further away, but the clinical manifestations also seem to be more severe [13]. National epidemiological studies of BS have been undertaken in Iran (6,500 cases)[14], Japan (3,316) [15], China (1,996) [16], Korea (1,527) [17] and Germany (590) [18], in addition to large case series from several countries [19-23]. From these reports, prevalence seems to vary from 80–421 per 100,000 individuals in Turkey, 80 in Iran, 8.6 in the USA and 7.2 in France to 4.2 in Germany [14-24]. Precise figures are not available for the UK, but the UK Behçet's Syndrome Society (the national patient group) have in excess of 600 members who report having 'confirmed Behçet

syndrome', leading to a possible prevalence of 10 per 100,000 individuals [25]. The male to female ratio in the larger national studies shows some variability, but overall no outright worldwide gender predominance is evident (Iran, 1.19; Japan, 0.97; China, 1.34; Korea, 0.57; Germany, 1.4; and Turkey, 1.03) [14-26]. The onset of BS is typically in early–middle adult life (that is 20–40 years of age) but, in rare instances, it also presents during childhood or in the elderly [27].

1.2 Clinical manifestations

BS is a multisystemic disorder, with mucocutaneous involvement being almost universal. Musculoskeletal symptoms are also common. Less commonly, ocular, vascular, gastrointestinal and/or neurological involvement occurs, with potentially serious consequences. The frequency of manifestations shows geographical variation. Additionally, at least two clusters of disease manifestations have been observed: that of superficial vein thrombosis, deep vein thrombosis and dural sinus thrombus; and that of acne, arthritis and enthesitis.

1.2.1 Orogenital ulceration

Recurrent oral aphthae are the hallmark of BS; they are almost always evident and can precede other manifestations by months or years. Ulcers can be small (minor ulcers, <10 mm), large (major ulcers, >10 mm) or, occasionally, herpetiform. Ulcers normally occur in crops on the buccal mucosa and mucosa of the inner lips but can also occur on the tongue, hard and soft palate and in the pharynx. Ulcers in BS do not typically form on the external lips, and lesions there should lead one to think of other disorders such as herpes virus or Stevens–Johnson syndrome. The ulcers are usually painful and occasionally scar. The most common differential is recurrent aphthous stomatitis, which affects up to 10% of the population. Oral ulcers in BS may be surrounded by an erythematous halo, although this observation is not sufficiently specific or sensitive to aid in diagnosis. Importantly, many other conditions can be associated with oral ulceration (a non-exhaustive list is shown in Table 2). Most oral ulcers in BS resolve within 10–14 days. Occasionally oral ulcers in BS can be persistent, but non-healing ulcers should be considered for biopsy to exclude an alternative diagnosis, such as Crohn's disease or

carcinoma. Interestingly, cigarette smoking may provide some protection from oral ulceration [28].

Genital ulcers occur less frequently than oral ulcers (Iran, 65% of patients; Japan, 73%; China, 76%; Korea, 83%; Germany, 64%; and Turkey, 88%)[14-26]. They resemble oral ulcers in appearance, are usually painful and, when deep, may heal with scarring. Genital ulcers in females affect the vulva and vagina and, occasionally, the cervix. In males, ulcers occur classically on the scrotum. Ulceration on the penis may occur in BS but should raise the possibility of HLA B27-related spondyloarthritis, particularly if situated around the glans penis. As with oral lesions, non-healing ulcers should be biopsied.

Table 1-2. Differential diagnosis of recurrent oral ulcerations

Differential diagnosis of recurrent oral ulcerations	
Aphthous ulcerations ('recurrent aphthous stomatitis') affects 10% population	
Rheumatological disease	Systemic Lupus Erythematosus Spondyloarthropathy Rheumatoid Arthritis
Gastrointestinal disease	Inflammatory Bowel Disease Coeliac Disease
Autoinflammatory conditions	HIDS
Infectious disease	Herpes Simplex HIV
Dermatological disease	Stephens Johnson Pemphigoid Pemphigus Lichen Planus
Haematological	Cyclic neutropenia
Other	Drugs methotrexate, other chemotherapy, nicorandil nutritional deficiencies Vitamin B12, Iron, Folic acid

Table adapted from paper by Ambrose, Haskard[11]

1.2.2 Pathergy

Pathergy refers to an exaggerated response to minor trauma, and is often experienced as a prolonged cutaneous erythematous inflammatory response to day-to-day insults, such as needle or thorn pricks or nettle stings. Pathergy is a hallmark feature of BS and can be very helpful in

differentiating BS from other conditions with overlapping clinical features such as Crohn's Disease (CD) and Systemic Lupus Erythematosus (SLE). Examining the pathergy response in BS was one of the main arms of this research project. The Pathergy test and histology of the pathergy response is discussed in more detail later in this chapter and in Chapter 3.

1.2.3 Skin lesions

Cutaneous lesions are very common in BS, occurring in 65–87% of patients (Iran, 65%; Japan, 87%; China, 69%; Korea, 84%; and Germany, 81%) [14-26]. Usual features include papules and pustules resembling acne, although care must be taken during diagnosis to consider whether pustules are an adverse effect of corticosteroid therapy. Acneiform lesions are more common in patients with BS with arthritis and are indistinguishable from acne vulgaris except that lesions may also occur on limbs [29]. The lesions are not sterile and have a similar microbiologic flora to acne [30]. Erythema nodosum may occur in BS, but is also a strong indicator of inflammatory bowel disease or sarcoidosis. Subcutaneous nodules may also be caused by superficial thrombophlebitis.

1.2.4 Ocular inflammation

The onset of ocular inflammation is usually within 2–3 years of the diagnosis of BS and is the presenting manifestation in 10–15% of cases. The eye is the most commonly involved vital organ (Iran, 57%; Japan, 69%; China, 35%; Korea, 51%; and Germany, 58%) [14-26]and tends to be most severely affected in males and in individuals less than 30 years of age [31]. Ocular disease is characterized by unpredictable, repetitive inflammatory attacks, which can subside spontaneously without treatment, with little inflammation between attacks. Bilateral panuveitis and retinal vasculitis are the typical manifestations of ocular disease [31]. Recurrent attacks eventually result in complications such as cataract, posterior synechiae, macular oedema, optic atrophy and glaucoma. Isolated anterior uveitis occurs in only ~10% cases, and is more common in females (22.3% patients) than males (5.8% patients) but is more typical of HLA B27-related diseases than BS [31]. It may present with a hypopyon (that is, an accumulation of white blood cells in the anterior chamber of the eye), which is visually dramatic but is rarely sight

threatening. Conjunctivitis, scleritis, episcleritis and sicca syndrome are uncommon and should also lead one to consider alternative diagnoses.

1.2.5 Neurological system

Headache is a common symptom in BS, occurring in over 80% of patients [32]; the vast majority of instances are a result of primary headache syndromes rather than detectable structural central nervous system (CNS) disease. Migraine is the most common problem (characterized by unilateral throbbing pain, visual or sensory aura), although a small number of patients have tension type headaches (characterized by a dull, constant, occipitovertebral ache)[32]. In patients complaining of headache, symptoms that warrant further investigation include severe or incapacitating pain, a change in character of the headache and particularly the presence of neurological signs [33].

Excluding headaches, neurological complications of BS (nBS) occur in less than 12% of cases (Iran, 3.5%; Japan, 11%; China, 6.5%; Korea, 4.6; and Germany, 11%) [14-26], often a few years after the onset of the other systemic features. Parenchymal disease occurs more frequently than neurovascular, and both types are more common in males than females [34]. By contrast, involvement of the peripheral nervous system is uncommon, and was seen in only 0.8% of patients in one large case series [33]. In parenchymal disease, meningoencephalitis occurs, with a mixed inflammatory cell infiltrate leading to necrosis and apoptotic neuronal loss [35]. Inflammatory infiltration, rather than fibrinoid necrosis, is seen around small vessels [35, 36]. The brainstem and mid-brain are the most commonly affected areas, but spinal cord lesions and cerebral involvement may also occur and, occasionally, nBS presents as a pseudotumour cerebri. Brainstem involvement usually presents subacutely, with headache, cranial neuropathies or cerebellar or corticospinal tract dysfunction [37]. Sensorineural hearing loss can occur, resulting in sudden deafness, balance disturbances and dizziness [38]. The characteristic MRI lesion in parenchymal nBS is a unilateral upper brainstem lesion extending into the thalamus and basal ganglia [39]. Analysis of cerebrospinal fluid shows a neutrophilic (in early disease) or lymphocytic (in late disease) pleocytosis, but usually no oligoclonal bands [40].

Neurovascular disease accounts for approximately 20% of cases of nBS and symptoms include dural sinus thrombosis, intracranial aneurysm and extracranial aneurysm and/or dissection. Aneurysms of the cerebral, vertebral and carotid arteries can also occur. The prognosis of nBS varies—those with a nonparenchymal process, such as dural sinus thrombosis, are unlikely to have recurrent disease, disability, or premature death if they survive the initial event. By contrast, patients with parenchymal disease have a less certain outcome [33, 40].

1.2.6 Musculoskeletal system

Musculoskeletal symptoms are extremely common in BS (Iran, 37%; Japan, 57%; China, 30%; Korea, 38%; and Germany, 53%) [14-26] . Arthritis is asymmetric and palindromic, potentially affecting most joints, but particularly the knees, ankles, and wrists. Erosive arthritis is very unusual and should suggest another diagnosis, such as spondyloarthritis (SpA) [41]. At one stage BS was classified in the spectrum of SpA [42, 43]. The distinction created much debate, and prospective studies of joint involvement in BS showed sacroiliac joint involvement to be rare in BS [41, 43]. As discussed in more detail later in the chapter, recent genome wide association studies (GWAS) have identified overlapping single nucleotide polymorphisms (SNPs) of interest in the two conditions (e.g. ERAP1). Fibromyalgia coexists in many patients with BS and is associated with anxiety and depression, but not necessarily with the activity of other manifestations [44]. Although chrondritis is not a usual feature of BS, the rare mouth and genital ulcers with inflamed cartilage (MAGIC) syndrome overlaps with features of BS and relapsing polychrondritis [45]. Osteitis and hyperostosis are also not features of BS and their presence should lead one to suspect synovitis, acne, pustulosis, hyperostosis, osteitis (SAPHO) syndrome[46].

1.2.7 Gastrointestinal manifestations

Many patients with BS experience abdominal pain, and symptoms similar to irritable bowel syndrome. Demonstrable gastrointestinal ulceration, with lesions in the terminal ileum, caecum, and ascending colon are less common (Iran, 7%; Japan, 15%; China, 9%; Korea, 7%; and Germany, 12%) [14-26, 47]. Ulceration may also occur in the small intestine [48].

Distinguishing BS with gastrointestinal ulceration from inflammatory bowel disease (e.g. CD) with extra-enteric involvement can be difficult, unless granuloma are visible in biopsy samples (CD) [49].

1.2.8 Cardiovascular system

The vasculopathy of BS is distinct from other forms of vasculitis as it predominantly affects veins and pulmonary arteries, both of which transport deoxygenated blood under low pressure. Vascular involvement shows wide geographic variability (Iran, 8.9%; Japan, 8.9%; China, 7.7%; Korea, 1.8%; Germany, 23%; and Turkey, 17%) [14-26]. When present, vascular pathology is frequently the presenting complaint [50], most commonly in male patients and those with a positive pathergy test or ocular involvement [51]. Superficial thrombophlebitis presents as tender superficial nodules and can be difficult to distinguish clinically from erythema nodosum. Besides sometimes having a linear appearance localizing to a peripheral vein, superficial thrombophlebitis can also be recognized by ultrasonography showing a central lumen. Deep vein thrombosis (DVT) usually affects veins of the legs [52], but may involve the superior and/or inferior vena cava, or, less commonly, the mesenteric, portal, hepatic, splenic, iliac, subclavian, axillary and retinal veins and/or dural sinuses. Venous thrombosis is considered to be partially accounted for by activated vascular endothelium, and no intrinsic abnormality of coagulation has been reported.

Arterial aneurysm formation is much less common than venous thrombosis. Aneurysms have a predilection for the pulmonary arterial tree, although aneurysms of the systemic circulation also occur. The association of pulmonary artery aneurysms with peripheral DVT is known as Hughes–Stovin syndrome, and is important to distinguish from pulmonary embolism in any patient with BS presenting with haemoptysis and DVT, as anticoagulation could be fatal [53]. In fact, embolism of venous thrombi is thought to be unusual in BS, perhaps because clots tether along a length of inflamed vein.

Unlike other inflammatory conditions, acceleration of atherosclerosis does not seem to be a feature of BS [54].

1.2.9 Genitourinary system

Besides genital ulceration, urogenital pathology is uncommon, and, in particular, parenchymal renal disease is rare. Young men may experience attacks of epididymitis, which is often misinterpreted as infective. Bladder mucosal ulceration may be responsible for urinary symptoms, and is probably underdiagnosed [55].

1.2.10 Constitutional symptoms

Patients with BS often have constitutional symptoms. Fatigue is almost universal and has a huge effect on patient quality of life [25]. Fevers are often self-reported but less commonly substantiated, and demonstrable recurrent fevers should lead one to consider other conditions such as autoinflammatory disorders, cyclical neutropenia (characterized by monthly fevers, mouth ulcers, pharyngitis and cervical lymphadenopathy), or sepsis.

1.3 Reaching the diagnosis

No diagnostic test currently exists for BS and markers of inflammation such as C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) may be only modestly elevated or even normal in patients with active disease. One of the aims of this research project was to study skin models of inflammation to assess their potential usefulness for diagnosis or measuring disease activity.

HLA B51 expression occurs with increased frequency in patients with this syndrome, depending on ethnicity [56]. However, expression of this antigen is neither sensitive nor specific as a diagnostic test. The diagnosis therefore has to be decided on clinical grounds, with due diligence in excluding other conditions. The Classification criteria published by the International Study Group in 1990 are helpful for this purpose, and require the presence of recurrent oral aphthous ulcerations plus two other major manifestations (Table 3) [57]. It is important to note that these criteria are intended for research and should not be applied dogmatically to clinical management. A thorough history and examination is essential.

Table 1-3. The International Study Group Criteria for diagnosis of BS

The International Study Group Criteria for diagnosis of BS	
Symptom	*The detail*
Oral ulcerations	3 times a year
Plus 2 of the following:	in the absence of other systemic diseases
recurrent genital aphthae	
eye lesions	including anterior or posterior uveitis, cells in vitreous on slit lamp examination, or retinal vasculitis, observed by an ophthalmologist
skin lesions	including erythema nodosum, pseudo vasculitis, papulopustular lesions, or acneiform nodules consistent with BS
a positive pathergy test	a papule 2 mm or more in size developing 24 to 48 hours after oblique insertion of a 20 to 25 gauge needle 5mm into the skin, generally performed on the forearm

Table adapted from paper by Ambrose, Haskard [11].

1.4 The pathergy test and monosodium urate crystal test

The pathergy test is usually performed by inserting a 20 gauge needle into the forearm skin and is considered positive if a papule or pustule is present after 48 hours. In national studies, pathergy phenomenon was seen in 34–57% of cases (Iran, 53%; Japan, 44%; Korea, 15%; Germany, 34%; and Turkey, 57%) [14-26]. Pathergy seems to be less common in non-endemic regions with only 10% of UK patients having a positive response [58]. Furthermore, the frequency of a positive test has declined over time, possibly owing to more effective prior cleaning of the test site [59]. Despite the low sensitivity, the pathergy test has a high specificity (>90%) and remains useful [59]. The neutrophilic dermatoses, such as Sweet's syndrome and pyoderma gangrenosum, and pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome may also give a positive pathergy test. Additionally, a positive pathergy test has been reported in patients with chronic myeloid leukaemia treated with IFN-α [60]. The pathology of pathergy reactions resembles that of other skin lesions in BS, with neutrophils, monocytes and lymphocytes present in large numbers, together with increased mRNA transcripts for the cytokines IFN-γ, IL-12 p40 and IL-15; the chemokines CCL20, CXCL10, CXCL9, and CXCL11; and adhesion molecules ICAM-1, and VCAM-1, at 48 hours in BS versus HC [61, 62]. Cytokine response to needle injury in the skin in BS is qualitatively different from that occurring in healthy individuals [62].

An alternative method to elicit an exaggerated response is to inject monosodium urate crystals (2.5 mg) into the dermis. The usual response consists of erythema and induration, which are maximal at ~24 hours. In healthy individuals there is an erythematous reaction at 24 hours which has usually largely resolved by 48 hours. In contrast, a prolonged response is observed in BS [63], with a sensitivity for BS of 78%, whereas the classical pathergy test had a sensitivity of 28% [64]. Moreover, the cutaneous response to MSU crystals appears to be more reproducible than pathergy (95% concordant response after one year, compared to 73% for pathergy) [64]. This unit has been able to show that UK patients also demonstrate a prolonged inflammatory response to MSU crystals [65]. The great advantage of the MSU crystal reaction over the pathergy reaction as an investigational approach is that it is a graded rather than a categorical (present/absent) response and can be quantified in all individuals. Moreover, the large literature on the inflammatory response to MSU crystals in the context of hyperuricaemia and gout provides an excellent mechanistic frame-work for exploring how the response is abnormal in BS [66, 67]. This is discussed in detail in Chapter 3.

1.5 Prognosis

Severity is generally greater in men, the young and those with genetic links to endemic regions [13].The greatest morbidity and mortality occurs with ocular, vascular and CNS disease. Young Turkish males (15–24 years of age) with ocular, vascular or CNS disease can have as much as a tenfold increase in mortality when compared with age and sex matched controls. In certain patients, disease may become less severe over time. Females and those with isolated mucocutaneous disease have a relatively good prognosis [13].

1.6 Inflammation and genetics

Most complex diseases are caused by the combination of genetic risk variants and environmental factors. Although there are many different types of genetic variation, current state of the art technology is most effective at identifying single nucleotide polymorphisms (SNPs), sites that differ by only one nucleotide among individuals in a population.

Early genetic work (1980s) was based on candidate gene association studies (CGAS) to investigate genes of interest. Recent technological advances now allow us to study thousands to millions of SNPs along the entire genome – Genome Wide Association Studies (GWAS), in large human samples. The advantage of the CGSA is its affordability and minimization of false negatives, whereas GWAS has the advantage of producing fewer false positives, being unbiased with regard to gene function, having the ability to correct for population stratification, and having coverage of variations across the genome. GWAS have a good track record of reproducibility. GWAS fail regarding identification of associated genes rather than genomic regions, identification of associated rare variants and studies can still have problems related to selection and misclassification bias. It also remains expensive, although costs continue to come down. Table 4 shows a simplified summary of some available genetic tests.

In general, genetic studies have revealed that regulatory variants are much more likely to cause disease than non-synonymous coding single nucleotide polymorphisms (SNPs) (i.e. SPNs that code for an alternative amino acid), and may be transcriptional or posttranscriptional (e.g. polymorphisms in post transcriptional regulatory elements related to micro RNAs (miRNA)).

Although genome wide association studies (GWAS) offer powerful means for identifying common variants associated with complex disease, they have been generally ineffective in identifying associations resulting from rare (minor allele frequency <1%) and low frequency (1-5%) variants. Next generation sequencers (HT-NGT) are becoming ever more sophisticated, offering analysis of single molecules of DNA (no PCR required), and using longer read lengths, which in combination with deep sequencing (reading the same strand of DNA multiple times) and advanced technology tools for re-assembly and analysis, result in ever more accurate and informative information becoming available for complex genetic syndromes such as BS.

Table 1-4 A summary of some common genetic tests

Genetic test	*Description*	*Advantages*	*Disadvantages*
Classic linkage studies of multicase families	Selected markers co-segregate with disease	Good for rare mutations with strong/obvious effect.	Need predictable inheritance pattern for analysis.
Allele sharing methods (e.g. affected sibling pairs, ASP)	Markers shared more than expected between disease siblings	No controls required. Model of inheritance not required.	Large numbers of siblings required. Low power to detect genes of modest risk.
CGAS	Assessment for presence of SNPs or VNTRs (e.g. microsatellite markers) in area of interest	Cheaper than GWAS. Less false negatives than GWAS.	Selection bias. False positives.
GWAS: HT and HT-NGS	1000s of SPNs studied at regular intervals throughout the genome	Relatively unbiased. 3rd generation sequencing: single DNA molecule, increasing accuracy (no PCR), longer read lengths.	Large study numbers. Miss rare variants. Multiple hypothesis testing. False positives (control selection).
Ultra-deep sequencing of genomic DNA	Combined with GWAS or CGAS: Depth = number of times a nucleotide is read during sequencing.	Identification of rare single-nucleotide polymorphisms (SNPs). Enables distinction between sequencing errors and true SNPs.	

Abbreviations: CGAS, candidate gene association studies; SNP,single nucleotide polymorphism; VNTR, variable number of tandem repeats; GWAS, genome wide association study; HT, high throughput; HT-NGS, high throughput next generation sequencing

1.6.1 Genetics and BS

There is strong evidence for a genetic contribution to BS. Having a sibling with BS confers a 10-50 fold increased risk of developing the syndrome relative to the general population [68]. Affected offspring of patients may have an earlier onset of disease (genetic anticipation) [69]. No single gene mutation is responsible for BS; rather, there appears to be a complex polygenic predisposition to its development, which reflects the heterogeneity of the clinical presentation. There is geographical variation in the distribution of symptoms, such as a high incidence of gastrointestinal involvement in the Far East [16, 17]. These observations support the hypothesis that BS may entail several distinct genetic mechanisms and that 'Behçet syndrome' is an umbrella term for clinically similar, but not identical illness, with the common denominator of oral ulceration.

In such a complex polygenetic disorder, numerous genes may be involved, regulating pro- or anti-inflammatory pathways. As BS is rare in children, it is likely that a trigger or burden of triggers in the environment is encountered in genetically predisposed individuals, resulting in development of the syndrome. This trigger(s) may differ, depending on the underlying genetic susceptibility. While genetics and gene regulation may hold the key to understanding this complex syndrome, there is also much to learn from hypothesis-driven research as well as from analysis of environmental interactions.

1.6.2 Innate immune dysfunction

The innate immune system is primed to recognise pathogen associated molecular patterns (PAMPs, e.g. lipopolysaccharide (LPS)) and danger associated molecular patterns (DAMPs, e.g. heat shock proteins (HSP)) via cell membrane receptors such as Toll-like receptors (TLRs), scavenger receptors, and intracellular receptors such as the NOD-like receptors (NLRs e.g. NLRP1 which activates the inflammasome, resulting in IL-1β and IL-18 production). Cells include phagocytic populations (e.g. dendritic cells, macrophages, neutrophils) and the natural killer cells (NK cells). Phagocytic cells ingest and display antigens, and secrete cytokines such as IL-1β, TNF-α, IL-6, IL-8 and IL-12 which up-regulate the response and recruit inflammatory cells. NK cells recognise infected or damaged cells displaying stress molecules and having down-regulated MHC Class I molecules, via killer activation receptors (KARs) and killer inhibition receptors (KIRs) respectively. B-1 B cells and non-activated B cells secrete natural antibodies and also take part in innate immunity.

1.6.2.1 Innate immune dysfunction in BS

Clinical manifestations of BS, such as episodic inflammation and clinical overlap with auto-inflammatory diseases, as well as the histological feature of neutrophil recruitment to sites of inflammation suggest that the innate immune response is important in BS. Recent genetic studies, discussed below, support the hypothesis that dysregulation of innate immunity is involved.

1.6.3 The inflammasome / IL-1β pathway

The inflammasome / IL-1β pathway is one of the cardinal pathways of innate immunity. In BS, studies have demonstrated polymorphisms within the IL-1β gene [70-72] as well as identifying polymorphisms within the Mediterranean fever 1 gene (MEFV1) [73-77]. In a study published in 2013, non-synonymous variants (NSVs) identified by deep exonic re-sequencing of candidate genes, in pooled samples of almost 2,500 BS patients and controls (48 samples per pool), found that carriage of the MEFV mutation Met694Val conferred BS risk in the Turkish population (OR 2.65). This gene is also implicated in Ankylosing Spondylitis (AS) [78] and Inflammatory

Bowel Disease (IBD) [79] in Turkish populations. They also found several Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) SNVs (Arg702Trp; Gly908Arg; Leu1007fsinsC) associated with BS, which were present in Turkish but not Japanese cohorts. These SNVs associated with reduced response to Muramyldipeptide (MDP) (a peptidoglycan constituent of gram positive and negative bacteria). These NOD2 SNVs have been shown to be associated with risk in Crohn's Disease (CD) but interestingly were found to be protective in BS. The same study assessed several other 'inflammasome' genes without finding any significant association, including CASP1 and PYCARD. Clinical studies support the role of IL-1β pathways in BS, with elevated IL-1β observed in the sera [80] and synovial fluid [81] of BS subjects.

1.6.4 Toll-like receptors and cytokines

TLR-4 stimulation (e.g. with LPS) results in activation of Interferon regulatory factor (IRF)/ Interferon (IFN), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen-activated protein kinase (MAPK)-activator protein (AP)-1 pathways resulting in activation of multiple cytokine pathways, including IL-6, TNF-α, IL-1 and IL-12. Two TLR-4 NSVs identified by deep exonic re-sequencing (D299G & T399I) were found to be protective for BS, and resulted in a reduced response to LPS. Interestingly, the same markers are risk SNVs for CD [73]. A separate study, found that monocytes of active BS subjects demonstrated increased activity of toll-like receptors (TLR)-2 & -4 and furthermore, monocytes from intestinal lesions in BS subjects expressed increased TLR-2 and -4 mRNA compared to health controls (HCs) [82].

TNF-α polymorphisms were reviewed in a meta-analysis and this report substantiated the association of BS with the -1031C, -857T and the - 238A TNF-α promoter polymorphisms in multiple ethnic groups [83]. Clinical studies have found elevated TNF-α in serum [80] and aqueous fluid [84] and certainly anti-TNF-α monoclonal antibodies have an established role in the treatment of BS [85].

Significant differences in IL-6 variable nucleotide tandem repeats (VNTRs) and allele frequencies were found in BS subjects. These differences were particularly apparent in the HLA-B51 negative and female subjects [86]. IL-6 protein has been shown to be elevated in serum and CSF fluid [87, 88]. Serum from BS subjects stimulate a classic pro-inflammatory response in macrophages [89].

1.6.5 Matrix metalloproteinases

Matrix metalloproteinase-2 and -9 (MMP-2, MMP-9) are secreted enzymes that regulates cell-matrix composition. Their main substrate is gelatin (a denatured collagen). They are produced by keratinocytes, monocytes, tissue macrophages, and neutrophils. They cleave denatured collagen (gelatins) and type 4 collagen which facilitates lymphocyte recruitment. They are also involved in cytokine and chemokine processing [90, 91]. These enzymes have been implicated in multiple inflammatory conditions including vasculitis [92], aggressive periodontitis [93] and aneurysmal formation [94]. It is thought that MMP-2 and -9 have anti- and pro- inflammatory roles respectively. MMP-2 has been shown to maintain gut barrier function [95]. Candidate gene analysis of MMP-2 and MMP-9 polymorphisms found the lower MMP-2 -1575 *G/*G and -735*C/*C which correlated to lower levels of MMP2 in erythema nodosum (EN) lesions on immunohistochemistry. Furthermore, they found significantly higher MMP-9-1562-*C/*C which correlated to more MMP-9 in EN biopsies. A clinical study has found serum MMP-9 levels to be higher in active BS versus HCs [96].

1.6.6 Natural killer cells and receptors

Natural killer (NK) cells are a group of circulating cytotoxic lymphocytes that play a role in nonspecific toxicity against virus-infected cells and tumour cells [97]. They are considered cells of innate immunity, but share some receptors with NK T cells and γδ T cells. These later two cells are described as transitional cells, straddling innate and adaptive immunity. NK cells also share some receptors with cytotoxic T cells, the natural killer cells of adaptive immunity.

A SNP (KLRC4, rs2617170) was identified by GWAS within the KLRK1-KLRC1 locus on chromosome 12 [98]. This haplotype block (the 'NKG2D' haplotype) contains 5 natural killer

cell receptor genes. The KLRC4 gene codes for the NKG2F C-type lectin receptor. Two non-synonymous variants in KLRC4 encoding KLRC4pIle29ser and pAsn104Ser alterations respectively were found on this BS protective haplotype of this Linkage disequilibrium (LD) block [98]. This haplotype has been associated with reduction of peripheral blood leukocyte cytotoxicity [99]. Furthermore, the LD NKG2D gene encodes an activating homodimeric C-type lectin receptor, which is expressed on NK cells, CD8+αβ T cells, γδ T cells, and activated macrophages. The NKG2D receptor triggers cell-mediated cytotoxicity upon the recognition of self-ligands, such as MICA and MICB (induced by cellular stress, e.g. virus / tumour). Interestingly these MICA and MICB genes are in LD with HLA B*51 [99]. In support of this haplotype's importance in BS, a Turkish whole genome linkage analysis study in multi-case BS families (using 395 microsatellite markers), found the strongest linkage peak (OD 3.94) was on chromosome 12p12-13 which includes the KLRK locus [100].

Non-genetic research also supports a role for these genes/pathways. CD69+ activated NK1 cells mainly produce IFN-γ and IL-10, two cytokines found in abundance in BS, and these cells express high levels of IL-12β receptors. NK2 cells produce IL-5 and express low levels of IL12-Rβ [101]. A study found CD69+-activated NK cells were significantly increased in active BS compared to inactive BS and healthy controls. Inactive disease was associated with the NK2 phenotype. Also of interest was that NK cells from inactive BS subjects, but not NK cells from controls, suppressed IFN-γ expression by CD4+ T cells from active BS subjects *in vitro*, suggesting that NK cells control a Th1 response in BS [102].

1.6.7 γδ T cells

γδ T cells represent a small subset of T cells that possess a distinct TCR on their surface. These cells are found at their highest abundance in the gut mucosa, skin and the respiratory tract. They do not require antigen processing and MHC presentation of peptide epitopes although some recognize MHC class IB molecules. They have a very limited set of V, D and J genes, but respond quickly. The antigens recognized by γδ T cells include heat shock proteins (HSP) and the non-peptide antigens prenyl pyrophosphates (PPP). The PPP isopentenyl pyrophosphate

(IPP) is a precursor for a variety of biological molecules, such as cholesterol and some vitamins. Peripheral blood γδ T cells have been found to be elevated in BS, with a polyclonal activation [103, 104]. They have been associated with active disease, with higher expression of CD69 and production of IFN-γ and TNF-α [105]. IPP-specific TCRVγ9Vδ2+ Th1-like cells from intra-ocular fluid have been isolated from BS uveitis subjects [106]. TCRVγ9δ2+ T lymphocytes are relatively expanded in active disease [107]. A recent paper found significantly increased CD16 expression, and less CCR7 expression in the peripheral blood (PB) γδ T cells of BS versus HC. CD16 expression represents an active phenotype (high cytolytic activity, inflammatory cytokines production) of the γδ T cells. CCR7 is used to distinguish tissue-homing effector (CCR7-) from lymph node-homing non-effector memory cells (CCR7+). γδ T cells also express inhibitory and activating members of the NK receptor family. TCRVγ9Vδ2+ T cells can be activated through NKG2D by stress ligands such as MICA. In this study, NKG2D receptors on the γδ T cells were decreased in subjects with BS compared to HC [108].

Table 1-5. Summary of innate immunity genetic markers identified in BS

Inflammasome pathway	*Gene marker*	*Study type*	*Population*	*Reference*
IL-1α and IL-1β	IL-1α-889C; IL-1β+3953 T; IL-1α -889C/IL-1β +5887T	CGAS	Turkish	[70-72]
MEFV	Met694Val, Met680Ile	CGAS; DER of NSVs	Multiple	[73, 74]
NOD2	Arg702Trp; Gly908Arg; Leu1007fsinsC	DER of NSVs	Turkish, not Japanese	[73]
Non-inflammasome pathway				
TNFA	-1031C (OR 5 1.35,); - 238A (OR 5 1.51); - 857T (OR 5 0.76)	Metanalysis GCAS	Multiple	[83]
IL-6	VNTR in 3'region IL-6 gene	CGAS	Korean	[86]
TLR4	D299G; T399I; rs1927906	DER of NSVs	Multiple	[73, 109]
Other pathway				
MMP2	-1575G, -735C	CGAS	Korea	[110]
MMP9	- 1562 C	CGAS	Korea	[110]
Natural Killer genes				
KLRC4 /NKG2F	rs2617170	GWAS	Turkish, Japanese	[98]
KLRK	12p12-13	Familial linkage study	Turkish	[100]
NKG2A	NKG2A c.-4258*C, c.338-90*G, and CD94 c.-134*T alleles	CGAS	Korea	[111]
NKG2C	NKG2C c.305*T (risk)	CGAS	Korea	[111]

Abbreviations: CGAS, candidate gene association studies; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats; GWAS, genome wide association study; HT, high throughput; HT-NGS, high throughput next generation sequencing

1.6.8 Adaptive Immunity

The immune system has to defend the organism against ever evolving pathogens, necessitating an almost unlimited repertoire of receptors to recognise invading pathogens. Antigen-specific recognition by receptors on B and T cells leads to clonal expansion. The receptors are generated in a random process and T cells with receptors against self are usually eliminated in the thymus. T cell receptors (TCRs) recognize a specific peptide epitope contained within a MHC molecule. B cells synthesize immunoglobulin of a single specificity that binds a single epitope. Engagement of BCR or TCR leads to initiation of signal transduction pathways and expression of cytokines, chemokines and other proteins.

1.6.8.1 HLA B51 and other HLA markers

Human Leukocyte Antigens (HLA) are a cluster of genes on chromosome 6, which, amongst other functions, code for the HLA class I and II cell surface antigen recognition molecules. Expressed on the surface of virtually all cells, class I molecules (HLA A, B and C) display antigens from inside the cell. If a cell is infected or damaged, antigen is displayed via class I molecules and the cell is targeted by circulating cytotoxic T cells (CD8+).

HLA B5 has 2 alleles: HLA B51 and HLA B52. HLA B51 allele binds peptides with 8-11 amino acids [112] and differs only by 2 amino acids from B52, where asparagine and phenylalanine are replaced by glutamic acid and serine in B52 [113]. These two amino acids are located in the B pocket of the antigen binding grove of the HLA molecule, which holds the second amino acid of the peptide being presented. HLA B51 may only hold a small amino acid in this grove (alanine or proline). HLA B51 is the strongest known genetic susceptibility factor for BS [6, 114-116]. The pooled odds ratio for developing BS associated with the presence of HLA B51 is 5.8 [117], but shows geographical variation (1.5 in Portugal to 16 in Italy [12, 118]). HLA B51 may contribute to disease severity, but its presence does not confirm BS [119]. 28% of Amerindian Eskimo population are HLA B51 positive, yet there are no reported cases of BS in this population [3].

The mechanisms by which HLA B51 may contribute to BS remains unknown, although there are a number of theories including linkage disequilibrium (LD) with genes such as MICA [6, 120]; misfolding (B51 is a slow folding molecule, which binds low affinity peptides. This binding may cause slow assembly and misfolding of the HLA B51 molecule possibly causing endoplasmic reticulum stress thereby inducing an inflammatory response) [121]; Low affinity binding of peptides (leading to enrichment of CD8+ and γδ T cell repertoires) [122, 123]; HLA B51 interacting with Natural Killer (NK) cells, CD8 and γδ T cells, through its HLA Bw4 epitope which it shares with HLA B27 [124, 125]; or peptides derived from digestion of the HLA B51 protein being presented by other HLA class II molecules and inducing an adaptive immune response (e.g. retinal s antigen, shares amino acid sequence homology with B27PD which is also found in B51) [126, 127].

Recent genetic work has made two exciting discoveries which may shed some light on the mechanism for pathogenesis of HLA B51. Endoplasmic reticulum aminopeptidase 1 (ERAP1) encodes an endoplasmic reticulum aminopeptidase, responsible for trimming peptides that are 9-17 amino acids long to 9 in preparation for HLA Class I presentation. It is also involved in cytokine receptor shedding. It was identified previously in GWAS studies in AS and psoriasis [128, 129]. A GWAS using a recessive model and fine mapping recently identified ERAP1 in a Turkish BS population. The mutations identified have been shown to confer protection against psoriasis (in HLA C06 individuals) and AS (in HLA B27 individuals) but conferred risk for BS in HLA B51 individuals.[98].

Another recent paper genotyped over 8000 variants in the extended HLA locus, and carried out imputation and metalysis of 24,834 variants in 2 BS cohorts from ancestry groups. Using this fine mapping they identified the most significant association to be rs116799036 (OR 3.74) which was located approximately 24kb upstream of HLA B and 18kb upstream of MICA[130]. To determine whether the genetic effect of rs116799036 can be explained by the association with HLA B51, they performed pairwise conditional analysis to control for HLA B5101. The genetic association remained significant independent of HLA B5101. Notably, when adjusted by conditioning on rs116799036 the genetic association with B5101 disease was completely

abrogated. Therefore this study strongly suggests that the association between HLA B51 and BS can be explained by rs116799036, found upstream of B51. This data is also consistent with a previous microsatellite study which identified the region to a 46kb DNA segment centromeric to HLA B [130]. The exact function of this region is not known. Some other HLA associated markers that have been identified are shown in Table 6.

Table 1-6. Summary of some HLA genetic markers identified in BS

Inflammasome pathway	*Gene marker*	*Study type*	*Population*	*Reference*
HLA B*51	rs116799036	GWAS, dense genotyping	Multiple	[130] [5, 6]
ERAP1	rs10050860 (encoding p.Asp575Asn) and rs17482078(p.Arg725Gln)	GWAS discovery, fine mapping validation	Turkish	[98]
PSORS1C1	rs12525170	GWAS, dense genotyping	Turkish& Italian	[130]
HLA-F-AS1	rs114854070	GWAS, dense genotyping	Turkish& Italian	[130]

Abbreviations: CGAS, candidate gene association studies; GWAS, genome wide association study.

1.6.8.2 T helper cells

There has been extensive interest in the role of T cells, and their interactions with APCs in BS**.** Drugs such as cyclosporine, mycophenolate, thalidomide and azathioprine, all of which act at least in some way through T cell inhibition, can be highly effective treatments. T helper (Th) cells, upon activation, can be directed towards a Th1, Th2, Th17 or the more recently described Th9 skew depending on their environment.

1.6.8.3 Th1 cells

Th1 cell defence is directed against intracellular pathogens. Initially cells require IFN-γ to upregulate IL-12Rs. IL-12 then stimulates differentiation towards a Th1 skew. Transcriptional factors involved include Signal Transducers and Activators of Transcription (STAT) 1, T-box transcription factor TBX21 (T bet) and STAT4. Th1 cells secrete IFN-γ and lymphotoxin, and induce chemokines such CXCL9, CXCL10 and CXCL11 which attract more T cells to the area and aid development of cytotoxic T cells.

STAT4 polymorphisms have been found to be associated with BS in the Turkish, Japanese, Han Chinese and Korean populations [98, 131, 132]. Furthermore the IL23R-IL12RB2 is a common variant SNP that was identified in the first two GWAS studies published in 2010 [6, 116]. In

clinical studies, IL-12 was found to be elevated in peripheral blood of BS subjects and correlated with disease activity[133]. High levels of IFN-γ were found in BS aqueous humour fluid as compared with fluid from subjects other causes of uveitis [84]. High levels of CXCL9, CXCL10 and CXCL11 have been observed in BS associated uveitis versus other forms of uveitis, and high CXCL10 was found in CSF fluid of BS subjects [134, 135].

1.6.8.4 Th17 cells

Th17 differentiation initially requires the presence of Transforming growth factor beta (TGFβ) plus either IL-6 or IL-21. Later IL-17 is required and then IL-23 is required for terminal differentiation and effector function. Signalling pathways involve STAT3, Interferon regulatory factor (IRF) 4, RAR-related orphan receptor gamma (RORγT), TLR2/4 and pSTAT3. IL-17 elicits an innate response with neutrophil recruitment, and promotes growths. It also induces secretion of CXCL9, CXCL10 and CXCL11, attracting Th1 cells [136, 137]. Both Interferon-α and Cylcosporin A inhibit the production of IL-17 in BS, and both have efficacy in the treatment of BS [138, 139].

The IL-12R/IL-23RB2 and STAT4 as well as the TLR4 SNPs already discussed in the Th1 paragraph above, may equally be implicated in the Th17 pathway [6, 73, 98, 116, 132, 140]. Additionally, a recent GWAS study in a Korean population identified interactions between IL-17a, IL-23R, and STAT4 polymorphisms, which confer susceptibility to intestinal BS [132]. A SNP (rs2397084) was identified in a Korean population within the IL-17 gene [141], and a STAT3 SNP (rs2293152) was found to be a risk factor for BS in Han Chinese population [142]. This SNP is also a risk factor for IBD.

In clinical studies, serum concentrations of IL-17, and production of IL-17 and IL-23 by stimulated peripheral blood derived mononuclear cells (PBMCs) has been shown to be elevated in active BS [143, 144].

1.6.8.5 Co-stimulatory molecules

T cell activation is enhanced by some co-stimulatory molecules e.g. CD28 (on T cells) interacting with CD80/CD86 (on APCs) and CD40L (on T cells) with CD40 (on APCs) and

inhibited by others, e.g. cytotoxic T lymphocyte antigen-4 (CTLA-4, on T cells) interacting with CD80 (on APCs), programmed death-1 (PD-1, on APCs) and programmed death-ligand 1 (PG-L1) [145].

Two candidate SNPs were identified in the CD40 gene (rs4810485TT, rs1883832TT) in Han Chinese population [146]. Candidate CD28 and CTLA4 SNPs were investigated in a Turkish cohort. The CTLA-4 +49AGG allele was found to be significantly lower in BS versus HC. This G allele has been found to be associated with the impaired control of T cell proliferation. The CD28 IVS3 +17T/C allele was found to be more prevalent in the BS population [147]. Clinical studies have shown a reduced expression of three co-stimulatory inhibitors (CTLA-4, CD86 and PL-L1) in active disease, as well as high levels of Inducible co-stimulator (ICOS), a member of the CD28 co-stimulator family [148].

Table 1-7. T helper cell populations

Th cells	*Stimulus*	*Signalling pathway*	*Effect*
Th1	IFN-γ initially, then IL-12	STAT1 ; T bet; STAT 4	Secrete IFN-γ and lymphotoxin, MIP-1, CXCL9/10/11. Attract and activate macrophages. Defence against intracellular pathogens.
Th2	IL-4 initially, then IL-5/IL-13	STAT6, GATA3	Defence against extracellular pathogens e.g. parasites; helminths. Stimulate IgE and Mast cells, B cells to produce antibodies.
Th17	TGFβ and IL-6/IL-21 initially, then IL17, IL23	STAT3, IRF4, RORγT; TLR2/4; pSTAT3	IL-22; IL-17, acts on endothelial and parenchymal tissues, illicit an immune response including acute phase response, and promoting growth. Induce CXCL9/10/11 which attract Th1.

1.6.9 Chemokines

Chemokines are involved in leukocyte recruitment to sites of inflammation. The CCR1-CCR3 locus contains a cluster of chemokine receptor genes within a LD block. Haplotype analysis of this region revealed 3 SNPs (rs13085057, rs13092160CC and rs13075270) associated with BS. rs13092160 TT was associated with less CCR1 and CCR3 expression [98, 149]. SNPs have also been found in association with the chemokine CCR5 in Italians [150]; and the chemokine CCL4 (MIP-1) [151]. In clinical studies CCL2 (MCP) levels in the blood of BS subjects were found to be significantly higher than HC or thrombotic controls [152, 153]. CCR5 and CXCR3 receptors have also been shown to be highly expressed in BS biopsies from oral ulcers[154]. The pathology of pathergy reactions shows increased mRNA transcripts for the chemokines CCL20, CXCL10, CXCR3, and CXCL11 [61, 62].

Table 1-8. Summary of T helper and chemokine markers identified in BS

Adaptive immunity	*Gene*	*Study type*	*Population*	*Reference*
Th1				
IL23-IL12RB2	rs924080 ; rs1495965 ; rs17375018, rs11209032	GWAS	Multiple	[5, 6]
STAT 4	rs7574070, rs7572482	GWAS	Multiple	[73, 132, 140]
Th17				
STAT 3	rs2293152 GG	CGAS	Chinese	[142]
IL23-IL12RB2	rs924080 ; rs1495965 ; rs17375018, rs11209032	GWAS	Multiple	[5, 6]
IL-17 gene	rs7771466	CGAS	Korean	[141]
Costimulatory molecules				
CD40	rs4810485TT, rs1883832TT	CGAS	Chinese	[146]
CD28	rs1181389	C GAS	Turkish	[147]
CTLA4	rs10497873	CGAS	Turkish	[147]
Chemokines				
MCP-1	-2518A/G	GWAS	Chinese	[151]
CCR1-CCR3	rs7616215; rs13084057; rs13092160, rs13075270	GWAS	Multiple, Chinese	[73] [149]
CCR5	ccr5-403AA, ccr5 delta32	CGAS	Iranian, Italian	[150]

Abbreviations: CGAS, candidate gene association studies; GWAS, genome wide association study.

1.6.10 Dampening inflammation

The immune system has developed many mechanisms to avoid sustained, over-zealous and auto-reactive inflammatory responses. These include thymic positive and negative selection; feedback loops; co-activation of pro- and anti- inflammatory signalling pathways; requirement of second signals; as well as anti-inflammatory pathways involving IL-10; TGFβ; resolvins; lipoxins; T-regs; Tr1 cells; T suppressor cells; debris clearance mechanisms; de-activated / M2c macrophages; glucocorticoids; transcriptional and post-transcriptional control pathways, to name just a few. In BS, there is a prolonged or sustained activation or possibly a failure to deactivate responses. The clinical hallmark of sustained inflammation in BS is the pathergy response.

1.6.10.1 IL-10

GWAS studies have found SNPs associated with IL-10 [6, 116]. The IL-10 disease allele (rs1518111 AA) was associated with a reduction in IL-10 mRNA expression and protein production in purified monocytes 24hr after LPS stimulation [6].

1.6.10.2 Treg cells

A recent study identified a SNP within the TGF-β gene [155]. Although no functional data is yet available regarding this TGF-β SNP, less TGF-β production would reduce Treg differentiation. A cellular study showed increased Th17 cells and decrease in frequency of CD4+foxp3+ Tregs in peripheral blood, which correlated with BS activity. BS serum causes a shift to high Th17 and towards lower numbers of TregsFoxp3 cells [156].

1.6.10.3 MicroRNAs

MicroRNAs (miRNA) play a role in post-transcriptional repression of protein production. A miRNA-146a polymorphism (rs2910164GG>CC) was identified in a Chinese BS cohort, and this was associated with higher IL-17, TNF-α and IL-1β, suggesting the disease associated SNP resulted in less negative feedback [157]. A clinical study found decreased miRNA-155 expression in PBMCs and DCs from BS subjects with active uveitis. Overexpression of this miRNA was shown to inhibit production of IL-6 and IL-1β; furthermore miRNA-155 transfected DCs significantly inhibited intracellular IL-17 expression in CD4+ T cells. Therefore, the lack of repression by miRNA-155 in BS may contribute to the pro-inflammatory / Th-17 response [158].

1.6.10.4 Apoptosis

Apoptosis is an important mechanism to dampen inflammation. A study has shown that activated T cells from BS showed resistance to CD95 induced apoptosis, with high level expression of B-cell lymphoma-extra large (Bcl-xl) in BS T cells. Thalidomide, a suppresser of NF-κB via IKK, was able to reverse this [159].

1.6.10.5 Ubiquitin and SUMO4

Ubiquitin and Small ubiquitin-related modifier 4 (SUMO4) (an ubiquitin like protein) SNPs have been found to be associated with BS in 3 genome wide association studies [160-162]. Ubiquitins are small regulatory proteins found in almost all tissues of eukaryotes. Amongst other roles, they direct proteins to the proteasome for destruction.

Table 1-9. Summary of anti-inflammatory markers identified in BS

Dampening inflammation	*Gene*	*Method*	*Population*	*Reference*
IL-10	rs1518111, rs1800872, rs1800871	GWAS	Multiple	[5, 6]
TGFbR3	rs1805110(Chinese)	GWAS	Chinese	[155]
Other				
MicroRNA-146a	rs2910164GG	CGAS	Chinese	[157]
SUMO4	rs237012	GWAS	Chinese	[160]
UBAC2	rs3825427,rs9517668,rs9517701 (Chinese), rs9513585,	GWAS	Chinese, Turkish	[161, 163]
UBASH3B	rs4836742	GWAS	Chinese	[163]

Abbreviations: CGAS, candidate gene association studies; GWAS, genome wide association study.

1.6.11 The role of neuropeptides in inflammation

Two of the best characterised neuro-immune systems are: (i) The hypothalamic-pituitary-adrenal (HPA) axis: Acute physical or psychological stress activates the HPA axis resulting in increased plasma ACTH and cortisol concentrations. Cortisol is a potent anti-inflammatory agent. (ii)The cholinergic anti-inflammatory pathway: The nervous system has been shown to act via the parasympathetic vagus nerve to inhibit cytokine release. Afferent vagal nerves sense pathogens, ischaemia and cytokines. Efferent nerve activation of the vagus nerve results in local release of acetylcholine (ACh) in organs including the spleen. Macrophages have ACh receptors which upon activation inhibit pro-inflammatory cytokine synthesis and release. The efferent neural signalling pathway is termed the cholinergic anti-inflammatory pathway [164-169]. The nervous system has been shown to play a role in several inflammatory conditions from psoriasis to arthritis [170-175].

There is ample clinical evidence suggesting that emotional stress can influence the course and disease activity of BS [176, 177]. The association between stressful life events and various dermatological diseases is explained by the concept of neuro-immuno-cutaneous system [178-182]. It was reported that neuropeptides, especially Substance P (SP), Calcitonin gene related peptide (CGRP) and neurotrophins such as nerve growth factor (NGF) affect the pathogenesis of skin disorders like atopic dermatitis and psoriasis vulgaris.

Recent genome wide association studies in BS have suggested a possible neuropeptide link. In one study, they identified near genome-wide significance for one non-MHC SNP, rs936551

(P=5.29 x 10 – 8) located at the telomeric end of the short arm of chromosome 4 within the promoter region of CPLX1, which encodes complexin-1 [6]. Complexins play a critical role in the control of fast synchronous neurotransmitter release. They operate by binding to trimeric SNARE complexes consisting of the vesicle protein Synaptobrevin and the plasma membrane proteins Syntaxin and SNAP-25, which are key executors of membrane fusion reactions [183]. SNARE complex binding by complexins is thought to stabilize and clamp the SNARE complex in a highly fusogenic state, thereby providing a pool of readily releasable synaptic vesicles that can be released quickly and synchronously in response to an action potential and the concomitant increase in intra-synaptic Ca(2+) levels [184]. Genetic elimination of complexins from mammalian neurons causes a strong reduction in evoked neurotransmitter release.

One study has formally investigated neuropeptides in BS [185] using immunohistochemistry of skin biopsies and protein quantification of serum samples. Strong immunoreactivity of SP was seen in the epidermis, panniculitis lesion and vasculitis lesion of BS subjects versus HCs. The concentration of CGRP measured by ELISA was decreased in BS subjects. CGRP was recently revealed to promote IL-10 production and suppress LC antigen-presenting function, T lymphocyte proliferation and production of IL-2, IL-12 or IFNγ [186].

1.6.12 Environmental Triggers

It is very unlikely that genetic risk wholly accounts for BS development. BS is a syndrome that typically develops in the 3rd or 4th decade, implying a further trigger is required to cause disease in those who are genetically susceptible. Environmental agents have been extensively investigated including bacteria (especially the Streptococcal species), viruses and other environmental agents such as chemicals, heavy metals and smoking. It is possible that the development of BS is related to the burden of triggers rather than one specific trigger for disease, as no superantigen has been identified to date. Alternatively, different triggers may result in the development of BS, depending on the presence of specific genetic abnormalities.

1.6.12.1 Streptococcal species

Streptococcal species have been extensively studied. *Strep. sanguinis* is a gram positive facultative aerobic coccus and member of the viridans streptococcus group. It is a normal inhabitant of the human mouth, especially in plaque, where it modifies the local environment making it less hospitable to cavity forming bacteria such as *Strep. mutans*. *Strep. sanguinis* antigens have been found in the skin and monocytes of BS subjects, but not in healthy controls. These antigens share a sequence of amino acids (homology) with the stress induced heat shock proteins (HSPs). Studies have shown that *Strep. sanguinis* and HSP (HSP 60/65kDa) activate γδ T cells in BS, but not in healthy controls. Studies suggest that following bacterial exposure, cells express HSPs that are antigenic and reactivate γδ T cells in susceptible individuals (molecular mimicry model) [187]. The relationship between streptococcal infections and BS is also suggested by clinical observations such as the aggravation of BS symptoms by dental treatment [188] and that oral ulcers precede the establishment of BS by months to years. In Turkish populations there is a higher rate of *Strep. mutans* salivary colonization compared with controls and colonization is associated with low levels of serum mannose binding lectin (MBL) compared to controls [189]. Elevated levels of antibodies against epitopes of mycobacterial HSPs, some of which have significant homology to human HSPs, have been observed amongst subjects with BS [190]. Exacerbations of ocular disease coincided with enhanced levels of these autoimmune antibodies. Furthermore these peptides can induce uveitis when injected into Lewis rats [191].

1.6.12.2 Intestinal permeability

Increased intestinal permeability may lead to chronic immune stimulation [192]. The gastrointestinal epithelial cells normally form a relatively impermeable physical barrier to luminal contents, facilitated by tight junctions [193]. A thick layer of mucus overlies the epithelial cell layer. Several studies have reported increased intestinal permeability in CD subjects [194]. Diversion of the faecal stream in such subjects is associated with the induction of remission. Intestinal permeability in BS subjects without gastrointestinal disease have been

assessed using Cr-EDTA and found to be significantly increased compared to healthy controls [195].

1.7 Summary

BS is a complex, heterogeneous condition. In addition to orogenital ulceration, pathergy is one of the hallmark features of the condition. There is no diagnostic test and there are no reliable biomarkers to monitor response to treatments or disease activity. Owing to the current limitations of our understanding of the underlying disease process, treatments are nonspecific. Further basic research is required to improve our understanding of this condition and more clinical research is needed to provide support for our current approach to patient management. Young males are most likely to develop severe ocular, CNS and vascular complications and need to be closely watched and aggressively treated.

BS is a complex disorder. There appears to be a polygenetic susceptibility, and in susceptible individuals a trigger or burden of triggers activates the immune system which becomes hyperactive and disordered, resulting in disease. This is not a 'pure T cell' or ' pure inflammasome' syndrome, but appears to be one characterised by generalised hyperactivity of many components of immune system, both innate and adaptive, and a slowness switching off inflammation. Rather than grouping all patients together as one disease, this is a syndrome that has a variety of genetic abnormalities and a variety of triggers all of which result in a similar, but not identical, clinical syndrome in individuals.

Dissecting the abnormal inflammatory response may potentially open window to the pathogenesis of the condition and may also provide the potential to discover a diagnostically useful tool or identify useful biomarkers.

1.8 Project Aims overview

This project aimed to dissect abnormal inflammatory responses in BS to gain an understanding as to which pathway(s) are responsible for the phenomena.

In Chapter 3, the hypothesis that the abnormal cutaneous inflammatory response to "non-specific" stimulation in BS is associated with a failure of deactivation of macrophages during the resolution phase of inflammation was explored using human *in vivo* models of inflammation (MSU injections and skin blisters). Secondary aims were to classify what other cells and cytokines were present in blister fluid. We studied cantharidin-induced skin blisters in 12 BS and 12 Healthy controls (HC), analysing blister fluid cells and cytokines using flow cytometry and multiplex bead cytokine analysis respectively.

In Chapter 4, the hypothesis that the abnormal cutaneous inflammatory response may involve neuropeptides was explored. We applied capsaicin topically to BS and HC, and measured the resultant change in dermal blood flow using laser doppler imaging to evaluate non-invasively if neuropeptides such as CGRP may play a role in the abnormal cutaneous inflammatory response in BS. The primary question was to assess if there is a difference in capsaicin response between HC and BS. We also wished to assess if subjects with a large MSU skin reaction and subjects who had an erythematous response to the cantharidin blisters also had an abnormal capsaicin skin test, as this could offer the possibility of a quicker and simpler test than the MSU injection. Finding a simpler test to replace MSU /pathergy testing would be a useful advance.

Chapter 5 summarizes key *in vitro* experiments undertaken alongside *in vivo* experiments. The IFN-γ / chemokine pathways were explored in detail in CD14+ monocyte populations in BS, HC and disease control subjects. We found significant differences in response in BS as compared to other groups and CXCL-10 was a chemokine of interest identified by these experiments. We found that following IFN-γ stimulation, monocytes from BS produced equivalent mRNA but more CXCL10 protein, indicating differential post-transcriptional regulation. This was further explored using sucrose density gradients.

Detailed introductions to each of these tests are found in the relevant chapters.

2. Materials and Methods

2.2 Study subjects and study design

2.2.1 Ethics

The study was conducted according to the principles of the Declaration of Helsinki and all participants gave written informed consent. Ethical approval was obtained from the Hammersmith, Queen Charlottes and Chelsea Research Ethics Committee.

2.2.2 Subject evaluation

Both male and female individuals aged over 18 years were included. Subjects with significant co-morbidities (e.g. asthma, diabetes mellitus) were excluded. (i) Behçet Syndrome (BS) subjects were recruited from the Hammersmith Hospital rheumatology clinic, and through the UK Behçet's Syndrome Society. All subjects satisfied the 1990 ISG Behçet Diagnostic Criteria [57]. A clinical history including medication history was obtained and disease activity was assessed using the Leeds Behçet's Disease Activity Form, which gives an activity score on a 0-12 point scale (>4 indicates active disease) [196]. (ii) Age- and sex-matched healthy volunteers were recruited locally by word of mouth and were required to be free of significant acute or chronic inflammatory disease, and to be on no medications (women taking oral contraception were not excluded). (iii) Rheumatoid arthritis (RA) subjects were recruited from Hammersmith Hospital and Charing Cross Hospital general rheumatology clinics. All subjects fulfilled the 2010 American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) criteria for RA [197]. A clinical history including medication history was obtained and disease activity was quantified using DAS28 (ESR) scoring which gives a score on a 1-10 point scale (>5.1 indicates active disease) [198]; (iv) Systemic lupus erythematosus (SLE) subjects were recruited from a combined renal-rheumatology clinic at the Hammersmith Hospital. All subjects fulfilled the ACR criteria for SLE having at least the presence of 4 of the 11 diagnostic criteria[199]. A clinical history including medication history was obtained and

disease activity was quantified using the SLE disease activity score (SLEDAI) which gives a score on a 1-105 point scale (>6 indicates active disease, rare to have score >20)[199].

2.2.3 Study design

The project used a cross-sectional observational approach to investigate cutaneous inflammation in healthy individuals, in subjects with BS and in subjects with other inflammatory diseases (RA, SLE).

2.2.4 Statistical methodology

Analysis was carried out using PRISM software. Medians with range were calculated unless otherwise stated. Mann-Whitney or Students t-tests were used to compare two outcomes. Kruskal-Wallis analysis of variance calculations were used to look for variance between groups. Where required, Dunn's test was used to correct for multiple comparisons in non-parametric groups.

2.3 Monosodium urate crystals (MSU)

2.3.1 Protocol for MSU crystal preparation

To eliminate confounding due to inter-batch variability of MSU crystals, a batch was made prior to commencement of the study (batch 12/04/2010) and this was used for all experiments. MSU crystals were prepared by a modification of the protocol used by Denko and Whitehouse, 1976 [200]. All equipment was sterilized overnight with 1% E-toxa (Sigma) clean solution then autoclaved, following which all other steps were undertaken under a laboratory hood. 12.2ml 1N NaOH was added to 400ml sterile water (endotoxin free, Sigma) and brought to boil on a hot plate. 1g Uric acid (Sigma) was added, stirring constantly with magnetic stirrer to dissolve. The solution was cooled on a hot plate to 60^{o}C and pH adjusted to 8.9, while stirring constantly. The mixture was then allowed to cool to room temperature for 4-5 hours while stirring, during which time it turned milky white. It was then kept at 4^{o}C overnight. The following day, crystals were poured over Whatman filter paper using a filter bottle attached to a suction pump. Filter

papers were kept in Petri dishes and dried at room temperature for 24 hours. The crystals formed thin brittle wafers, which were broken up and stored in a glass container. A portion of crystals was baked at 200ºC, either for 2 hours or overnight to ensure they were LPS free. Batches were compared by *in vitro* monocyte stimulation, looking at TNF production, to ensure they were equally pro-inflammatory post-baking. This was found to be the case, and all crystals were then baked overnight. Crystal sizing was performed by light microscopy using a Zeiss Photomicrograph III (Oberkochen, Germany) equipped with polarizing filters. The average size of crystals was 7.96 μm, standard deviation 3.67 μm.

2.3.2 Endotoxin testing

Endotoxin levels were tested using an endotoxin assay purchased from Bio Whittaker, UK. Levels were < 35 pg/ml per 1mg/ml of solution. Crystals were stored in sterile containers.

2.3.3 MSU administration and size measurement

Following cleansing of the skin with alcohol, sterile MSU crystals (2.5 mg in 100μl sterile water) were injected into the upper dermis of the volar aspect of the forearm. At 24 and 48 hours the injection site was digitally photographed together with a scale marker and maximal diameter of erythema measured.

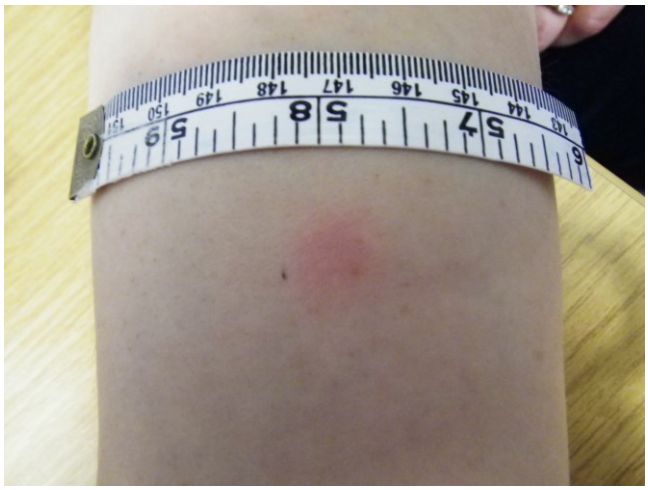


Figure 2-1 Erythema at the site of a MSU injection, 24 hours after the crystal was injected.

2.4 Skin blister models

2.4.1 Suction blisters

Skin was injected intradermally with 2.5mg MSU. 24-48 hours later the site was photographed, following which a suction cup was placed over the injection site and secured. Gentle suction (150-200mm Hg) was applied. Suction cups of 5mm, 7.5mm and 10mm diameter were used. Blisters took 60-180 minutes to form. The fluid was gently aspirated using a 200µl pipette tip into a siliconized ependorf, and placed on ice.

2.4.2 Cantharidin blisters

2.4.2.1 Cantharidin mode of action

Cantharidin is an acantholytic agent. On application to skin, it is absorbed by the lipid layers of epidermal cell membranes leading to release of serine proteases that cause degeneration of the desmosmal plaque, leading to detachment of tonofilaments from desmosomes. This in turn leads to intraepidermal blistering and nonspecific lysis of the skin. The blister causes the tissues containing the protein to separate from the surrounding skin. Since acantholysis is intraepidermal, healing occurs without scarring, although hyperpigmentation may occur, especially in darker skin.

2.4.2.2 Cantharidin skin blisters

Skin was cleaned with an alcohol wipe. A 25µl droplet of fluid containing 0.1% cantharidin collodion ('Cantharone' 0.7% Dormer Laboratories, USA, DIN 00619035 diluted in acetone) was applied to a 1cm circle of filter paper on the volar aspect of the subject's forearm. This was covered with a 2cm x 2cm piece of paraffin and a bandage. At 24-48 hours blisters were photographed, then gently punctured with a 200µl siliconised pipette tips and fluid was collected into siliconised ependorfs and kept on ice.

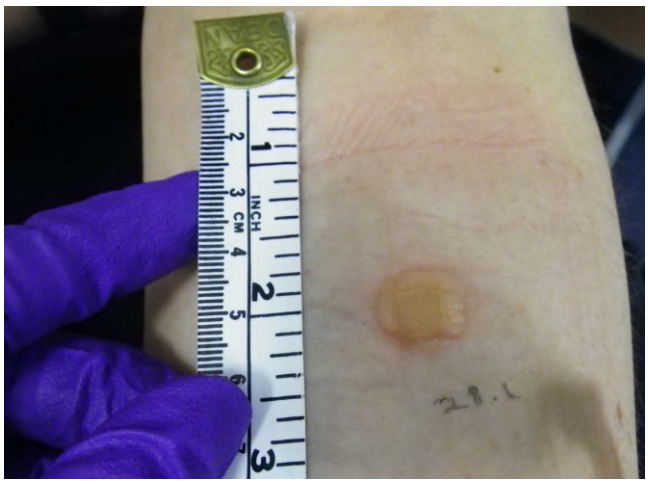


Figure 2-2 A cantharidin elicited skin blister.

2.4.3 Blister fluid processing

Fluid recovered from the suction or cantharidin skin blister was immediately transferred to a siliconized ependorf and placed on ice. The volume was recorded and then 10µl was aspirated and applied to a haemocytometer for a total cell count. The remainder was spun in a microcentrifuge at 300g, after which interstitial fluid was separated, aliquoted and stored in siliconized ependorfs at -80^{o}C for future analysis of blister fluid protein content. Cells were resuspended in FACS buffer (phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, UK) and 0.05% sodium azide (Sigma-Aldrich, UK)) for immediate characterization by flow cytometry. 70µl was set aside a for white cell differential slide.

2.4.3.1 Cytospin

A labelled slide was added to the cytospin metal container, along with filter paper and the plastic cover. 50 µL of fluid was applied and the slide was spun for 5 minutes at 300g. The slide was removed and left to dry, then stained with Quick Diff (IHCworld, USA), and then washed gently in water, then air dried. Once dry, a cover slip was applied with glue in the fume hood. Differential counts were performed by light microscopy.

2.5 Monocytes isolation and culture

2.5.1 Peripheral venous blood sampling

Peripheral venous blood was collected using an aseptic technique and anti-coagulated with 10% sodium citrate (Sigma-aldrich, UK). The blood sample was diluted (1:1) with Hanks Buffered Saline (Sigma-aldrich, UK), layered carefully onto Histopaque (Sigma Aldrich, UK) and then spun with deceleration turned to 0, for 20 min at 700g. The interface containing peripheral blood mononuclear cells (PBMCs) was removed and washed three times with PBS (500g for 10 min, then 400g x 10 min, then 300g x 10min). Deceleration was set to 9 for these 3 spins. The cells were then re-suspended in FACS buffer for immunostaining, or in MACS buffer for CD14+ cell isolation.

2.5.2 CD14+ monocyte positive selection

PBMCs were re-suspended in 80µL of MACs buffer (PBS with 0.5% BSA and 0.05% 0,5M EDTA (Sigma-Aldrich)) and 20µL CD14 microbeads (Miltenyi, Germany) per 10^{x7} cells. The solution was mixed well and incubated for 15 minutes then washed by adding 1.2mL buffer per 10^{x7} cells and centrifuging at 300g for 10 min. Up to 10^{x8} cells were resuspended in 500µL of buffer and applied to a MS column which had been pre-rinsed with 500µL of buffer (Miltenyi, Germany) in the magnetic field. The column was washed with 1500µL buffer, and once the reservoir was empty, the column was removed from the magnet and immediately placed on a 10mL falcon tube. 1000µL of buffer was applied to the column and immediately flushed by firmly pushing the plunger down. Cells were counted, washed and plated at 1million cells/mL in DMEM (Invitrogen, UK) with 10% fetal calf serum (FCS) (Biosera, UK), 1% penicillin-streptavidin (Sigma-aldrich, UK). Cell purity was confirmed as 98% pure by flow cytometry.

2.6 Flow cytometry

2.6.1 Flow cytometry theory

Flow cytometry is a cellular analysis technology in which a beam of light is directed onto a focused stream of fluid passing through the flow cytometer. The scatter of light is analysed to reveal characteristics of the cells passing through that stream of buffer. Cells labelled with a fluorescent antibody will cause a distinct pattern of light scattering at a specific wavelength which can then be detected by the flow cytometer. By using antibodies to specific cell markers, this method can be applied to determine whether the cell population is expressing a given antigen. If multiple antibodies are being used to assess a cell population, it is imperative to identify the threshold above which a value is interpreted as positive. This can be achieved with isotype control antibodies or by using 'Fluorescence minus one'. It is also useful to use compensation beads for single staining with each fluorescent antibody to aid adjustments for compensation during analysis.

2.6.2 Fluorescence minus one

The fluorescence minus one (FMO) technique controls for all fluorescent dyes for which the threshold is in question; that is, where spectral overlap correction may lead to greater uncertainty as to what constitutes background staining. In an FMO control stain set, all reagents used in a given multicolor stain are included except the reagent for which the threshold is to be determined. To show the threshold that distinguishes fluorescent cells from cells that do not express the determinant detected by the reagent omitted from the FMO control, data are collected for cells stained with FMO control stain sets and are compensated, displayed and gated in the same way as cells stained with the full stain set. The background fluorescence for each subset in the display is demonstrated by the upper boundary of the subset in the FMO control. An example is to assess for CD163, we had one tube of cells stained with all antibodies from row 1, and on tube stained with all antibodies except anti-CD163.

2.6.3 Antibodies for flow cytometry

Table 2-1. Antibodies used for flow cytometry experiments

Row	Antibody	Company	Country
1	Anti-human CD163 IgG1 FITC conjugate	Bachem	UK
	Anti-human CD16 IgG1 R-PE conjugate	Invitrogen	USA
	Anti-human CD14 IgG2a Pacific Blue conjugate	Invitrogen	USA
	Anti-human HLA DR (class II) IgG2b APC conjugate	Invitrogen	USA
2	Anti-human CD4 iGg2a biotin	Caltag	USA
	Anti-human anti-γ/δ TCR IgG1 PE conjugate	Pharmigen Becton Dickinson	USA
	Anti-human CD3 IgG1 PC7 PE Cy7 conjugate	Beckman Coulter	USA
	Anti-human CD8 IgG2a pacific blue conjugate	Invitrogen	USA
3	Anti-human CD56 FITC conjugate	Invitrogen	USA
	Anti-human CD16 IgG1 R-PE conjugate	Invitrogen	USA
	Anti-human CD3 IgG1 PC7 PE Cy7 conjugate	Beckman Coulter	USA
	Anti-human CD8 IgG2a pacific blue conjugate	Invitrogen	USA
Other	Streptavidin Alexa fluor 488 conjugate	Invitrogen	USA
	Live/dead fixable aqua dead cell stain kit	Invitrogen	USA

(i) Row 1: antibodies for examining monocyte/macrophage population. Anti-CD14 antibodies were used to identify the monocyte/macrophage population. Use of anti-CD16 (FcγRIII) was intended to separate CD14+ monocytes into CD14highCD16low and CD14lowCD16high. CD14+CD163+ cells and CD14+HLA-DR+ expression were measured as markers of deactivated or activated populations. (ii) Row 2: antibodies for examining T cell subsets. CD3 was used as the marker for all T cells, as it is present on cells expressing either αβ or γδ T cell receptors. γδ T cells was measured directly in the FL2 channel, whilst the proportion of CD3+ αβ T cells was deduced by subtracting the number of CD3+ γδ T cells from the total CD3+ population. (iii) Row 3: antibodies for examining NK cells. NK cells express CD56 (a homophilic adhesion molecule of the immunoglobulin superfamily (NCAM)) and CD16. The absence of CD3 but the presence of CD56 and CD16 were deemed the most reliable markers for pure NK cells in humans. Two functional subsets of NK cells exist: the more mature CD56dim CD16high population is potently cytotoxic, whereas the CD56bright CD16low/neg NK cells have low cytotoxicity but produce much greater amounts of cytokines.

2.6.4 Flow cytometry protocol

Flow cytometry was used to quantify and characterise leukocyte subsets in blister fluid and peripheral blood. Cells (from blisters or PBMCs) were re-suspended in FACS buffer and blocked with 10% human pooled plasma for 20 minutes on ice. $1x10^5$ cells were applied to wells pre-prepared with antibodies (1µl antibody per $1x10^5$ cells) and incubated on ice for 15 minutes. Wells were then washed for 5 minutes at 300g with 200µL FACS buffer, 3 times. The secondary antibody was applied and after a further 15 minute incubation, and repeat 3 washes the cells were transferred to FACS tubes for analysis. Cells were analysed with on a Beckman Coulter CyAn ADP 9 colour, 3 laser high performance analyser.

2.7 Neuropeptide investigation

2.7.1 Thermal threshold testing (TTT)

A thermal threshold testing system (NeuroSensory Analyzer, Merdoc Ltd Israel) with a rate of rise in temperature of 1 degree/second was used. The thermode was placed at on the volar aspect of the forearm. The baseline temperature was set at a neutral point (30^{o}C). Thermal thresholds are determined for cool and warm sensation, and for heat and cold perceived as pain. Four consecutive tests are carried out for each modality. The mean temperature was recorded as the threshold.

2.7.2 Capsaicin mode of action

Capsaicin occurs naturally as the pungent compound in hot chilli peppers. It is a highly selective and potent exogenous agonist for the TRPV1 receptor on nerve endings. The TPRV1 receptor (transient receptor potential cation channel subfamily V member 1, also known as the capsaicin receptor and the vanilloid receptor 1) is a non-selective transmembrane cation channel found on small nerve fibre terminals [201]. It is activated by a wide variety of exogenous and endogenous physical and chemical stimuli. The best-known activators of TRPV1 are: heat greater than 43°C, capsaicin and allyl-isothiocyanate (the pungent compound in mustard and wasabi). Activation causes neurogenic inflammation and vasodilation via the local release of vasoactive

mediators including CGRP and substance P and possibly also by the activation of dorsal root reflexes [201]. TPRV1 receptor activation causes vasodilation via the local release of vasoactive mediators including CGRP. This reaction can be blocked by CGRP antagonists.

2.7.2.1 Capsaicin preparation and administration

Capsaicin powder was obtained from Sigma-Aldrich N.V. (Bornem, Belgium) and was dissolved in a 3:3:4 mixture of ethanol 100%, Tween-20 and distilled water. Capsaicin was diluted so that 20mL of the mixture contained 100, 300 or 1000µg capsaicin. Capsaicin responses were assessed in BS and HC subjects. All subjects were asked to abstain from non-essential drugs including antihistamines for 3 days, as well as from chocolate, caffeine and alcohol for 12 hours before the test. All measurements were performed while the subjects rested in a semirecumbent position on a comfortable recliner in a quiet, temperature-controlled room. 10-mm rubber O-rings (McMaster-Carr, New Brunswick, NJ, USA; 8 mm inner diameter) were placed onto the volar surface of the forearm. The rings were positioned so that their distal edges were 20cm proximal to the wrist crease, within approximately 1 cm of the midline and avoiding visible veins. After placement of the O-rings, a laser Doppler imager was used to obtain baseline scans of the Dermal Blood Flow (DBF) in the areas defined by the rings. Subsequently, these O-rings served as reservoirs to contain the topically applied 20-ml capsaicin or placebo solutions.

2.7.3 Real-time blood flow imaging system: Laser-doppler

Real-time blood flow imaging (BFI) was undertaken with a Perimed Laser Doppler Perfusion Imager (HR-LDPI system, PeriScan PIM II®; Perimed, Järfälla, Sweden) that had an X-Y scanning laser probe, allowing two-dimensional mapping of the blood flow variability over an extended skin surface using doppler technology. The red light of a 633-nm helium-neon laser penetrates the skin variably to a depth of about 1mm. The vasculature within these layers comprises vessels of variable size, orientation and function. For this reason, it is not meaningful to express the cutaneous flow in absolute terms, but rather as arbitrary ‘perfusion units’. These

calculations were performed using the built-in statistical function of the HR-LDPI system (LDPIwin; Perimed).

2.7.3.1 Real-time blood flow imaging system: Laser-Speckle Flowmetry

Laser speckle contrast imaging exploits the fact that a random speckle pattern is generated when tissue is illuminated by laser light (wavelength 780nm), and this speckle changes when blood cells move within the region of interest. When there is a high level of movement (fast flow) the changing pattern becomes more blurred and the contrast in that region reduces accordingly. Therefore low contrast is related to high flow, high contrast to low flow. The contrast image is processed to produce a colour-coded image that correlates with blood flow in the tissue. This technique gives a video frame rate of blood flow images (25 per second). The zoom function enables resolutions exceeding 1M pixels per cm. The resulting image is mainly of blood flow in the microvessels in the surface layers of the tissue being sampled. The effective sampling depth is small (0-2mm). Flux (PU) is the scale of blood flow (0-1000PU). The scale is arbitrary because it is impossible to be precise about the sample volume. Data may be represented as total or % change in flux once the area of interest is defined. Another output is to assess the flare area i.e. how many pixels are above the mean flow post intervention. Laser speckle flowmetry is effectively still a Doppler technique because high speed results in suppression of contrast and low speed is an expansion of contrast, in the same way that sound waves get compressed with higher speeds in the typical Doppler illustrations. Laser Doppler is a slightly different to laser speckle as different algorithms are used to calculate flow.

2.8 Protein analysis

2.8.1 ELISA

Inflammatory mediators in blister fluid were determined by capture ELISA using a matched antibody pair (DuoSet; R&D Systems, Abingdon, UK) according to the manufacturer's recommendations. Capture antibodies were diluted to the working concentration in PBS and 100µL was added per well. The plate was sealed and incubated overnight at room temperature.

Plates were washed 3 times then blocked for1 hour then washed again 3 times. Samples and standards were applied and incubated for 2 hours then plates were washed again 3 times. Detection antibodies were applied for 2 hours plates were washed 3 times. Streptavidin (R&D, UK) was applied for 20 minutes then plates were washed 3 times. Tetramethylbenzidine Substrate solution (TMB) (Sigma-Aldrich) was applied. Once an adequate blue colour had developed the reaction was stopped with H2SO4 (Acros organics) and Optical Densities (OD) were determined using the microplate reader set to 450nm. Standard curves for each cytokine (in duplicate) were generated by using the reference cytokine concentrations supplied in this kit. All samples were measured in duplicate and the results were calculated against the standard curve, and expressed as the mean ± SEM cytokine concentration (pg or ng/ml).

2.8.2 Multiplex bead analysis – Cytokine analysis of blister fluid

Soluble mediators in blister fluid were analysed using FlowCytomix beads (eBioscience, country) on the Cyan FACS machine. Multiplex bead analysis allowed the measurement of multiple cytokines in small volumes of blister fluid (25µl.) The following cytokines were assayed: IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-22, TNF-α and IP-10. Briefly, beads were labelled with antibodies which specifically react with each of the analytes to be detected in the multiplex system. The beads could be differentiated by their sizes and by their distinct spectral addresses. A mixture of coated beads for each analyte to be measured was incubated with the samples or standard mixture. The analytes present in the sample bound to the antibodies linked to the fluorescent beads. A biotin-conjugated second antibody mixture was then added, the specific antibodies binding to the analytes captured by the first antibodies. Streptavidin-Phycoerythrin was then added, binding to the biotin conjugate and emitting fluorescent signals. Fluorescence was measured by flowcytometry. Standard curves for each cytokine (in duplicate) were generated by using the reference cytokine concentrations supplied in this kit.

2.9 RNA extraction and cDNA generation

2.9.1 RNeasy protein extraction method

Cells were harvested and frozen in 350 µL RLT buffer (Qiagen, UK) containing 3.5 µL 2-mercaptoethanol (Sigma-Aldrich, UK). The lysate was passed 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe, or pipetted into a QIAshredder spin column (Qiagen, UK) and centrifuged x 2 min at full speed. One volume of 70% ethanol was applied to the homogenized lysate and mixed well. The sample was transferred to an RNeasy spin column (Qiagen) placed in a 2 mL collection tube, and centrifuged for 30 secs at 8000g. The flow-through was discarded. 700 µL buffer RW1 (Qiagen) were added to the RNeasy spin column. The column was centrifuged for 15 seconds at 8000g, and the flow-through was discarded. 500 µL of buffer RPE was added and centrifuged for 15seconds at 8000g, twice. The spin column was placed in a new collection tube and spun on full speed for 1 minute. The spin column was placed in a new 1.5ml collection tube and 30µL RNase free water was added and spun for 1 minute x 8000g.

2.9.2 Trizol RNA extraction

Homogenization: 1mL of Trizol Reagent (Invitrogen, UK) was added to sample and homogenized by pipetting the suspension up and down several times. The samples were incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Phase separation: Chloroform (200µL of chloroform for every 0.75mL of TRIzol LS) was added. Samples were vortexed for 15 seconds then incubated for 2-3 minutes. Samples were centrifuged at 12,000g for 15minutes at 4 ^{o}C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, and interphase, and a colourless upper aqueous phase. The upper aqueous phase, containing RNA, was separated into a fresh tube. RNA precipitation: RNA was precipitated by adding 1mL of isopropyl alcohol. Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA precipitate formed a visible pellet. The supernatant was completely removed

and the RNA pellet was washed twice with 1mL 75% ethanol. The pellet dried by incubating a 37°C incubator for 5-10 minutes. RNA was dissolved in 20µL RNase free water (Sigma) by passing the solution up and down several times then left on ice for 30 minutes.

2.9.3 Trizol LS RNA extraction

Homogenization: 0.75 ml of TRIzol LS Reagent (Invitrogen, UK) was added to each 0.25 ml of sample (0.75 ml of TRIzol LS Reagent per 5-10 x 10^6cells) and homogenized by pipetting the suspension up and down several times. The samples were incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Phase separation: Chloroform (200µL of chloroform for every 0.75mL of TRIzol LS) was added. Samples were inverted and mixed well for 15 seconds then incubated for 10 minutes. Next the samples were centrifuged at 12,000g for 15minutes at 4^{o}C to get phase separation. The aqueous phase of the sample was removed and placed in a new tube. RNA isolation: 2µL of glycoblue was added to samples to act as a carrier to the RNA. 800µL of 100% isopropanol was added and incubated at room temperature for 10minutes. The samples were centrifuged at 12,000g for 10 minutes at 4 ^{o}C. The supernatant was removed leaving only the RNA pellet. This was washed with 1mL 75% ethanol and the sample was vortexed. The samples were then centrifuged at 7,500 g for 5 minutes at 4 ^{o}C and the supernatant was discarded. The pellet was air dried for 10 minutes then resuspended in 20µL RNase-free water by passing the solution up and down several times then left on ice for 30 minutes.

2.9.4 RNA purification and concentration analysis

The purity and the concentration of the RNA were analysed using the Nanodrop spectrophotometer (Thermo Scientific).

2.9.4.1 RNA purification

At this step, the sample contained RNA and DNA. DNA-Free (Ambion, UK) was used to purify the sample. Samples were diluted in the provided buffer in a 1:10 dilution and incubated for 30minutes at 37^{o}C. The DNase Inactivation Reagent was vortexed before us then added to all

samples (10% volume). Samples were mixed and incubated for 2 minutes, then centrifuged at 10,000g for 1.5minutes. The supernatant was carefully aspirated into a fresh tube, leaving the beads pelleted at the bottom. Samples were nanodropped.

2.9.4.2 cDNA synthesis

cDNA was synthesised using the SuperScript® III RT kit (Invitrogen) following manufacturer's instructions. Briefly, 1ug of total RNA was added to 1µl oligo(dT)20 (50µM) (Eurofins, Promega) and 1 µl 10 mM dNTP Mix (Promega), and adjusted to a final volume of 13 µl with water. The mixture was heated to 65°C for 5 mins and incubated on ice for 5 mins. 4 µl of 5x first-strand buffer (Invitrogen), 1 µl of 0.1M dithiothreitol (DTT) (Invitrogen), and 1 µl of SuperScript III reverse transcriptase were added to the mixture. The reaction was incubated at 25°C for 5 min, 50°C for 50 mins and inactivated by heating it to 70°C for 15 mins then stored in freezer or on ice.

2.9.5 Quantitative real-time PCR

2.9.5.1 Quantitative real time PCR theory

Quantitative real time PCR (qRT-PCR) works via the generation of a fluorescent signal which is measured after each cycle to detect the replication of an amplicon [202]. The readout is given as the number of cycles required for the fluorescent signal to surpass the threshold level set by the user. This output is called the cycle threshold (Ct), and the fewer cycles required to surpass threshold, the more of the specific mRNA is present in the sample. Housekeeping genes allow normalisation of results. Data was analysed using the comparative Ct method, where the Ct of each sample for the gene of interest is normalised to its own housekeeping gene Ct, in order to calculate ΔCt. ΔCt = Ct (target gene) – Ct (Housekeeper)

2.9.5.2 Quantitative real time PCR protocol

5 µl of template cDNA (pre-diluted 1:20) was amplified using the following reagents; 12.5 µl SYBR green fastmix IQ (Quanta perfecta, Invitrogen); 6.5 µl H2O, 0.5 µL forward primer (pre-diluted 1:10), 0.5 µL reverse primer (pre-diluted 1:10). The cDNA or DNA was denatured by

heating the mixture at 95°C for 3 minutes, and amplified according to the following conditions, repeated for 40 cycles: 95°C for 10 secs, 56°C for 30 secs. Real-time PCR were performed using the iCycler thermocycler (Biorad). The results were analysed using iCycler IQ optical system software (Biorad).

Table 2-2. Oligonucleotides used for qRT PCR

Oligonucleotides used for qRT PCR		
CXCL-9	F	5' TTG CTG GTT CTG ATT GGA GTG 3'
	R	5' CCT TGG TTG GTG CTG ATG C 3'
CCL-2	F	5' AGT CAC CTG CTG TTA T 3'
	R	5' TCA AGT CTT CGG AGT T 3'
CXCL-10	F	5' GCT ATG TTC TTA GTG GAT GTT C3'
	R	5' AGG ATG GCA GTG GAA GTC 3'
HPRT	F	5' TTG GTC AGG CAG TAT ATT CC 3'
	R	GGG CAT ATC CTA AA CA AC 3'
ACTIN	F	GAG CTA CGA GCT GCC TGA CG 3'
	R	GTA GTT TCG TGG ATG CCA CAG GAC T 3'
GAPDH	F	5' CAA CAG CCT CAA GAT CAT 3'
	R	5' GAG TCC TTC CAC GAT ACC 3'
Oligo dt		5' CGA CTC GAG TCG ACA TCG ATT TTT TTT TTT TTT TT 3'

2.9.5.3 Validation of qRT-PCR product with agarose gel

On completion of the first qRT-PCT run for each new primer set, the product was validated with an agarose gel. Product with gel loading dye, and a ladder was loaded on to a 2.5% agarose gel (2.5g agarose in 100mls TAE buffer and 1.5µL ethidium bromide) and run at 100V x 20 minutes. Products were assessed to ensure only a single band was present and that the band was the expected size.

2.10 Sucrose-density-gradient polysome experiment

2.10.1 Sucrose-density-gradient polysome experiment theory

RNA from cells was isolated and layered on top of a tube containing a sucrose density gradient (sucrose 50% at the bottom to 20% at the top of the tube). It was then spun at very high speeds which resulted in 'heavy' polysomes travelling further down the tube then 80S or 40S subunits.

Free RNA travelled the least far. A needle pierced the bottom of the tube and very even denser sucrose was introduced slowly (60%) to push the contents of the tube into a collecting tube and past a laser (which fed to a graph tracer giving an image of passing fluid contents), and finally the sucrose was fed into collecting ependorfs, which allowed the sample to be collected in fractions and analysed.

2.10.2 Establishing the sucrose density gradient

Buffer was prepared as follows: 0.3 M NaCL (Ambion, USA), 15mM MgCl2 (Ambion, USA), 15mM Tris HCL 7.6 (Calbiochem, Germany) in molecular grade water (Sigma-Aldrich, UK). Falcon tubes of buffer were prepared with the following concentrations of molecular grade sucrose (Sigma-Aldrich): 50%, 44%, 38%, 32%, 26% and 20%. Polyallomer tubes (Beckman Coulter, USA) were placed into a secure rack. 1.5mL of the highest concentration of sucrose was added. Tubes were snap frozen in liquid nitrogen. 1.5mL of the reducing concentrations were added sequentially, freezing the tube between each one to establish a density gradient from 50% to 20% in the tube. Tubes were covered with parafilm and stored in -80^{o}C freezer until 4 hours before they were required.

2.10.3 Cell lysis and ultracentrifugation

Cell lysis buffer was prepared as follows: 0.3 M NaCL (Ambion, USA), 15mM MgCl2 (Ambion, USA), 15mM Tris HCL 7.6 (Calbiochem, Germany), 1% 1% Triton x-100 355mM (Sigma-Aldrich, UK), 10µL/mL superase (Superase.IN, Ambion, USA) and 10 µL/mL protease inhibitor (Protease Inhibitor cocktail, Sigma-Aldrich, UK). CD14+ monocytes were suspended in DMEM with 10% FCS and 1% Pen/strep at 1 million cells per mL concentration. 5mLs were plated in a 6 well plate and left to settle for 1 hour. Cells were stimulated with 20ng/ml IFN-γ for two hours, or else left unstimulated. Unstimulated cells were harvested once they had settled for one hour. 2.8µL of cyclohexamide was added to the 5ml plate for 5 minutes before harvesting to inhibit further translation. Supernatant was then aspirated and stored for later protein analysis. Supernatant was replaced with 2 mls of fresh medium and cells were scraped, aspirated into a falcon tube and spun at 500g for 10 minutes. Supernatant was completely

removed then 250µL of cell lysis buffer was added. The sample was pipetted up and down then left on ice for 15minutes. After 15 min the sample was spun in a microcentrifuge at 10,000g for 15minutes (4 ^{o}C). 10µL was aspirated for mRNA quantification. The remaining supernatant was pipetted carefully onto the sucrose density gradient and spun in the ultracentrifuge at 150,000g for 90 minutes at 4^{o} C with deceleration set to level 9 (0=max deceleration, 10=coasting).

2.10.4 Sucrose density gradient polysome experiment

Table 2-3. Equipment used for sucrose density gradient experiment

Sucrose density gradient equipment		
Brandel	Syringe pump	USA
Brandel	Tube piercer	USA
Brandel	Optical unit with reference flow cell	USA
Dataq	Acquisition starter kit	UK
Brandel	UV/VIS absorbance detector	USA

Equipment was set up and prepared by running 50mls of 3% hydrogen peroxide on the morning of the experiment. This was followed by 100mls of molecular grade water, and then by a preparation run of 50% sucrose to establish the baseline settings. Machine settings were as follows: fluid flow speed 0.75 mL/min, paper flow rate 60 cm/hour, sensitivity 0.5 ABS. Samples were collected in 1.5ml RNase free ependorfs. Trizol LS was used to extract mRNA.

2.10.5 Green Fluorescent Protein (GFP) spike

Following extraction, each sample was 'spiked' with a known quantity of GFP mRNA before RNA purification, cDNA conversion and RT-PCR analysis. The GFP spike was used to normalise values to account for any loss of product during processing. The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range and is was first isolated from the jellyfish Aequorea Victoria. It has not been conserved in humans.

2.10.5.1 Construction of the GFP protein from a Transcription Kit Theory

DNA from GFP had been inserted into a polylinker site of a transcription vector with a pSPT18. The promoters for SP6 and T7 RNA polymerases are located on either side of the polylinker. T7 RNA polymerases specifically transcribe DNA sequences downstream of the T7 promoters. Cloned DNA inserts within the polylinker region can therefore be specifically transcribed. T7 RNA polymerase uses the cloned DNA as template to synthesize complementary RNA in the presence of Mg2+ and ribonucleoside triphosphates. Spermidine stimulates enzyme activity. To obtain the DNA, the template DNA must first be linearized with a suitable restriction enzyme before the transcription reaction to obtain transcripts of a defined length.

2.10.5.2 The GFP construct

GFP had been cloned and stored in the DNA vector. The vector had a SP7/T7 promoter site. The vector was first linearized with pBBR1 (a medium copy replicon for Gram-negative bacteria) by adding 5µg DNA to 2.5µl buffer; 1µl enzyme and water to a final volume of 25µl and incubating for 1-2 hours at 37 ^{o}C. Alcohol precipitation of DNA was then undertaken by making the digest to 100µl with water and adding 1/10th volume of Na Acetate Ph5.3 and 2.5 volumes of 100% ethanol and then placing the mixture in a -80^{o}C freezer for 1 hour. Following a spin in microcentrifuge for 20minutes at full speed, the digest was then decanted and washed with 70% ethanol and spun again for 5 minutes in the microcentrifuge. The pellet was dried completely and the concentration was measured. Reverse transcription of DNA to RNA: The following was added to a microfuge tube on ice: 1µg DNA; ATP, GTP, CTP, UTP mixture 8µl; 10xbuffer (vial 8) 2µl; Water to final volume of 18µl and T7 polymerate (vial 12) 2µl. The mixture was briefly centrifuged then incubated for 2 hours at 37^{o}C. 2µl DNase 1 and RNase free (vial 9) was then added to remove template DNA and the mixture was incubated for a further 15 minutes at 37 ^{o}C. The reaction was then stopped by adding 0.8µL of 0.5M EDTA ph8 and then heating to 65 ^{o}C for 10 minutes. Alcohol precipitation was undertaken again, followed by measurement of the concentration. 40µg was added to each of the sucrose density gradient samples.

3. Examining the exaggerated cutaneous inflammatory response using a skin blister model of inflammation

This chapter describes experiments using two skin blister models of inflammation (suction blisters and cantharidin blisters) to investigate the exaggerated cutaneous inflammatory response in BS. The pathergy reaction, which forms part of the criteria for the diagnosis of BS [57] is not understood. Diagnosis of BS is still entirely clinical. Other than in helping to exclude other conditions, existing laboratory tests (e.g. serology) are not particularly useful in diagnosis or monitoring disease activity. Acute phase reactants (CRP, ESR) may be normal despite active inflammation. There is therefore a clear need for better diagnostic tools for this condition. Additionally, increasing our understanding of abnormal cutaneous inflammation may open a window into the underlying pathogenesis of this condition.

3.1.1 Abnormal inflammatory responses in BS

Pathergy was introduced in Chapter 1 as one of the hallmark characteristic features of BS. It manifests as an exaggerated responsiveness to immunologically "non-specific" inflammatory stimulation, with patients often reporting exaggerated skin responses to needles, nettles, insect bites, plant thorns and other day-to-day trauma. Patients with BS are often given antibiotics postoperatively by surgeons concerned by erythema around drip-sites or surgical wounds, and it is likely that in many such cases the erythema represents traumatic inflammation rather than infection. The "pathergy reaction" refers to the appearance of a papule or pustule 48 hours after insertion of a 20g needle into the skin. A positive pathergy reaction provides one of the criteria for the International Study Group classification criteria for BS [57]. Although pathergy appears to be a common feature of BS in Turkey, it is only demonstrable in ~10% of UK patients [58, 65, 203, 204]. Furthermore, the test necessitates a visit to clinic on 3 consecutive days, which is costly and cumbersome for both the health service provider and the patient. This, along with its low sensitivity limits its clinical usefulness.

The pathology of pathergy reactions resembles that of other skin lesions in BS, with neutrophils, monocytes and lymphocytes present in large numbers, together with increased mRNA transcripts for the cytokines IFN-γ, IL-12 p40 and IL-15; the chemokines CCL20 , CXCL10, CXCL9, and CXCL11; and adhesion molecules ICAM-1, and VCAM-1 at 48 hours in BS versus HC [61, 62]. Whether these abnormalities are due to the occurrence of an inappropriate Th1-driven immune response, to dysfunctional NK cells or some other immunological or non-immunological mechanism is not yet clear. However, importantly, the study of Melikoglu et al suggests that the cytokine response to needle injury in the skin in BS is qualitatively different from that occurring in healthy individuals [62].

3.1.2 Intradermal (id) injection of monosodium urate (MSU) crystals

Intra-dermal (ID) injection of MSU crystals is an alternative way of eliciting inflammation. The usual response consists of erythema and induration, which are maximal at ~24 hr. In healthy individuals there is an erythematous reaction at 24 hr which has usually largely resolved by 48 hr. In contrast, BS subjects exhibit a prolonged response [63], with a sensitivity for BS of 78%, whereas the classical pathergy test had a sensitivity of 28% [64]. Moreover, the cutaneous response to MSU crystals appears to be more reproducible than pathergy (95% concordant response after one year, compared to 73% for pathergy) [64]. Our unit have been able to show that UK subjects also demonstrate a prolonged inflammatory response to MSU crystals [65]. The great advantage of the MSU crystal reaction over the pathergy reaction as an investigational approach is that it is a graded rather than a categorical (present/absent) response and can be quantified in all individuals. Moreover, the large literature on the inflammatory response to MSU crystals in the context of hyperuricaemia and gout provides an excellent mechanistic frame-work for exploring how the response is abnormal in BS [66, 67].

3.1.3 Immunopathology of BS

As discussed in detail in Chapter 1, the immunopathology of BS is not clearly understood. There is evidence of involvement of multiple innate and adaptive pathways, resulting in an exaggerated or prolonged inflammatory response. There may also be a failure of the anti-inflammatory mechanisms of the immune system to switch off inflammation. Inflammatory tissues in BS typically show a mixed leukocytic infiltrate of neutrophils, macrophages and T lymphocytes [205-207]. Immunoglobulin and complement deposition do not appear to be marked features, suggesting that cellular immune mechanisms predominate [208].

There is evidence from analysis of peripheral blood and lesional tissues that the inflammatory response is skewed to a Th1 cytokine profile with increased expression of IFN-γ and IL-12, but the immunological drivers are still poorly understood [84, 133-135, 209, 210]. This is also supported by genetic studies [5, 6, 98, 132, 140]. Raised levels of TNFα are found in the sera of BS subjects, as well as IL-6, IL-8 and IL-18 [211-214]. The role of IL-23/IL-17 has also been creating interest [143, 215, 216]. Pathways that result in dampening inflammation have also featured in genetic studies, with particular interest in a SNP within the IL-10 promoter which resulted in less IL-10 protein production in monocytes of subjects with the disease associated allele [5, 6]. Other studies have highlighted a polymorphism within the TGF-beta gene [155]; low numbers of regulatory T cells in active BS[156] and a potential involvement of miRNAs [157, 158].

3.1.4 Inflammatory responses to MSU crystals *in vitro*

MSU crystals are thought to stimulate inflammation through activating mast cells, neutrophils and monocytes, inducing expression in the latter of pro-inflammatory genes including IL-1, TNFα, IL-6, IL-8, and Cox-2 [217-222]. These amplify inflammation through effects on blood flow, expression of endothelial cell (EC) adhesion molecules (e.g. E-selectin, ICAM-1, VCAM-1), chemotaxis of neutrophils and other leukocytes and stimulation of acute phase plasma protein generation [223-225]. Our laboratory and others have found that release of IL-1β and TNFα fully accounts for the ability of MSU crystal-stimulated monocytes to activate EC

adhesion molecule expression *in vitro*, with IL-1β release preceding and stimulating release of TNFα [223, 226]. It has been suggested that MSU crystals stimulate monocytes via a signalling mechanism involving CD14 and TLR-2, TLR-4 and MyD88 [227], although the involvement of TLRs remains controversial [228, 229]. Importantly, MSU crystals have been shown to stimulate IL-1β production by activating the NALP-3 inflammasome [226]. Our laboratory has shown that monocyte to macrophage differentiation can lead to reduced expression of pro-inflammatory factors (e.g. TNFα/IL-1β) in response to MSU crystals and to the acquisition of an anti-inflammatory response profile (e.g. TGFβ production) *in vitro*. Based upon these observations, we have published a series of papers proposing that macrophage differentiation contributes to the normal spontaneous down-regulation of MSU-induced inflammation in gout [230-232]. Thus, it is possible that the prolonged inflammatory response to MSU crystals in BS is related to a failure of macrophage differentiation to this anti-inflammatory phenotype.

3.1.5 Suction blister model of skin inflammation

We need to develop clinical research methodology to better understand the abnormal inflammatory response to MSU crystals in BS, as (i) there are major differences in the metabolism of uric acid between humans and sub-primates so animal models are not ideal, (ii) there is no accepted animal model of BS, and (iii) the complexity of the inflammatory response cannot be reproduced completely *in vitro*. Application of a small secure cup to the skin, attached to a gentle suction device (150-200mm Hg) leads to the gradual formation of a small blister. Suction blister fluid is largely derived from the interstitial fluid [202]. Compared to skin biopsies, suction blister fluid is obtained by means of a less invasive sampling technique. This is an important consideration in BS, where insults to the skin may lead to an aggressive inflammatory response and poor wound healing. Suction blister fluid can be used as body fluid to detect soluble mediators of inflammation [233, 234]. For several diseases, including epidermal necrolysis [235] and scleroderma [236], suction blister fluid has been used as a source to identify/ quantify potential protein biomarkers. Studies have also been published using suction blisters to study cellular content, including granulocytes [237]and monocytes [238].

These techniques varied in that blisters were left overnight, and then 'unroofed' and stimulated with serum before being covered again for 10 hours, following which fluid was collected.

For our protocol, 2.5 mg MSU crystals were injected intradermally to induce an MSU pathergy/inflammatory response. At 24-48 hours suction cups were applied over the erythematous area, blisters formed over the following 1-3 hours, following which fluid was gently aspirated using a 200μl pipette tip into a siliconized ependorf, and placed on ice.

3.1.5.1 Cantharidin skin blister model of inflammation

Our group had previously published studies examining the kinetics of inflammatory responses in human skin, using blisters elicited by application of the protein phosphatase inhibitor cantharidin [231, 239, 240]. This established the principle that detailed information could be obtained on small volumes (~100 μl) of blister fluid using multichannel flow cytometry and sensitive immunoassays for cytokines. We had found that there are marked differences in the profile of leukocytes and inflammatory mediators present at 16 and 40 hr after blister stimulation by cantharidin in healthy volunteers: (a) blister fluid contains significantly more leukocytes at 40 hr than at 16 hr, with Kimura staining showing significantly increased proportion of macrophages and reduced proportion of neutrophils; This gave us a model for studying early and late phases of the inflammatory response. For this study we used 24 hour blisters as an acute inflammation model and 48 hour blisters to reflect resolving inflammation.

3.1.6 Monocyte and macrophage biology

Abnormalities of macrophage differentiation could account for the phenomenon of prolonged inflammation, or failure to switch off inflammation, which is observed in BS. Furthermore, as outlined above, macrophages are central players in augmenting and dampening the inflammatory response to MSU crystals.

Myeloid progenitor cells in bone marrow give rise to monocytes/neutrophils. Monocytes give rise to tissue macrophages, osteoclasts and dendritic cells (although not all of these cells differentiate from monocytes) [241]. Monocytes constitute 5-10 % circulating leukocytes. They are heterogeneous, with variability in size, granularity and nuclear morphology. Human

CD14+ monocytes, which consist of CD16+ and CD16- subsets specialize in phagocytosis, production of reactive oxygen species (ROS) and the recognition of ligands for extracellular TLRs, in response to which they secrete inflammatory cytokines. Among this subset, CD16 may be a marker of activation. Monocytes that lack CD14 (CD14dim) but which express CD16 monocytes patrol the endothelium of blood vessels in an LFA-1-dependent manner, and respond to viruses and nucleic acid-containing immune complexes via a pro-inflammatory TLR7–TLR8–MyD88–MEK pathway. CD14dim cells may be involved in the innate local surveillance of tissues with regard to cell death and viral infection, the scavenging of immune complexes and the pathogenesis of autoimmune diseases such as lupus [242-245].

Macrophages also contain many subgroups. Classically Activated ('M1') macrophages are responsive to type 1 inflammatory cytokines (e.g. IFN) and microbial products; their function is microbicidal and inflammatory; and they have a 'core response to infection' with upregulation of transcription factors leads to pro-inflammatory cytokine (e.g.IL-1b/TNF/IL-6/IL-12) induction and upregulation of cytokine receptors (e.g. IL-7R/IL-15R). They also secrete chemokines (e.g. CCL2, CCL5, CXCL8 and CXCL10) and up-regulate the chemokine receptor CCR7. Uncontrolled M1 can lead to tissue damage and multiorgan failure [246, 247] .

M2 polarization is associated with chronic evolution of infectious disease is thought to be associated with macrophage reprogramming to M2; Alternatively Activated ('M2a') macrophages are responsive to IL-4 / IL-13; Their role is that of an immunomodulator, leading to increased endocytic activity; increased mannose receptor (MR),dectin1 and arginase, increased cell growth and increased tissue repair. They are involved in parasite killing. Innate activated ('M2b) macrophages are responsive to immune complexes and TLR agonists/IL1R agonists and are also immunomodulators, leading to increased iNOS and ROS and production of proinflammatory cytokines. Deactivated ('M2c') macrophages are responsive to IL-10; glucocorticosteroids; TGFβ; CD200-CD200R and act as an immunomodulator leading to increased production of IL-10; TGFβ; PGE2 and reduced expression of MHC2 molecules on the cell surface. CCR2, CCR5, and CD163 are cell surface markers for this phenotype [246, 247].

3.1.7 CD163 as a marker of deactivated macrophages

The development of anti-inflammatory responsiveness to MSU crystals of *in vitro* differentiated macrophages parallels the rise in macrophage expression of CD163 [240]. CD163 is a macrophage-specific member of the cysteine-rich superfamily, and functions as a scavenger receptor of haptoglobin: haemoglobin complexes [248, 249]. CD163 is known be upregulated by incubating monocytes with glucocorticoids or with the predominantly anti-inflammatory cytokine IL-10, and, conversely, CD163 expression is suppressed by TNFα, IL-4 or IFN-γ [250, 251]. On the strength of these observations, CD163 has been proposed as a marker of deactivated/anti-inflammatory macrophages involved in resolution [250, 252]. It should be noted that CD163 positive deactivated macrophages are not equivalent to the "alternatively activated"/M2 macrophage phenotype stimulated by IL-4/IL-13 [196, 246].

3.2 Hypothesis

We tested the hypothesis that a failure of macrophages to differentiate to the CD163+ 'deactivated' macrophage may contribute to the pathergy response observed in BS.

3.3 Aims

The primary aim of this project was to gain an understanding of the abnormal cutaneous inflammatory response to stimuli such as MSU in subjects with BS. Our specific question related to macrophage differentiation in BS and HC. A secondary aim was to find a useful diagnostic tool for this condition.

3.3.1 Research aims in detail

1. At the start of this study we aimed to compare the usefulness of two techniques of blister formation: (i) using a small suction cup placed over the MSU injection site at 24 or 48 hours, to obtain fluid and cells from the MSU lesion, (ii) using the cantharidin model of blister formation.

2. Once a skin blister protocol was established, we wished to explore the mechanisms underlying the delayed resolution of inflammation in BS, focusing the investigation on the following questions:

(1) Is there a delay in macrophage deactivation in BS and, if so, does this distinguish those with BS from healthy individuals

(2) What is the "bigger picture" of differences in cells and cytokines that distinguishes the inflammatory response in BS compared to controls?

(3) If macrophage parameters do not distinguish BS from controls, what other features of inflammation might be responsible?

(4) Could any of the observations be useful diagnostically?

3.4 Results

3.4.1 Assessment of a suction blister model to study cutaneous inflammation

Initially we wished to evaluate the usefulness of suction blisters to study the MSU reaction. For the method to be useful it would need to be a reliable way to obtain adequate cells for flow cytometry analysis, and fluid for protein analysis. We created two suction blisters over MSU-injected sites in 6 healthy control subjects. Subjects were injected on the forearm with MSU and 16 or 40 hours later 5mm suction cups were applied. We found that following application of a suction cup and negative pressure, blisters took between 1 and 2 hours to form. However, 3 of the 12 blisters burst during the suction process, and fluid was lost. In addition to this, a further 5 of the 12 blisters contained less than 1 x 10^5 cells - too few for flow cytometry analysis.

Table 3-1. Suction blister volumes and cell counts in 6 healthy controls

Suction blister volumes and cell counts in 6 HC subjects				
Subject	*Time point (hr)*	*Blister volume (µl)*	*Total Cell count (x10 ^{5})*	*Comment*
1	16	180	1.5	
	40	0	0	Burst at 80 min
2	16	0	0	Burst at 15min
	40	660	68.3	

3	16	140	135	
	40	160	7.7	
4	16	30	0.8	
	40	30	0.76	
5	16	80	0.2	
	40	70	0.4	
6	16	0	0	Burst at 60 min
	40	50	0.45	

To compensate for the low numbers of cells, we produced suction cups of a larger diameter. A 10 mm cup was found not to be effective at creating blisters as the skin was simply pulled to the roof of the cup. Although blisters did form with the 7.5 mm cup, less than 1 x 10^5 cells were recovered in both of two volunteers - again too few for multichannel flow cytometry analysis. Additionally, the time required for blisters to form increased, and in one of the subjects took 4 hours.

In contrast, cantharidin blister techniques were already established in the group and were known to be a robust model of skin inflammation, producing reliable quantities of fluid and cells. The decision was taken to proceed with this method for the study, but also to perform an MSU injection on the other arm to provide a link to a stimulation that had previously been shown to differentiate BS from healthy controls.

3.4.2 Establishment of a 3 day protocol for cantharidin skin blisters.

The final protocol that was used is shown below. On day one, following informed consent, a questionnaire was completed to record subject demographics, history, medications and disease activity. A blood sample was taken using an aseptic technique for *in vitro* experiments. Following this, cantharidin was applied to one forearm and an overlying paraffin film and bandage applied. MSU was injected into the dermis on the other forearm. On day two, the MSU reaction was measured and photographed and the second droplet of cantharidin was applied. On day 3, blisters (one being 48 hours old and one being 24 hours old) and the MSU reaction sites were measured and digitally photographed and the blister fluid was gently aspirated using a

pipette, into a labelled 1ml siliconised ependorf. Samples were kept on ice and brought immediately to the laboratory for analysis.

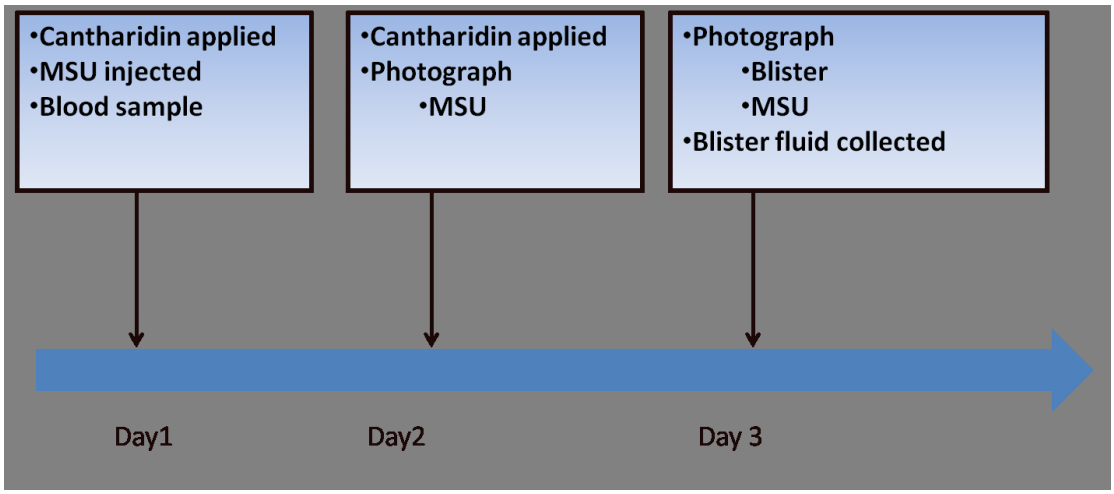


Figure 3-1. The final protocol to establish 24 hour and 48 hour cantharidin skin blisters, and an ID MSU injection test.



Figure 3-2. Cantharidin skin blister application.
Cantharidin solution is applied to a 1cm circular piece of filter paper on the volar aspect of the forearm. Parafilm and a bandage are placed over the filter paper. At 24 or 48 hours, the bandage is removed and the blister is photographed. Fluid is aspirated and collected for analysis.

3.4.3 Demographics of the BS and HC cohorts

We recruited 12 BS and 12 HC subjects. Demographics of the recruits are shown below. Seven of the BS were male (58%) versus six of the HC subjects (50%). The median age in the BS group was 43years (interquartile range 34.5-46.8) versus 32 years (interquartile range 28.3-36.0) in the HC group. Six of the BS group were taking steroids (prednisolone). Of those 6, the median dose was 10 mg. BS subjects were also taking a variety of other disease modifying

medications: namely: colchicine (n=3); azathioprine (n=6); an anti-TNF agent (n=5); mycophenolate moefetil (n=2) and tacrolimus (n=1). The median disease activity using the Leeds questionnaire was 4.1 (scale of 0-12) indicating mild-moderate disease activity. In the HC group, all subjects were in good health and taking no regular medications.

General Demographics	Healthy Controls		Behçet Syndrome	
Cohort size	12		12	
Median age (Interquartile range)	33	(31-38)	43	(37-46)
Male sex (%)	6	50%	7	58%
Clinical Characteristics				
Mucocutaneous	0		12	100%
Erythema nodosum			3	25%
Pathergy or poor wound healing			9	75%
Musculoskeletal – arthralgias	0		10	83%
Arthritis			3	25%
Cardiovascular	0		3	25%
Aneurysm			2	17%
Thrombosis			1	8%
Ocular	0		2	17%
Posterior/panuveitis	0		2	17%
Gastrointestinal inflammation	0		1	8%
Neurological	0		3	25%
Disease Activity Score *	0		4	
Current medications				
Colchicine	0		4	33%
Azathioprine	0		5	42%
Mycophenolate	0		2	17%
Anti-TNF therapy	0		4	33%
Cyclosporine	0		1	8%
Methotrexate	0		1	8%
Steroids **	0		6	(10mg)

*** Behçet: Leeds Behçet's disease activity form (2002 version) score of 0-12 (>4 indicates active disease).**

**** Number of subjects taking steroids, and the median prednisolone dose taken by those subjects**

Table 3-2. Demographics of BS and HC subjects recruited for cantharidin skin blister study.

Features listed include general demographics, disease manifestations and medications being taken at the time of the study.

3.4.4 Monosodium urate injections

Previous studies, outlined in Chapter one, have shown that ID MSU injections result in significantly more erythema in BS than HC at 48 hours. We injected all participants with 2.5mg MSU into the volar aspect of the forearm not being used for skin blisters. At 24 and 48 hours the maximum diameter of erythema was measured and photographed. We found that BS had significantly larger MSU responses at both 24 and 48 hours to the MSU injection than HCs.

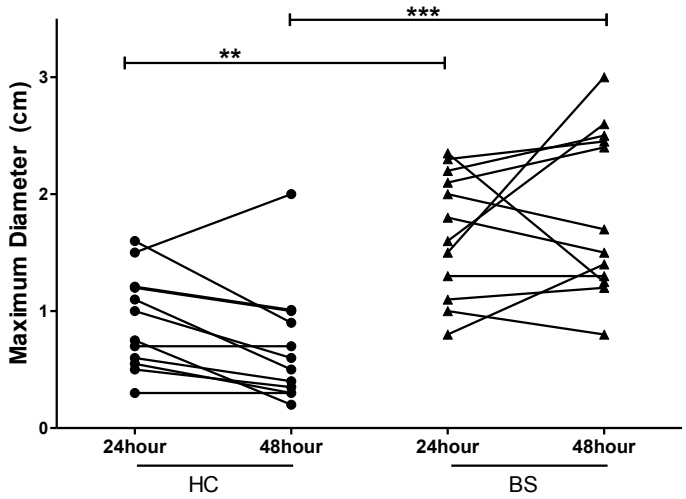


Figure 3-3. The maximum diameter of erythema in HC and BS subjects 24 and 48 hours after the intradermal injection of MSU crystals.

Kruskal-Wallis non parametric two-tailed testing of analysis of variance between the groups $P<0.001$. Mann Whitney non parametric testing two-tailed testing at 24 and 48 hours showed significantly differences between HC and BS (24 hour $P=0.0029$; 48 hour $P=0.004$)

3.4.5 Naked eye assessment of cantharidin skin blisters

Prior to collecting fluid, cantharidin skin blisters were photographed. Interestingly, many of those BS subjects who had a large reaction to the MSU, also developed an erythematous 'halo' around the MSU blister. We classified MSU responses in all subjects (BS and HC) as 'strong' or 'weak'. We found that the presence of the halo around the cantharidin blister strongly correlated with having a strong MSU response (Figure 3.5, Figure 3.6).



Figure 3-4. The typical appearance of cantharidin skin blisters in HC (left) and BS (right) subjects.

Note the surrounding 'halo' of erythema in the BS subject.

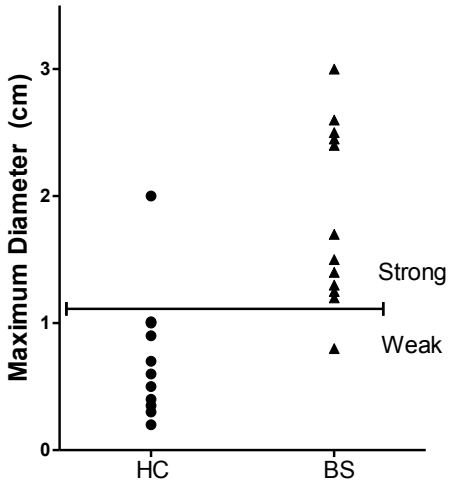


Figure 3-5. MSU responses classified as 'strong' or 'weak' depending on the size of erythema at 48 hours.

This grouping was used to correlate with erythema around the cantharidin blisters (see below)

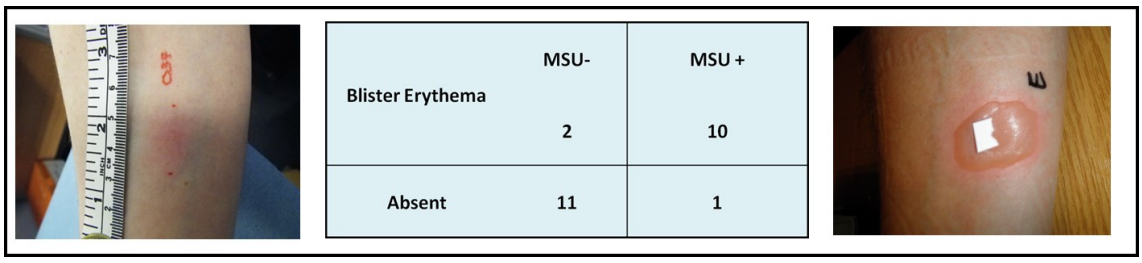


Figure 3-6. The correlation between having a large MSU response and development of erythema around the cantharidin skin blister.

Chi square test 13.2 df 1 P=0.0002 indicating a significant correlation.

3.4.6 Cantharidin volume and cell count

Fluid recovered from the cantharidin skin blister was immediately transferred to a siliconized ependorf and placed on ice. The volume was recorded and then 10µl was aspirated and applied to a haemocytometer for a total cell count. The remainder was spun in a microcentrifuge at 300g, following which interstitial fluid was separated, aliquoted and stored in siliconized ependorfs at -80ºC for future analysis of blister fluid protein content. Cells were resuspended in 'FACS buffer' (phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, UK) and 0.05% NaN3 (Sigma-Aldrich, UK)) for immediate characterization by flow cytometry. 70µl was set aside for white cell differential slide.

The median volume of fluid recovered from the HC blisters was 210µL (24hour) and 185µL (48hour); and from the BS blisters was 500µL (24 hour) and 180µL (48 hour). Although a trend was seen to having less fluid in the 48 hour sample, and more fluid in the BS samples, this did not reach significance.

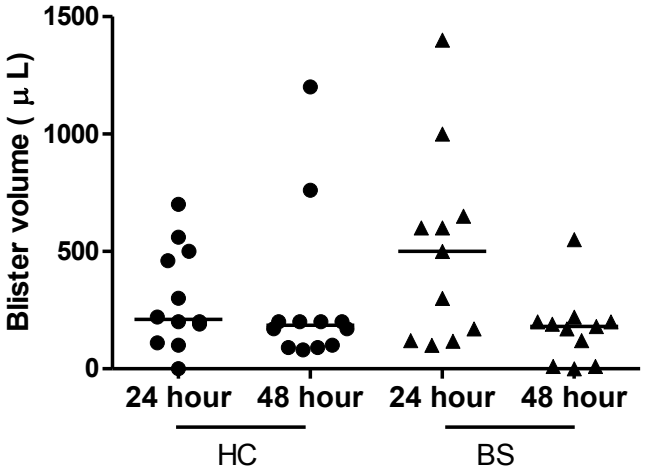


Figure 3-7. Cantharidin blister volumes in HC and BS at 24 and 48 hours.
The line represents the median values for each group. Kruskal-Wallis non parametric two tailed analysis of variance did not show significant differences between the groups (P=0.16).

A cell count was undertaken shortly following blister fluid aspiration using a haemocytometer. The median cell count (cells x 10^4 / mL) for HC blisters was 67 and 134, for 24 and 48 hour blisters respectively, and that for BS blisters was 110 and 150, for 24 and 48 hour blisters

respectively. There was a trend towards a greater density of cells in BS samples, and there appeared to be an increased cell number in later samples, but differences between groups did not reach significance.

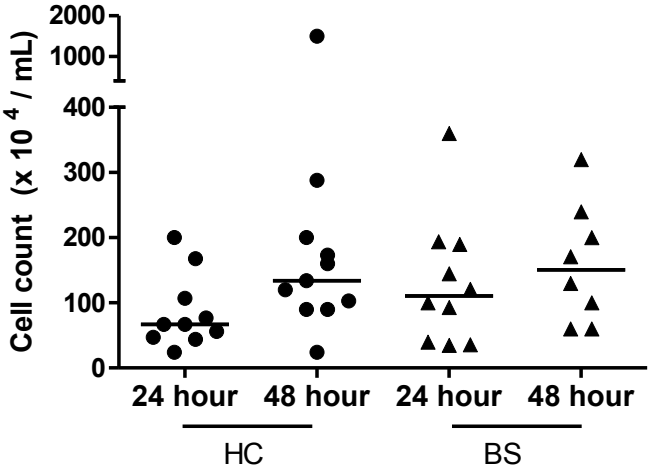


Figure 3-8. The cell count (cells x 10^4 / mL) of 24 and 48 hour blisters in HC and BS.
There was a trend towards more cells in the BS cohort, and in the later blisters but Kruskal-Wallis non parametric two tailed analysis of variance showed no difference between the groups (P=0.174).

The total cell count obtained was next calculated based on the volume and cell/mL count. The median total cell count (x 10^4) for HC blisters was 16 and 24 for 24 and 48 hour blisters respectively, and that for BS blisters was 36 and 25 for 24 and 48 hour blisters respectively.

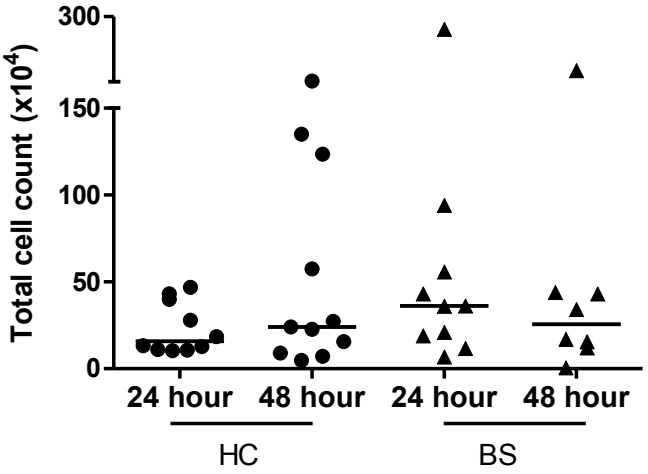


Figure 3-9. Cantharidin blister total cell counts (x10^4) in HC and BS at 24 and 48 hours. The line represents the median values for each group. Kruskal-Wallis non parametric two tailed analysis of variance did not show significant differences between the groups (P=0.66).

A slide was prepared and stained for each blister to obtain a white cell differential. The differential showed the expected differences between 24 hour and 48 hour blisters. Twenty four hour blisters showed a predominantly neutrophilic infiltrate (80% of BS and 86% of HC). Forty eight hour blisters show fewer neutrophils (55% in both BS and HC subjects). Macrophages were seen with a higher frequency in the 48 hour blisters versus 24 hour blisters as would be expected (HC 7.2% increasing to 35% by 48 hours, BS 12% increasing to 29%). Percentages of lymphoctyes and other leukocytes were low in all groups with no clear differences observed between any of the groups.

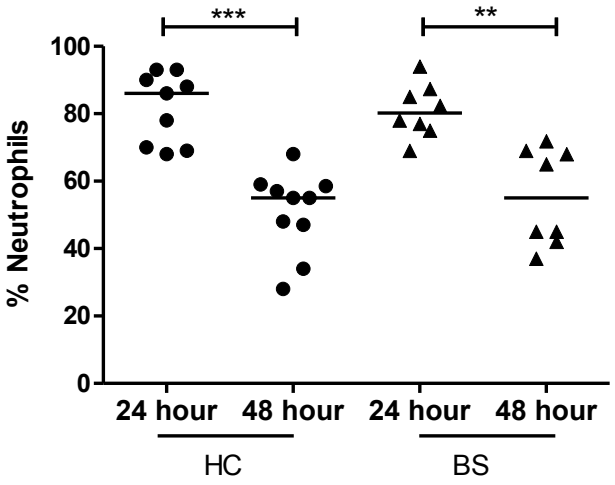


Figure 3-10. The percentage of neutrophils present in cantharidin skin blisters.

A higher % of neutrophils were present in the 24 hour sample versus the 48 hour sample in both groups (Mann Whitney P=0.0003 HC; P=0.0016 BS). The Kruskal-Wallis non parametric two tailed analysis of variance confirmed significant variance between the time points but not between the HC and BS groups (P<0.0001).

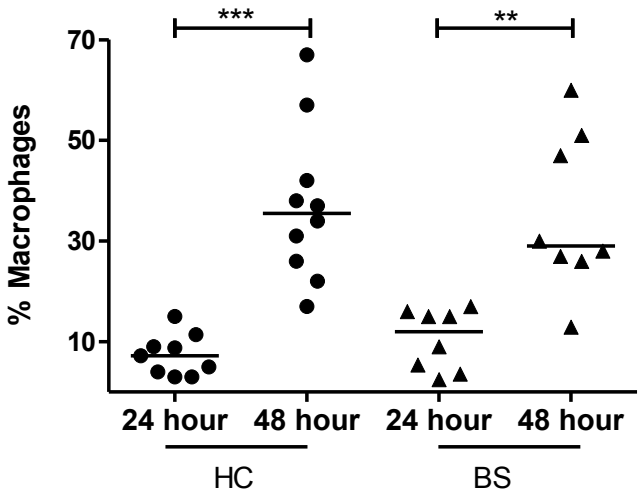


Figure 3-11. The percentage of macrophages present in cantharidin skin blisters.

A higher % of macrophages were present in the 48 hour sample versus the 24 hour sample in both groups (Mann Whitney P=0.0003 HC; P=0.0039 BS). The Kruskal-Wallis non parametric two tailed analysis of variance confirmed significant variance between the time points but not between the groups (<0.0001).

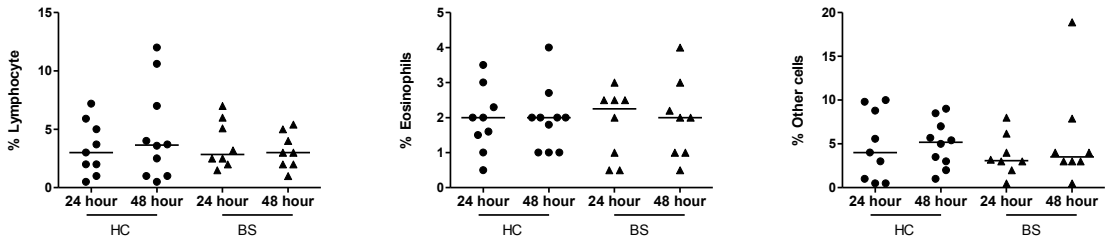


Figure 3-12. The percentage of lymphocytes; eosinophils and 'other' cells present in cantharidin skin blisters.

The Kruskal-Wallis non parametric two tailed analysis of variance found no significant variance within any group.

3.4.7 Flow cytometry

Flow cytometry is a cellular analysis technology in which a beam of light is directed onto a focused stream of fluid passing through the flow cytometer. The scatter of light is analysed to reveal characteristics of the cells passing through that stream of buffer. Cells labelled with a fluorescent antibody will cause a distinct pattern of light scattering at a specific wavelength which can then be detected by the flow cytometer. As outlined in Chapter 2, fluorescence minus one (FMO) was the technique applied throughout the following experiments.

3.4.7.1 Establishment of a flow cytometry protocol

The final protocol used for multichannel flow cytometry analysis of blister fluid and PBMCs from peripheral blood was as follows. Row 1: antibodies for examining monocyte/macrophage population. Anti-CD14 antibodies were used to identify the monocyte/macrophage population. Use of anti-CD16 (FcγRIII) was intended to separate CD14+ monocytes into CD14highCD16low and CD14lowCD16high. CD14+CD163+ cells and CD14+HLA-DR+ expression were measured as markers of deactivated or activated populations. (ii) Row 2: antibodies for examining T cell subsets. CD3 was used as the marker for all T cells, as it is present on cells expressing either αβ or γδ T cell receptors. γδ T cells was measured directly in the FL2 channel, whilst the proportion of CD3+ αβ T cells was deduced by subtracting the number of CD3+ γδ T cells from the total CD3+ population. (iii) Row 3: antibodies for examining NK cells. NK cells express CD56 (a homophilic adhesion molecule of the

immunoglobulin superfamily (NCAM)) and CD16. The absence of CD3 but the presence of CD56 and CD16 were deemed the most reliable markers for pure NK cells in humans. Two functional subsets of NK cells exist: the more mature CD56dim CD16high population is potently cytotoxic, whereas the CD56bright CD16low/neg NK cells have low cytotoxicity but produce much greater amounts of cytokines. The figure below shows an example of 'row one' gating of PBMCs.

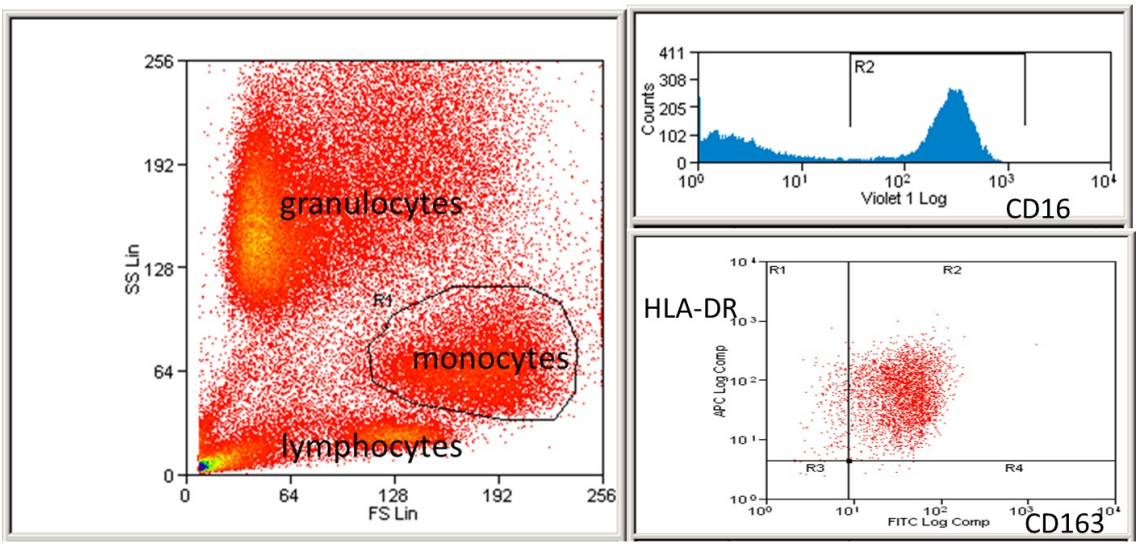


Figure 3-13. A sample of gating of flow cytometry sample prepared from peripheral blood derived mononuclear cells.

Cells were gated to include monocyte populations. They were then gated for include only CD16+ populations. Lastly cells were assessed for CD163 or HLA DR.

3.4.7.2 Macrophage phenotype: CD163 and HLA DR expression

The primary research question was to assess whether in HCs there may be an increase in CD163high CD14+ cells at 48 hours compared to 24, and whether this increase in CD163high CD14+ cells was reduced in BS. Using flow cytometry, we gated cells by forward and side scatter and by CD14 positivity to isolate the monocyte/macrophage population. These cells were also co-stained with both CD163 and HLA DR antibodies and relative fluorescent intensities (RFIs) were calculated. In HCs, CD 163 tended to be low in peripheral blood and early blisters, and rose in late blisters. We found that CD163 was quite variably expressed in BS subjects. This may have been related to the

fact that 50% of our BS subjects taking steroids (a known inducer of CD163 expression [248]). We could draw no significant conclusions about differences in expression between the two groups. HLA DR expression was low in peripheral blood and increased significantly in early blisters in both groups. The late blister showed a continued HLA DR expression in both groups. Interestingly, the majority of HC samples were stabilising or starting to reduce HLA DR expression at 48 hours – the time point of 48 hours may have been too early to show advanced resolving inflammation. In contrast, in the BS groups, expression in a few individuals continued to increase at 48 hours.

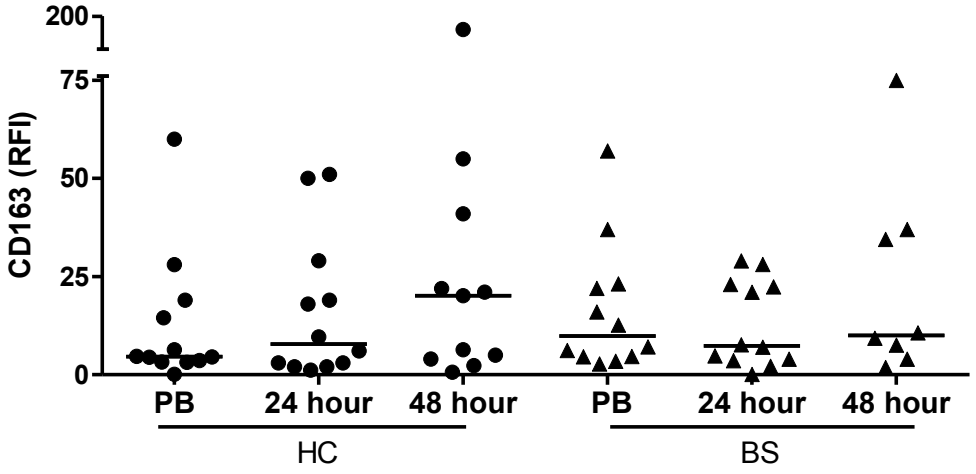


Figure 3-14. CD163 expression by flow cytometry of peripheral blood derived monocytes (PB), 24 hour blister cells and 48 hour blister cells.

CD163 is used as a marker of deactivated macrophages. There is a trend in HC for low CD163 expression in PB monocytes, low expression in 24 hour blisters and higher expression in late blisters. In BS samples there is relatively high CD163 expression in PB, possibly secondary to steroid medication. There appears to be less CD163 expression in the BS 48 hour samples versus HC. Kruskal-Wallis non parametric two tailed analysis of variance shows no variance between groups.

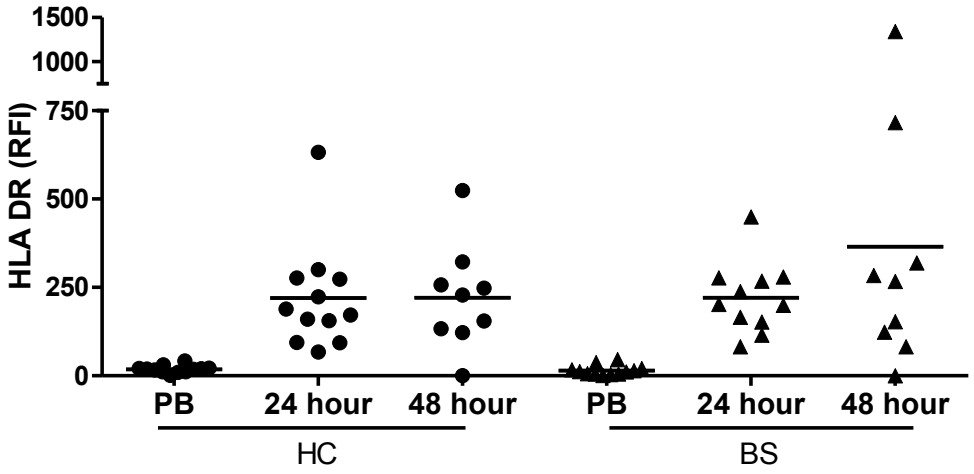


Figure 3-15. HLA DR Expression by flow cytometry of peripheral blood derived monocytes (PB), 24 hour blister cells and 48 hour blister cells.

HLA DR is used as a marker of activated macrophages. There is significantly lower HLA DR expression in PB monocytes relative to blister samples (Kruskal-Wallis non parametric two tailed analysis of variance $P<0.001$). However no significant differences were observed between the blister groups.

3.4.7.3 T cells subsets and NK cells

In addition to examining macrophage phenotypes we wished to gain a broader picture of cells present in early and late blisters. Using multichannel flow cytometry we examined T cell subsets and compared these over time between our two groups. Cells were also gated by forward and side scatter and CD3 positivity. We found that CD3+CD4+ T cells formed approximately 75% of the lymphocyte population in all groups. A higher proportion of CD3 T cells were CD4+ in the blister fluid relative to peripheral blood samples, indicating CD4+ cells were recruited preferentially. Cells were gated by CD56+ and CD3 negativity or positivity to isolate natural killer cells and NK T cells. As low levels of these cell types (<1%) were found in all groups, numbers were insufficient for further analysis.

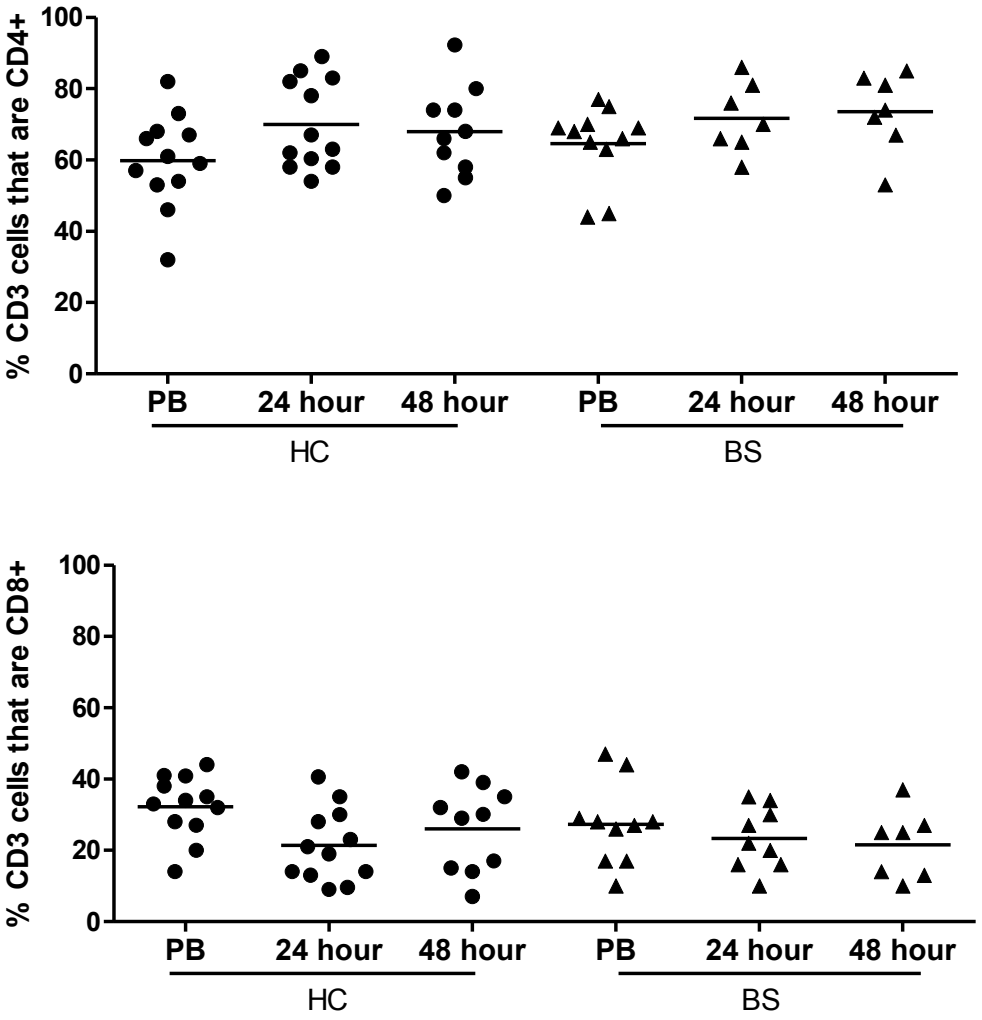


Figure 3-16. The percentage of CD3+ cells that co-stained for CD4 or CD8.
No significant differences were observed between the groups. The overall number of CD3+ cells was low in blister fluid.

3.4.8 Blister fluid cytokine and chemokine concentrations

Blister fluid that had been stored at -80^{o}C was analysed by multiplex bead analysis on the flow cytometer to quantify candidate cytokine and chemokine concentrations in all blisters.

Cytokines were chosen to assess Th1/Th2/Th9/Th17/Th22 skew by measuring human IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, IL-22 and TNF-α in an immunoassay analysed on a flow cytometer.

Th-1 cytokines include IL-2, IFN-gamma, IL-12 and TNF-beta, while Th-2 cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13. Th-17 cytokines include IL-22; IL-6 and IL-17A. Th-9 cytokines include IL-9 and acute phase cytokines include IL-1β and TNF-α.

Results of the cytokine analysis are shown below. Disappointingly, results revealed low concentrations of many candidate proteins. Before correction for multiple analysis, CXCL10 levels were significantly different between the two groups, with 24 hour BS blisters having significantly lower CXCL10 concentrations than HCs (p=0.014). This was no longer a significant difference with multiple analysis corrections (Dunn's test for multiple analysis). Trends of differences were seen in other groups, with trends towards lower IL-10; IL-1β; IL-4; IL-13 and IL-17A but none of these results reached statistical significance. Given that concentrations of most cytokines were quite low, and that corrections for multiple analysis was undertaken, our study was not powered to find small differences between groups.

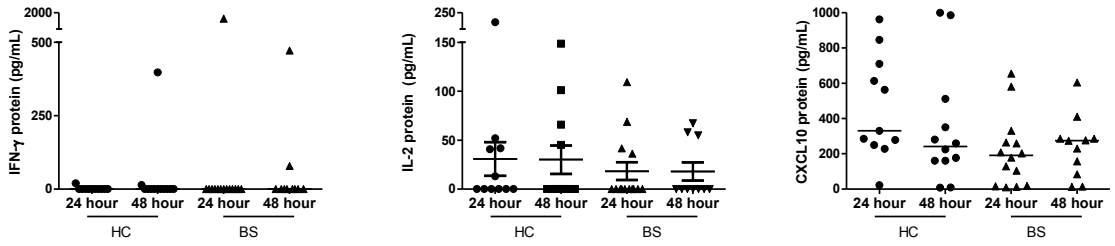


Figure 3-17. The Th1 skew associated cytokines present in blister fluid at 24 and 48 hours. IFN-γ, IL-2 and CXCL10 are associated with a Th1 skew. Very little IFN-γ protein was observed in any sample. There was a non-significant trend towards less IL-2 in BS samples. There was less CXCL10 protein in 24 hour BS blister samples than in HC samples (Mann-Whitney test P=0.015), however significance was lost following correction for multiple analysis (Dunn's test for non-parametric analysis).

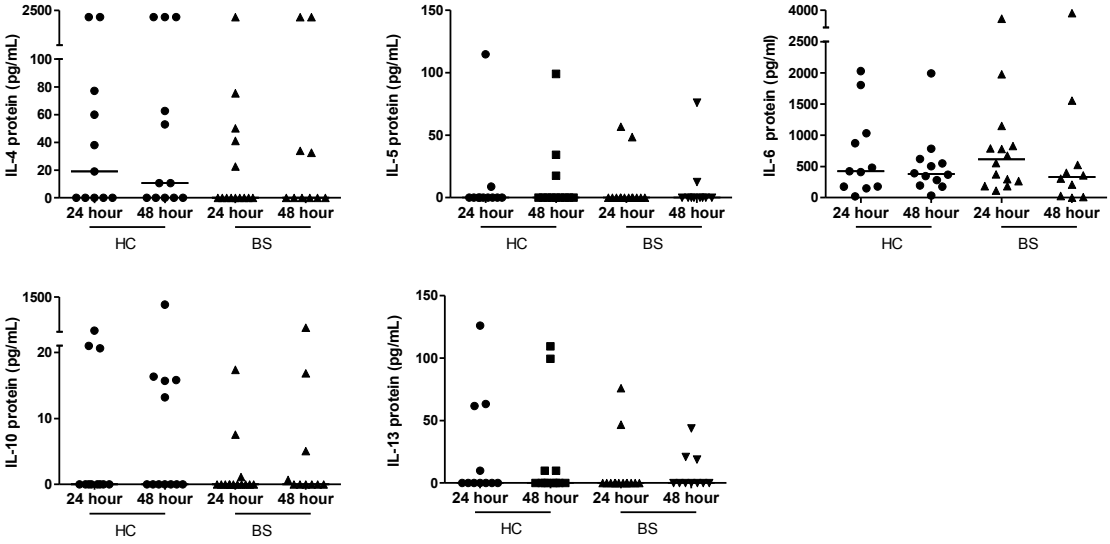


Figure 3-18 The Th2 skew associated cytokines present in blister fluid at 24 and 48 hours.
IL-4, IL-5, IL-6, IL-10 and IL-13 are associated with a Th2 skew. Very little protein was observed most samples apart from IL-6. There was a non-significant trend towards less IL-4 and IL-10 in BS samples. There was also a trend towards more IL-6 in 24 hour BS blister samples than in HC samples however no observations were statistically significant.

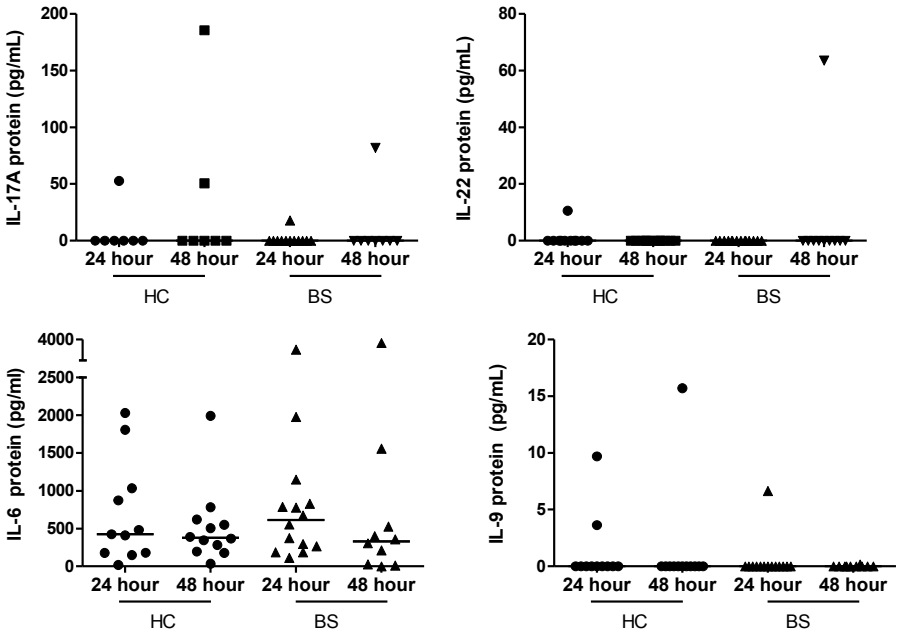


Figure 3-19 The Th17/9 skew associated cytokines in blister fluid at 24 and 48 hours.

IL-17A, IL-22 and IL-6 are associated with a Th17 skew. IL-9 is associated with a Th9 skew. Again, very little of these cytokines was observed in most samples apart from IL-6.

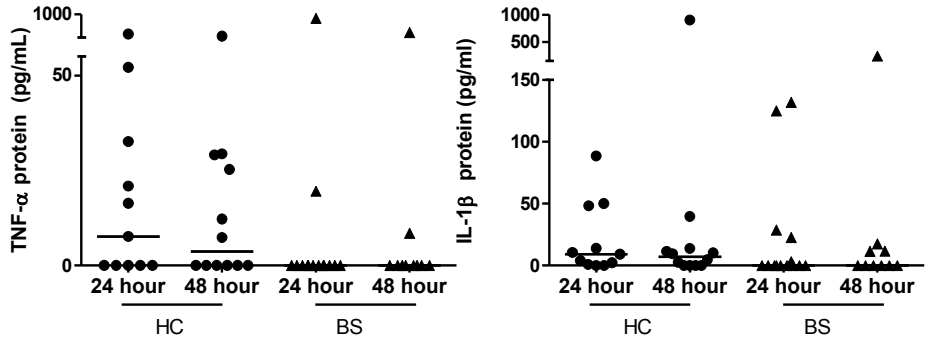


Figure 3-20. The acute phase response associated proteins present in the blister fluid at 24 and 48 hours.

TNF-α and IL-Iβ are secreted during an acute phase response. There was a non-significant trend towards less TNF-α and IL-Iβ protein in BS blister samples.

3.5 Discussion

3.5.1 Skin blister models of inflammation

Generating suction blisters over inflammatory-responses using the MSU blister model did not turn out to be a useful approach for obtaining inflammatory tissue from BS subjects. There was a large variation in the quantity of cells and fluid obtained, the procedure was lengthy and the incidence of blisters bursting was too high. However, this technique may still have a use for protocols requiring fewer cells and offers the benefit of directly sampling cells and fluid from the site of the MSU reaction without the need for a biopsy.

The cantharidin skin blister method was found a useful and robust model of skin inflammation, giving reliable volumes of fluid and adequate cell numbers for all analysis. We found it very reassuring that a large MSU reaction correlated strongly with the presence of a halo around the blister. This implies that the cantharidin model is a valid method of studying the abnormal inflammatory response in BS.

3.5.2 Macrophage activation states

We found trends but no significant differences between BS and HC with regard to our primary aim, assessing markers of macrophage activation/deactivation. In both HC and BS, subjects had low percentages of macrophages in early blisters (where neutrophils predominate) which increased in late blisters, and there was a non-significant trend towards BS having less deactivation in later blisters. However this finding was not statistically significant. Most subjects had low HLA DR in peripheral blood which increased in the early blister and started to settle in the late blister. It is possible that with higher numbers of participants, and by excluding BS taking corticosteroids we may have found greater differences.

3.5.3 Other cells and cytokines in early and late blisters

Both HC and BS had high percentages of neutrophils in early blisters and fewer in late blisters. Low levels of lymphocytes and natural killer cells were observed in both groups at all time

points which made it difficult to look for differences between the groups with our numbers. We were surprised that we found no differences in any cell type or cytokine level between the two groups apart from CXCL10 levels which were significantly different but lost that significance with multiple testing adjustments. CXCL10 is released by multiple cells (including macrophages, endothelial cells and dendritic cells) in response to IFN-γ and this finding is explored in detail in Chapter 5. We found no differences in IFN-γ levels between the two groups, but cytokine levels in general were very low, making it difficult to find significant differences between our groups.

3.6 Concluding remarks

The erythema surrounding blisters in BS subjects was a very interesting finding. This correlated with the size of erythematous responses to MSU crystals, but was not associated with any obvious differences in immune cells or cytokines. CXCL10 was highlighted as a chemokine of interest and this chemokine is investigated in detail in Chapter 5. We also reflected on what other mechanisms could be resulting in this erythematous response. Neuropeptides came to mind for several reasons, as discussed in Chapter 4, as agents that could be playing a role in this setting. Chapter 4 investigates a potential role for neuropeptides in BS.

4. Investigation of the role of neuropeptides in BS

4.1 Introduction

In Chapter 3, we found that BS subjects had a significantly larger reaction to MSU crystals than HC at both 24 and 48 hour time points. Subjects with a large MSU reaction also developed a ring of erythema around capsaicin blisters. We did not find obvious differences between the two groups in cell blister volume, cells or cytokines which led us to consider alternative causes of this erythema. In this chapter we explored the possibility that neuropeptides may contribute to the abnormal cutaneous inflammatory response seen in subjects with BS.

4.1.1 The neuro-immune system

4.1.1.1 Interplay between the nervous system and inflammation

The intensity and duration of an inflammatory process depends on the local balance between pro- and anti-inflammatory mediators. Peptides of neuronal origin exert specific effects on immune cells and have been shown to regulate inflammation. It is logical that the neurological system would play a role in controlling the immune system when we consider that it coordinates other major physiological responses from heart rate and blood pressure to blood glucose levels.

Two of the best characterised neuro-immune systems are: (i) the hypothalamic-pituitary-adrenal (HPA) axis: acute physical or psychological stress activates the HPA axis resulting in increased plasma adrenocorticotrophic hormone (ACTH) and cortisol concentrations. Cortisol is a potent anti-inflammatory agent [253, 254]. Interleukin-6 also enhances the HPA axis response to inflammation and other stressors [255]; (ii) the cholinergic anti-inflammatory pathway: this is an extremely fast, reflex-like anti-inflammatory pathway controlled by brain networks, e.g. through the vagus nerve system, designed to control cytokine release and functions [166, 167, 169, 256, 257].

The vagus nerve innervates principal organs, including those that contain the reticuloendothelial system (liver, lung, spleen, kidneys and gut). Experimental activation of the cholinergic anti-

inflammatory pathway by direct electrical stimulation of the efferent vagus nerve inhibits the synthesis of TNF in liver, spleen and heart, and attenuates serum concentrations of TNF during endotoxaemia [258, 259]. Animals subjected to hypophysectomy or adrenalectomy are significantly sensitized to the lethal effects of endotoxin [260]. Stimulation of either the right or the left cervical vagus nerves protects against the development of hypotension and inhibits serum TNF responses to ischaemia reperfusion [259]. In a standardized model of experimental murine arthritis induced by the application of carrageenan, vagus nerve stimulation inhibited the inflammatory response and suppressed the development of paw swelling, indicating that the cholinergic anti-inflammatory pathway can inhibit localized inflammation specifically [164]. The molecular dovetail between the cholinergic nervous system and the innate immune system is a nicotinic, a-bungarotoxin- sensitive macrophage acetylcholine receptor [258]. Exposure of human macrophages, but not peripheral blood monocytes, to nicotine or acetylcholine inhibits the release of TNF, IL-1 and IL-18 in response to endotoxin but not anti-inflammatory cytokines (such as IL-10) [258]. Acetylcholine inhibits the expression of TNF protein in macrophages, but not the induction of TNF messenger RNA levels, indicating that activation of the cholinergic receptor transduces intracellular signals that inhibit cytokine synthesis at a post-transcriptional stage [258].

4.1.2 Neuropeptides

Neuropeptides are neuronal signalling peptides, released from nerve endings to perform a similar communication/messenger role for nervous tissue that hormones undertake for glandular tissue, although usually in a more localized capacity.

The association between stressful life events and various dermatological diseases is explained by the concept of neuro-immuno-cutaneous system involving neuropeptides. It has been shown that neuropeptides especially calcitonin gene-related peptide (CGRP), substance P (SP), and neurotrophins such as nerve growth factor (NGF) affect the pathogenesis of skin disorders such as atopic dermatitis and psoriasis vulgaris [178, 261, 262] .

4.1.2.1 Calcitonin-gene-related-peptide (CGRP)

CGRP is one of the most abundant peptides produced in both peripheral and central neurons with receptors found throughout the body including throughout the cardiovascular systems and in organs of the immune system [263, 264]. CGRP is a 37-amino acid neuropeptide generated by tissue-specific alternative processing of the calcitonin gene [265, 266]. It is generally co-expressed with either somatostatin or substance P in sensory neurons [267]. CGRP exists in two forms, α-CGRP and β-CGRP. The less-studied β-CGRP differs in three amino acids (in humans) and is encoded in a separate gene in the same vicinity (on chromosome 11). CGRP mediates its effects though 2 receptors: a heteromeric receptor composed of a G protein-coupled receptor called calcitonin receptor-like receptor (CALCRL) and a receptor activity-modifying protein (RAMP1). Although part of the calcitonin family, GCRP's effect on bone is unclear. It is known to inhibit osteoclasts *in vitro* [268].

CGRP is perhaps best known for its cardiovascular effects. It is the most potent vasodilator of the neuropeptides, both of arterial and venous vessels [269, 270]. Additionally, the microvasculature is very sensitive to the effects of CGRP, with a potency 10 times greater than the prostaglandins [270, 271].

The interaction of CGRP with the immune system is complex. There is evidence that many neuropeptides such as the neurotrophins (e.g. nerve growth factor) and tachykinins (e.g. Substance P) play a pro-inflammatory role and CGRP is co-secreted with these peptides. CGRP increases vascular permeability leading to increased access of immune cells to sites of inflammation. CGRP has also been shown to induce production of IL-8, a well-known chemotactic factor for neutrophils [272]. However there is growing evidence for an anti-inflammatory role for this peptide.

Specific receptors for CGRP are present in T and B lymphocytes and in macrophages [273, 274]. GCRP been shown to promote macrophage IL-10 production, suppress Langerhans cells antigen-presenting function, T lymphocyte proliferation and production of IL-2, IL-12 and IFN-γ [186, 262]. CGRP is also an inhibitor of antigen presentation by macrophages [275]. CGRP inhibits LPS induced production of chemokines CXCL8, CXCL1 and CCL2 by human dermal

microvascular endothelial cells [276]. It also prevents LPS induced IL-1β, IL-6 and TNF-α protein production, IkBα degradation and NF-κB activation [276-279]. Additionally, systemic treatment of mice with CGRP has been found to reduce blood neutrophilia induced by systemic administration of LPS and also protected against a lethal dose of LPS [280]. After intradermal administration, CGRP inhibited the induction of contact hypersensitivity at the injected site in mice [281]. APCs and human monocytes are able to synthesize CGRP, and CGRP expression is enhanced after LPS stimulation. Endogenous CGRP synthesis in monocytes is regulated by NGF. Monocytes produce their own NGF and its synthesis is up-regulated on activation and the neutralization of endogenous NGF using anti-NGF antibodies markedly reduces the synthesis of CGRP induced by LPS [278].

4.1.2.2 Tachykinins

Tachykinins are a group of neuropeptides that include SP. In addition to exciting neurons, in the vascular system they increase vascular permeability and are potent vasodilators [282], they promote angiogenesis and cell growth [283] and in some situations promote healing [181]. In normal human dermal microvascular endothelial cells SP has been shown to induce production of IL-8 [272]. SP stimulates hydrogen peroxide production by neutrophils and also stimulates pro-inflammatory cytokines such as TNF-α, IL-1 and -6, histamine, leukotriene B4 and prostaglandin D2, and immunomodulatory cytokines such as IL-2. It induces adhesion molecules such as intercellular adhesion molecule (ICAM)-1, vascular cellular adhesion molecule (VCAM)-1 and endothelial leucocyte adhesion molecule-1 dermal microvascular endothelium, and causes neutrophilic and eosinophilic infiltration in cutaneous tissue [272].

Interestingly SP has been shown to correlate with pro-inflammatory cytokine concentrations in synovial fluid in RA subjects, but although CGRP is also present in synovial fluid, there is no correlation to cytokine concentrations [284, 285].

4.1.2.3 BS and neuropeptides

There is ample clinical evidence suggesting that emotional stress can influence the course and disease activity of BS [176, 177], implicating a neuro-immune interaction, similar to that

observed in dermatological diseases described above. As neuropeptides such as CGRP and SP are potent vasodilators, they could potentially be involved in the erythema observed with during the pathergy response, MSU response, or the erythema surrounding the cantharidin skin blisters.

Recent genome wide association studies in BS identified a SNP with near genome-wide significance (rs936551) located at the telomeric end of the short arm of chromosome 4 within the promoter region of CPLX1, which encodes the peptide complexin 1 [6]. Complexins play a critical role in the control of fast synchronous neurotransmitter release. They operate by binding to trimeric SNARE complexes consisting of the vesicle protein synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25, which are key executors of membrane fusion reactions [183]. SNARE complex binding by complexins is thought to stabilize and clamp the SNARE complex in a highly fusogenic state, thereby providing a pool of readily releasable synaptic vesicles that can be released quickly and synchronously in response to an action potential and the concomitant increase in intra-synaptic calcium levels [184]. It is possible that a under or over functioning Complexin-1 in BS may alter the dynamics or quantity of neuropeptides with downstream effects on inflammation.

Complexin I and II knockout mice (KO) have been investigated, primarily with regard to neurological function. Complexin I KO mice have seizures from birth, are unable to reproduce and are lethal by 2-4 months [286]. Modified KOs display marked deficits in social behaviours, ataxia and coordination deficits [287] , and have profound motor and cognitive deficits [286]. Nothing is documented in these papers about inflammatory or vascular abnormalities. Complexin involvement has been implicated in Alzheimer's Disease [288], Schizophrenia [289] and Hungtington's Chorea [290].

Intriguingly, recently a human whole-genome microarray of over 50 thousand genes was used to analyse mRNA expression profiles of peripheral blood mononuclear cells obtained from four BS at baseline and at 22 weeks after initiation of infliximab. Quantitative polymerase chain reaction analysis was performed for selected up- or down regulated genes, to confirm the microarray results. TNF blockade resulted in not only reduced expression of many cytokines but also reduced expression of complexin II (CPLX2) in all BS subjects [291].

In one study of BS volunteers and healthy and psoriasis controls, skin lesion punch biopsies revealed strong immunoreactivity for SP in the epidermis, panniculitis lesion and vasculitis lesion of BS subjects versus HC. The concentration of SP trended towards more in BS (active BS 39.28 pg/ml, inactive BS 33.40 pg/ml) than in normal controls (26.21 pg/ml) but findings were not significantly different. The concentration of CGRP in active BS (23.12 pg/ml) and inactive BS (19.94 pg/ml) subjects was significantly lower than that of HCs (67.09 pg/ml) [185].

A second study of BS and HC showed different findings. They found serum levels of CGRP to be higher in BS than HC, and higher in active versus inactive BS. Serum SP values and IL-8 were also higher in active BS versus inactive and disease controls [292]. Both of these studies were small (15 and 30 BS subjects respectively) making it difficult to draw a conclusion.

4.1.3 Methods of studying neuropeptides

Several mechanisms exist to study neuropeptides, the most common being to biopsy and study samples by immunohistochemistry. Given the poor wound healing experienced by BS subjects, a non-invasive mechanism is preferable.

4.1.3.1 Thermal threshold testing

Quantitative sensory testing (QST) describes quantitative neurological assessments. Proprioception, light touch and vibration sense are conveyed in large, fast –conducting fibres in the dorsal columns to the thalamus whereas pain, temperature and pinprick sensation are carried by small, slow conducting fibres in the spinothalamic tract (C and Aδ fibres). For the purpose of our investigations we were interested in small pain fibres as stimulation of these are understood to result in reflex axonal vasodilatation in response to pain. Thermal threshold testing using contact heat is a mechanism to assess small fibres without relying upon punch biopsy and has been shown to correlate with TRPV1 staining in skin biopsies [293, 294]

4.1.3.2 Indirect CGRP measurement using topical capsaicin

Capsaicin is the naturally occurring pungent compound in hot chilli peppers. It is a highly selective and potent exogenous agonist for the TRPV1receptor (transient receptor potential cation channel subfamily V member 1, also known as the capsaicin receptor and the vanilloid receptor 1) and topical application of capsaicin results in local vasodilation [295-300]. The TPRV1 receptor is a non-selective transmembrane cation channel found on small nerve fibre terminals [201] . It is activated by a wide variety of exogenous and endogenous physical and chemical stimuli. The best-known activators of TRPV1 are heat greater than 43°C, capsaicin, allyl isothiocyanate (the pungent compound in mustard and wasabi). Activation causes neurogenic inflammation and vasodilation the local CGRP release and also by the activation of dorsal root reflexes [201]. Capsaicin induced vasodilation can be blocked by CGRP antagonists [301]. Upon TRVP1 activation, depolarization results in action potentials, which propagate into the spinal cord and brain, and may be experienced as warming, burning, stinging or itching. A normal neurotransmitter is broken down or reabsorbed pre-synaptically very quickly. However, capsaicin is chemically stable and can generate a biochemical signal with a persistent effect.

Capsaicin induced increase in dermal blood flow (DBF) assessed by laser Doppler imaging is a novel phamacodynamic assay to measure CGPR activity non-invasively. Changes in blood flow can be measured by laser imaging following the topical application of capsaicin solution [297, 301, 302].

4.2 Hypothesis and aims

4.2.1 Rationale for investigating capsaicin induced blood flow in BS

We studied CGRP release indirectly by applying capsaicin topically to the forearm of volunteers and measuring the resultant change in blood flow. The primary aim was investigate if neuropeptides played an obvious role in the exaggerated cutaneous erythematous response in BS.

In addition to investigating if neuropeptides play a prominent role in pathergy, capsaicin testing offers a non-invasive, well tolerated, rapid assessment tool. No return visits are required and the whole test can be undertaken in under 2 hours. Therefore, the secondary aim of this chapter was to evaluate the potential usefulness of capsaicin induced DBF as a diagnostic test.

4.3 Results

4.3.1 Pilot study of 5 BS and 5 HC to evaluate the usefulness of thermal threshold testing

Initially 5 BS and 5 HC subjects underwent thermal threshold testing to assess if this may be a useful test to study. Following written consent, a full history and examination (including a full neurological examination) was undertaken in all participants. No neurological deficits were discovered in either group. All participants then underwent thermal threshold testing. A thermode was secured to the volar aspect of their arm and subjects were asked to press a on a mouse keypad when the thermode felt hot; painfully hot; cold and painfully cold. Subjects tended to describe the thermode as hot by 34^{o}C, and as painfully hot by 41^{o}C. Subjects experienced the thermode as cold from 28 ^{o}C. The groups differed as to their perception of cold as painful, with BS finding the thermode painful from 23 ^{o}C versus HC who experienced cold pain at 14 ^{o}C. Given the lack of overall differences, and some logistical difficulties accessing this equipment out of hours, thermode temperature testing was not undertaken as part of the main experiment.

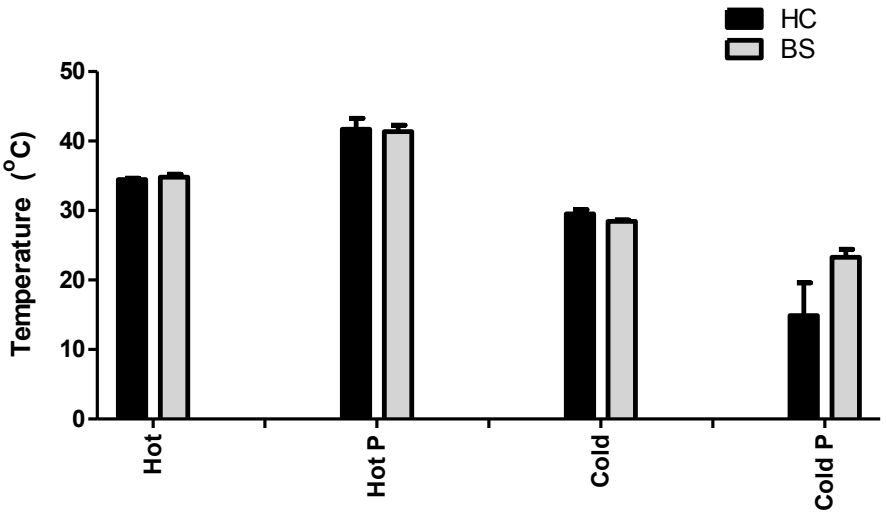


Figure 4-1. The results of thermal threshold testing in 5 BS and 5 HCs.
Results are expressed as mean with standard deviation. Participants were asked to state when they perceived the thermode to be hot, painfully hot (Hot P), cold, painfully cold (Cold P). Results were similar between the two groups.

4.3.2 Establishing a protocol for laser blood flow measurement

All preliminary capsaicin dose finding experiments were undertaken on the Perimed laser doppler machine. The aim was to evaluate if 300µg or 1000µg of capsaicin was a more effective and reliable dose to administer. The lower dose was administered to the non-dominant arm and 1000µg to the dominant arm in 4 BS and HC. Images were taken at 10 minute intervals over the next 2 hours to form a picture of the dynamics of the reaction. The total flux and the area under the curve were the values calculated. Several subjects produced no response or a minimal response with 300µg dose. In contrast the 1000µg dose was found to be a more reliable dose and was chosen for future experiments.

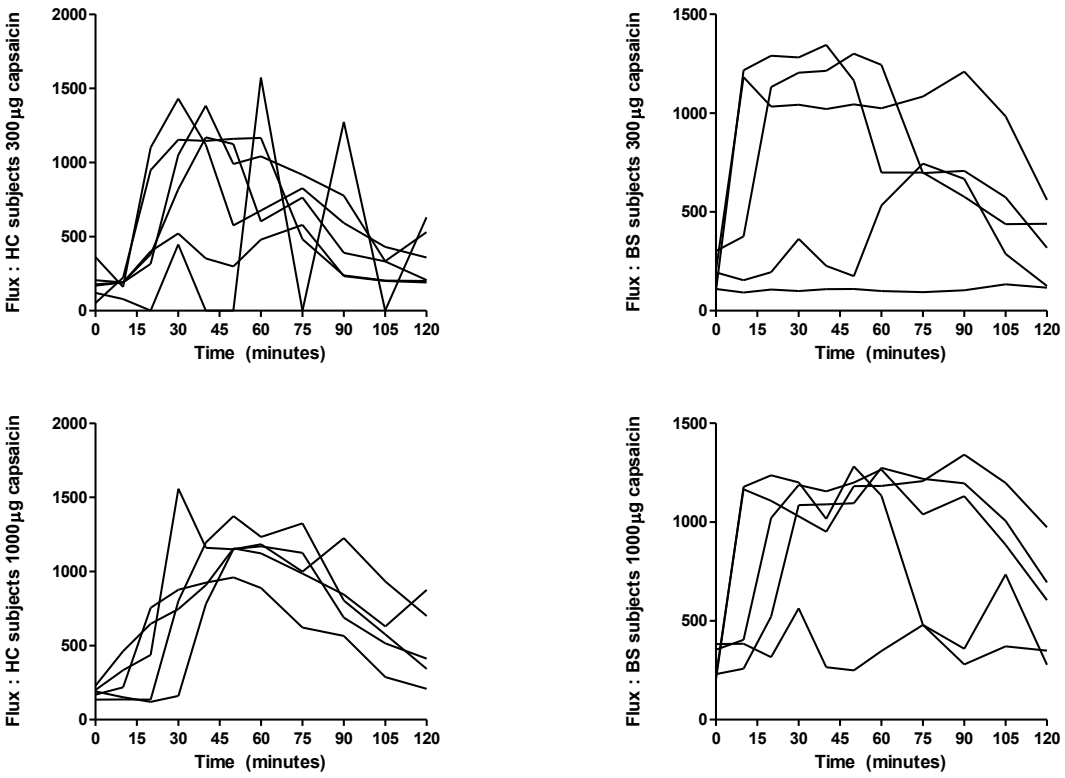


Figure 4-2. The preliminary dose finding experiment showing doppler flux values in HC and BS subjects following the application of either 300µg (top row) or 1000µg (bottom row) of capsaicin.

The higher dose elicited more robust results and was selected for future experiments.

4.3.3 Perimed Laser Doppler Imager versus Moor Laser Speckle Photometry Imager

During preliminary experiments we noted that the threshold of the Perimed laser doppler had to be set at very high setting to exclude background noise (6.4 out of 10) and therefore although output values were accurate, images were of low quality. We had the opportunity to use a Moor Laser Speckle Photometry scanner (Moor Imager) on loan from Moor Instruments Ltd. which produced images of high quality, and therefore the main experiment was undertaken using the newer machine.

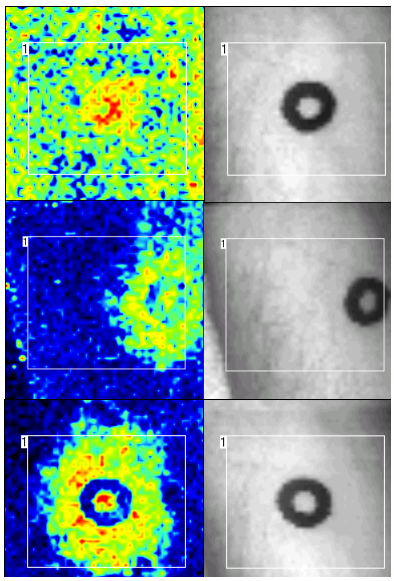


Figure 4-3. A typical image obtained by the Perimed Imager.

Following application of capsaicin increased blood flow can be seen in the area surrounding the rubber O ring over time. The images were deemed of low quality relative to the Moor imager (below).

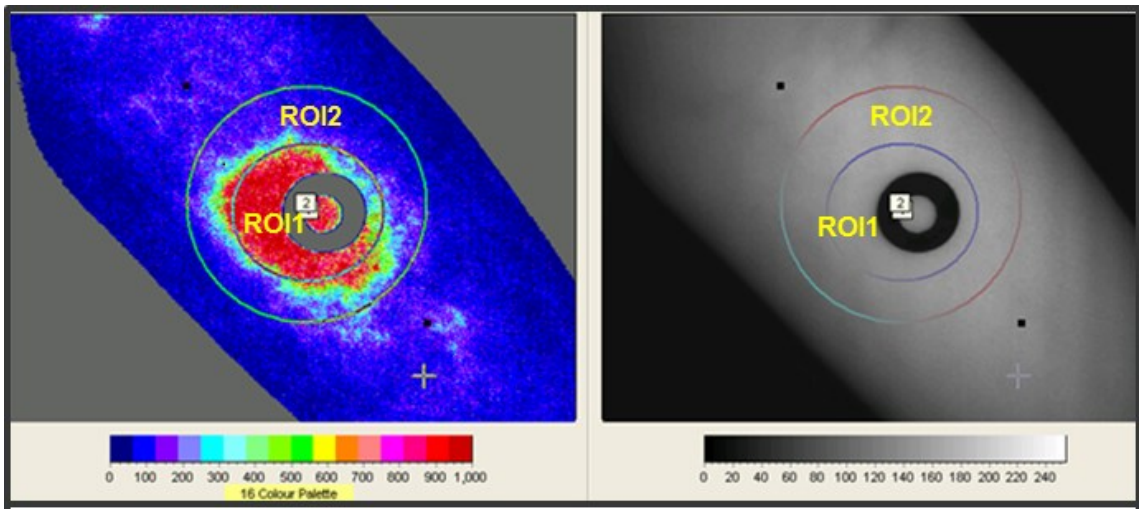


Figure 4-4. A typical image obtained by the Moor Imager.

This newer machine produced images of impressive quality. This image also highlights examples of gating regions of interest (ROIs).

4.3.4 Establishing a final protocol for the use of capsaicin

13 HC and BS subjects were recruited for this study. All were asked to abstain from alcohol, caffeine and all non-essential medications prior to testing, especially pain killers and antihistamines.

Following written consent, baseline scanning was performed. Then 20µL of the liquid containing 1000µg capsaicin was applied 22cm proximal to the wrist crease on the volar aspect of the dominant forearm. The imager recorded at 10 second intervals. Subjects were recorded for three 20minute periods with 15 minute breaks in-between. This allowed us to measure the initial speed of onset of the flare, the peak of the flare and assess the degree of resolution of the response by 90 minutes.

Output measures were (i) the mean point flux within areas of interest (ROI1 and ROI2), and (ii) the pixel count within in the entire image after subtracting pixels with values below a cut-off of flux intensity (threshold set at 200 or 500 flux units (PU)). ROI1 was a circular area, which was twice the size of the rubber O ring. ROI2 was a circular area 3 times the size of the rubber O ring. These AOIs were chosen as the O ring was a fixed value on all images making comparison between subjects straight forward.

4.3.5 Interpretation of results

Flux (PU) represents blood flow values. The scale is arbitrary because it is impossible to be precise about the sample volume. The scale is 0 to 2000 on the Perimed laser doppler and 0-1000 on the Moor imager. The scale is linear – so 500PU represents twice the flow of 250PU. Data may be presented as total flux or as a % change. A typical image is seen below (Figure 4-5). We expressed results as the % increase from baseline values. We chose a small ROI (ROI1) to evaluate the intensity of the response and a larger ROI (ROI2) to form an impression about the lateral spread of the response.

An alternative method to assess the flare response is to calculate the number of pixels above the baseline pixel threshold number (Figure 4-6). By selecting a low threshold (e.g. 200 PU) one gets an idea of the lateral spread of the flare response, and by selecting a high threshold (e.g. 500 PU) one gets an impression of the intensity of the flare response.

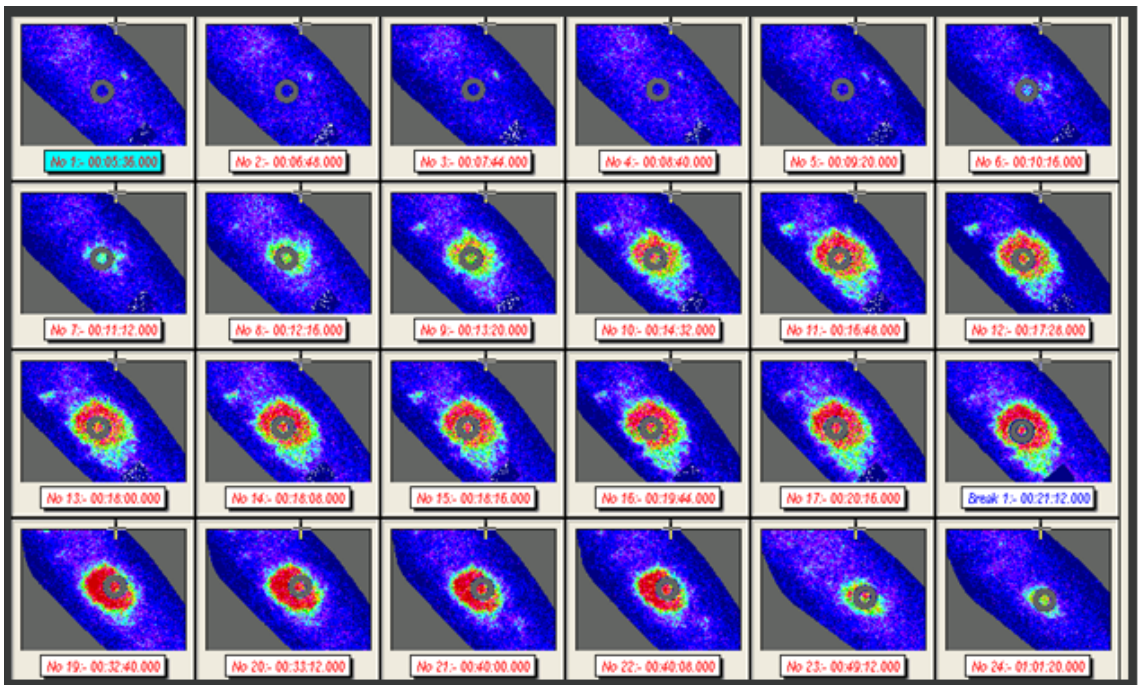


Figure 4-5. A sample of Moor images following the application of capsaicin into the rubber O ring.

Images are obtained over 90 minutes. The mean flux (PU) within regions of interest are calculated for each time point to assess changes in local blood flow over time. This image shows the flux values.

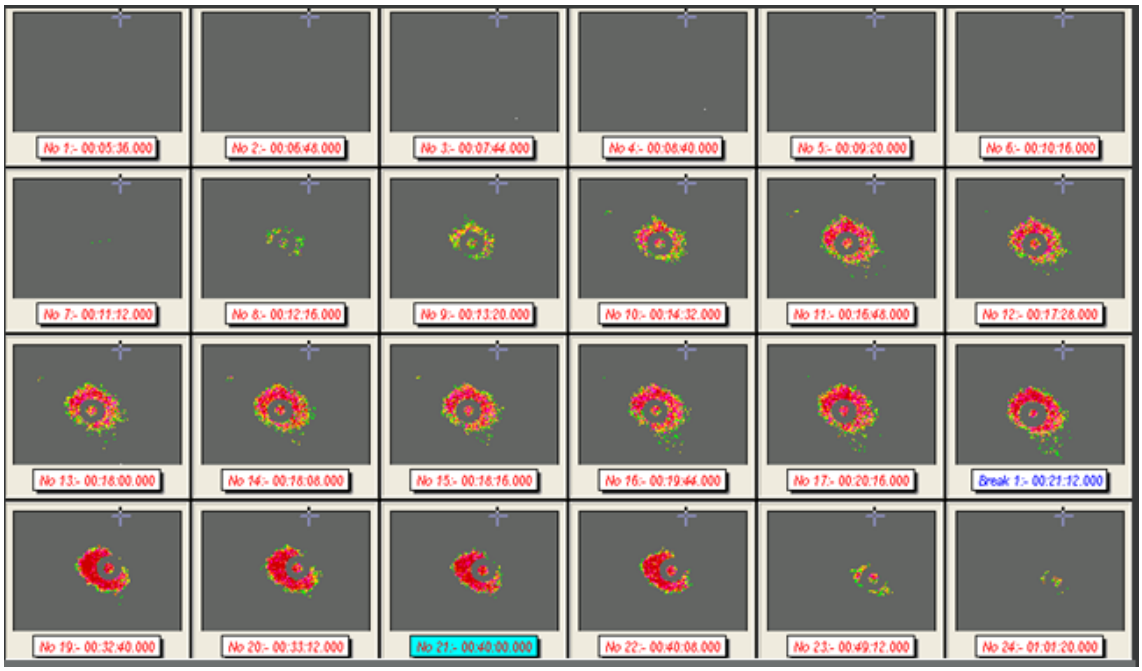


Figure 4-6. A sample of Moor images following the application of capsaicin into the rubber O ring.

Images are obtained over 90 minutes. An arbitrary threshold is set of 500 flux units in these images, meaning that areas of low flux are excluded, allowing areas of high flux (and high blood flow) to be quantified. The pixels remaining in the image are counted.

4.3.6 Study cohort

We recruited 13 HC and 13 BS subjects. Demographics of the recruits are shown below. Six of the HC were male (46%) versus 7 of the BS subjects (55%). The median age in the HC group was 33 years (interquartile range 27-44) versus 46 years (interquartile range 33-51) in the BS group. Nine of the BS group were taking steroids (prednisolone). Of those 9, the median dose was 5mg. BS subjects were also taking a variety of other disease modifying medications. The median disease activity using the Leeds questionnaire was 3 (scale of 0-12) indicating mild/low disease activity. In the HC group, all subjects were in good health and taking no regular medications.

General Demographics	**Healthy Controls**		**Behçet Syndrome**	
Cohort size	13		13	
Median age (Interquartile range)	33	(27-44)	46	(33-51)
Male sex (%)	6	46%	7	54%
Clinical Characteristics				
Mucocutaneous	0		13	100%
Erythema nodosum			8	61%
Pathergy/poor wound healing			11	85%
Musculoskeletal – arthralgias	0		10	77%
Arthritis			5	38%
Cardiovascular	0		5	38%
Aneurysm			1	7%
Thrombosis			4	31%
Ocular	0		5	38%
Posterior/panuveitis	0		3	23%
Gastrointestinal inflammation	0		2	15%
Neurological	0		4	31%
Disease Activity Score *	0		3	
Current medications				
Colchicine	0		1	7%
Azathioprine	0		6	46%

Mycophenolate	0	3	23%
Anti-TNF therapy	0	4	31%
Cyclosporine	0	0	
Methotrexate	0	2	15%
Steroids **	0	9	(5mg)

*** Leeds Behçet's disease activity form (2002 version) score of 0-12 (>4 indicates active disease).**

**** Number of subjects taking steroids, and the median prednisolone dose taken by those subjects**

Table 4-1. Demographics of BS and HC subjects recruited for capsaicin skin blood flow testing.

4.3.7 Change in blood flow within 'Region of interest 1' (ROI1)

In each data set, a region of interest was defined as a circle twice the size of the rubber O ring (ROI1). The mean flux within this region was recorded at baseline and over time (0-90 minutes). Results were expressed as the % increase from baseline flux. Over a 90 minute period results increased then returned to near baseline, allowing an area under the curve (AUC) to be calculated. The ROI1 is a small area, and the results reflect the intensity of the flare response over time.

Figure 4-7 shows the mean results of % increase in flux over time (90minutes) for all HC and BS subjects. The two graphs show a significant variance (F 2.09, DFn 137, and $P < 0.0001$). Interestingly it was the HC cohort that showed the greater increase in blood flow. We then calculated the area under the curve for each individual (Figure 4-8) but this failed to show a significant difference between the two cohorts. The trend remained for BS to show a smaller increase in blood flow versus HCs.

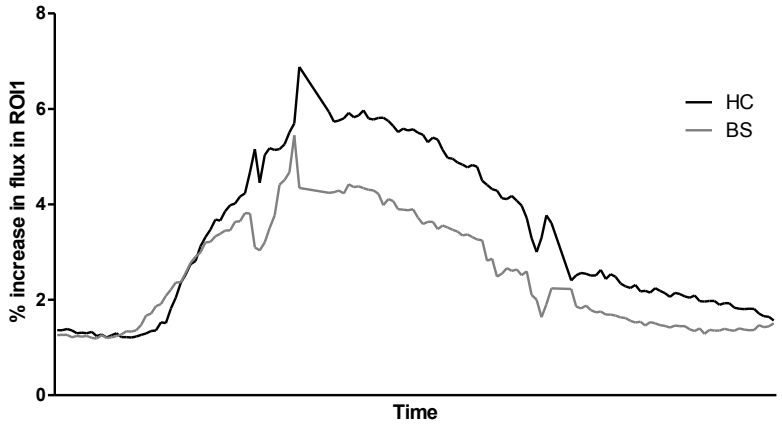


Figure 4-7. The mean values of HC and BS for % increase in flux over time within ROI1.
The HC cohort show a significantly greater % increase versus BS (P<0.0001).

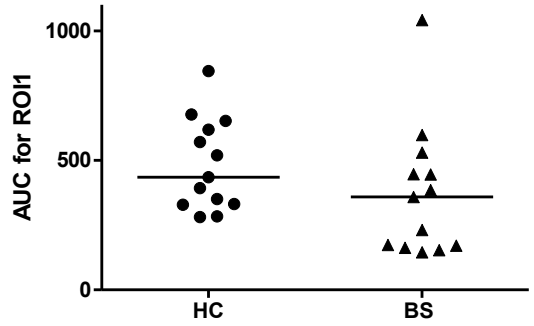


Figure 4-8. The percentage increase in mean flux from baseline within ROI1 was calculated for each subject at each time point allowing an area under the curve (AUC) value to be calculated.

There was a trend towards a lower change in AOC in BS, indicating less increase in blood flow following capsaicin administration. This finding was not statistically significant (Mann-Whitney P=0.12).

4.3.8 Change in blood flow within 'Region of interest 2' (ROI2)

In each data set, a region of interest was next defined as a circle three times the size of the rubber O ring (ROI2). The mean flux within this region was recorded at baseline and over time (0-90 minutes). Results were expressed as the % increase from baseline flux. Over a 90 minute period results increased then returned to near baseline, allowing an area under the curve (AUC) to be calculated. The ROI2 is larger than ROI1 and therefore results give an impression of the lateral spread of the flare response.

Figure 4-9 shows the mean results of % increase in flux over time (90minutes) for all HC and BS subjects. The two graphs show no statistically significant differences. Interestingly again it was the HC cohort trended towards showing a greater increase in blood flow. We then calculated the area under the curve for each individual (Figure 4-10) but this also failed to show a significant difference between the two cohorts. The trend remained for BS to how a smaller increase in blood flow versus HCs.

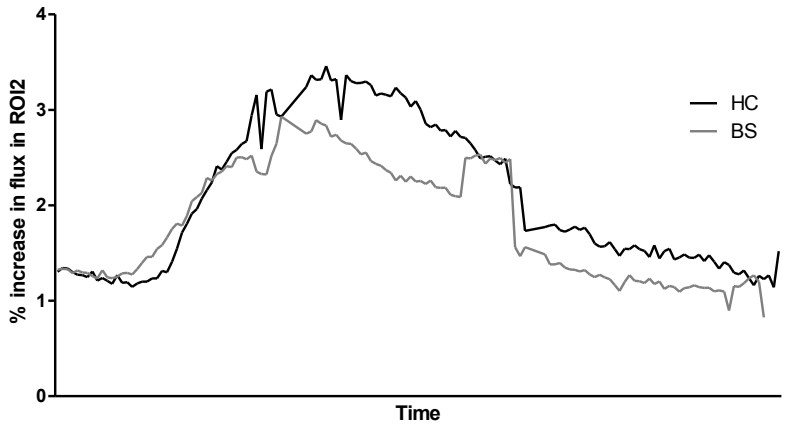


Figure 4-9. The mean values of HC and BS for % increase in flux over time within ROI2. The two groups show no significant variance although the trend remains for a smaller increase in blood flow in the BS cohort.

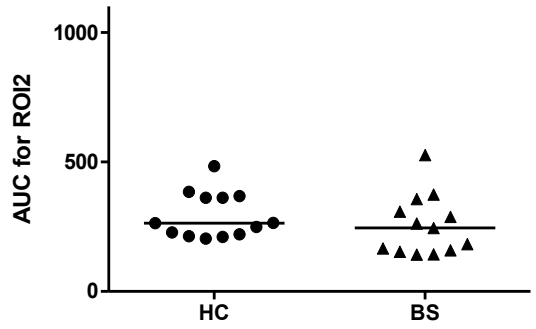


Figure 4-10. The percentage increase in mean flux from baseline within ROI2 was calculated for each subject at each time point allowing an area under the curve (AUC) value to be calculated.

There was a trend towards a lower change in AOC in BS, indicating less increase in blood flow following capsaicin administration in BS. This finding was not statistically significant (Mann-Whitney P=0.34).

4.3.9 Flare response assessment using total pixel counts

An arbitrary threshold may be set which eliminates pixels below a specified flux intensity (example shown in Figure 4.6). This allows areas of low flux to be excluded, and areas of high flux (and hence high blood flow) to be quantified. The pixels remaining in the image are counted. We arbitrarily choose two cut off thresholds of 200 and 500 flux. A threshold of 200PU does not exclude most areas with high blood flow and therefore allows for an estimation of the lateral spread of the flare response. A 500PU threshold excludes all but the most intense pixels, and therefore allows for an estimation of the intensity of the blood flow.

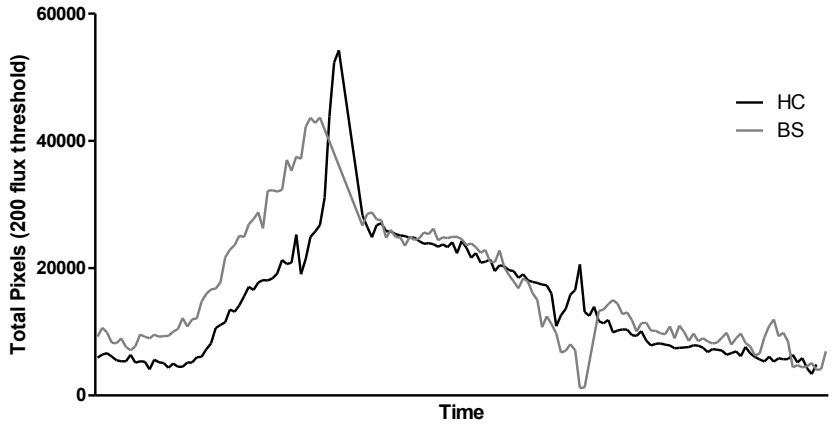


Figure 4-11. The mean total pixel counts over time in HC and BS cohorts.
The BS cohort appear to flare early but the HC reach a greater peak. There was no statistically significant variance between the two curves.

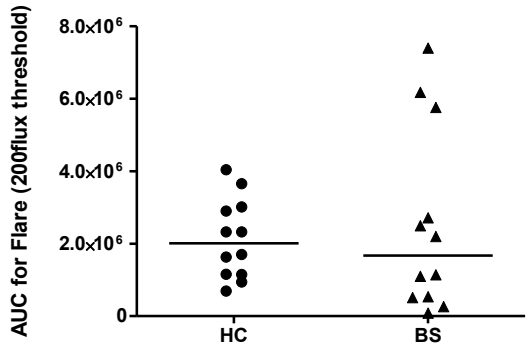


Figure 4-12. The total pixel counts over time were plotted and an area under the curve (AUC) was calculated for each subject.
There was a trend towards fewer total pixels in the BS cohort following capsaicin administration. This finding was not statistically significant (Mann-Whitney P=0.58).

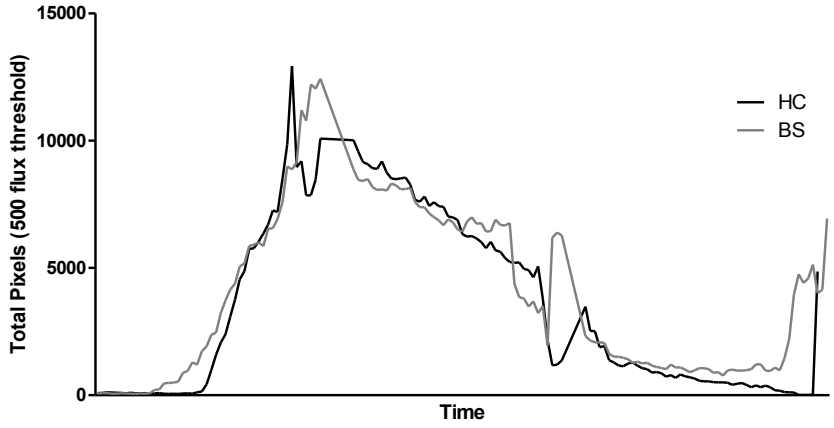


Figure 4-13. The mean total pixel counts over time in HC and BS cohorts.

For this analysis a threshold of 500 flux units was set. The number of pixels above this threshold were counted. The HC and BS traces of total pixels over time overlap indicating no difference between the groups. As 500 flux units is a high threshold, this result reflects the intensity of the flare response.

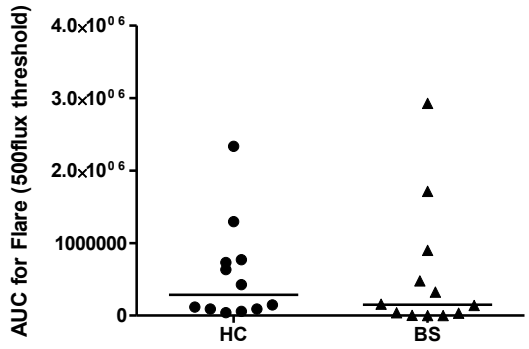


Figure 4-14. The total pixel counts over time were plotted and an area under the curve (AUC) was calculated for each subject, using a threshold of 500 flux units.

There was a trend towards fewer total pixels in the BS cohort following capsaicin administration. This finding was not statistically significant (Mann-Whitney P=0.40).

4.4 Discussion

In Chapter 3, we found that cantharidin blisters displayed an erythematous halo that was not easily explained by any obvious differences in immune cells or cytokines. This led us to investigate neuropeptides as a possible contributing agent.

As BS flare ups may be triggered by stress, it seems plausible that neuro-immune interactions may be important in this syndrome. Furthermore, neuropeptides play a role in cutaneous

vasodilation and in inflammation. We found previous publications regarding neuropeptide levels in BS subjects showed conflicting results, with one paper finding high CGRP in serum and the other finding lower concentrations [185, 292]. We decided to study CGRP in this chapter as it may be readily investigated non-invasively through the topical application of capsaicin and measurement of the subsequent cutaneous vasodilation. We also hoped that the capsaicin test may offer a potential diagnostic test for BS.

4.4.1 BS capsaicin flare responses appeared to be smaller than that of HC

BS subjects who experience pathergy tend to describe an exaggerated response to any stimuli, from thorn pricks and nettle stings to surgical cuts and MSU injections. Therefore we were surprised when, contrary to our expectations, the BS cohort did not mount an exaggerated flare response to capsaicin. In fact the BS cohort displayed a smaller flare response than HCs. This experiment therefore did not find evidence of an exaggerated flare in BS subjects and did not find the capsaicin skin test to be of any diagnostic use.

4.4.2 Study limitations

Possible reasons for the smaller flare response found in the BS cohort are either study limitation factors (a false negative) or else that BS subjects do release less CGRP upon stimulation (a true positive). Study limitations include (i) the small sample size: however all trends went in the same direction of the BS cohort having a smaller flare response; (ii) the BS cohort chosen: 85% of the BS cohort reported experiencing symptoms suggestive of the pathergy phenomenon, and in this respect were representative for the syndrome; (iii) medications: All of the BS subjects were on immunosuppressive medications and 9 were taking prednisolone. However, the median dose of prednisolone was only 5mg, and subjects were receiving a wide variety of medications making an effect of a particular medication small. All subjects in both groups were asked to abstain from painkillers, caffeine and alcohol prior to the study. These substances are known to interfere with neuropeptide release.

4.4.3 Reasons why it may be plausible that BS subjects secrete less CGRP than HCs

The findings of this chapter may reflect a true representation of CGRP release in BS. CGRP differs from SP in that, in addition to vasodilation, there is evidence of it having anti-inflammatory properties. As discussed in detail in the introduction to this chapter, CGRP leads to less antigen presentation, inflammatory cell recruitment and also directly stimulates IL-10 release. A SNP in IL-10 which results in less IL-10 protein production in the disease-associated-allele was discovered in the first two GWAS studies of BS [5, 6]. Less IL-10 may slow down the resolution phase of inflammation, and therefore it is possible that if BS subjects secrete less GCRP in response to insults such as MSU injections, then this may result in less activation of or slower activation of cutaneous anti-inflammatory pathways leading to a prolonged cutaneous inflammatory response, as seen following MSU injections.

4.5 Future work

Capsaicin is chemically stable and can generate a biochemical signal with a persistent effect. The TRPV1 channel is highly calcium permeable which allows significant amounts of calcium to flow down its steep electrochemical gradient into nerve fibres. TRPV1 is also expressed on intracellular organelles and prolonged external capsaicin application (e.g. with a capsaicin skin patch) results in release of calcium from endoplasmic reticulum and induction of additional intracellular calcium release from internal stores via calcium dependent calcium release. Sustained high levels of intracellular calcium activate calcium dependent enzymes such as proteases and can induce depolymerisation of cytoskeletal components such as microtubules. Moreover, osmotic swelling due to the chloride accumulation that accompanies influxes of positively charged ions also occurs. An additional effect of high concentrations of capsaicin, is a direct inhibition of mitochondrial respiration. Numerous mitochondria are present in peripheral terminals of nociceptors and may congregate there in response to nerve growth factor. In this setting mitochondria will release pro-apoptotic factors such as cytochrome C. As all of this is happening in the nerve cell process rather than in the nucleus, this results in localized

degeneration and apoptosis in a nuclear free zone. All of this leads to impaired local nerve function for extended periods [297, 303]. Topical low strength capsaicin formulations have been widely used for neuropathic [304]and musculoskeletal [305] pain management since the 1980s.

If CGRP was truly involved in the observed differences in the ID MSU response between HC and BS, then it would be expected that the MSU response would be equalized in both groups if it was repeated on a skin site that had recently been treated with a prolonged application of capsaicin. In this instance neuropeptide release would be abrogated in both groups and it may be possible that in this case HCs showed a result similar to their BS counterparts. It was beyond the timeframe of this research project to undertake this further experiment.

5. Exploring the IFN-γ–Chemokine pathway in BS *in vitro*

5.1 Context

In chapter 1 we reviewed inflammation in BS and found among other things, that a Th1 skew and IFN-γ signalling had been implicated. In Chapter 3 we used a cantharidin skin blister model of inflammation in an attempt to dissect the abnormal cutaneous inflammatory response of BS. A chemokine CXCL10 was noted to be potentially interesting because lower levels were noted in BS versus HC. While this finding was no longer statistically significant once corrections were made for multiple comparisons we felt it was worth investigating given that it is one of the 3 IFN-γ inducible chemokines. Throughout all *in vivo* experiments, blood samples were also collected for *in vitro* work. The beginning of this chapter introduces IFN-γ signalling pathways, IFN-γ induced chemokines, and provides a brief review of mechanisms of translational control, as these are relevant to the results.

5.2 Background

5.2.1 Interferons

Interferons (IFN) are ubiquitous cytokines produced by immune cells in response to infection by a DNA or RNA virus. There are three major classes. Type I IFNs in humans consist of 13 IFN-αs, IFN-β and others. They are encoded by 17 non-allelic genes that lack introns and are located on chromosome 9 in humans. These IFNs are glycosylated proteins containing 160-200 amino acids and sharing 30% homology. They can usually be produced by all cell types. Stimulation via the TLR receptors including TLR 3, 4, 7 and 9, leads to IFN I secretion. IFN binds to ubiquitous surface receptors composed of 2 membrane spanning proteins IFNα R1+2 that form a ternary complex with the ligand which leads to activation of intracellular signalling involving

the Janus kinase 1 (JAK1) and tyrosine kinase (TYK2) pathways and STAT1/STAT2-IRF9 pathways of intracellular signalling with the resultant activation of the IFN-stimulated response element (ISRE). Type II is IFN-γ, and is mainly produced by NK cells and T cells and is discussed in detail below. Type III consists of 3 IFN λ molecules, also known as IL-28A, B and IL-29. They are co-produced with IFN-β but act at different receptors, the IFNLR1 and IL-10R2. Each type is characterized by a specific receptor and signal transduction pathway and is subject to strict regulation. The main role of IFNs is in co-ordinating an anti-viral response, but they also play a role in defence against bacterial infections and TB, decreasing tumour growth, and angiogenesis. Type I interferons are considered players in innate immunity, and Type II are associated with adaptive immunity [306]. They are implicated in several autoimmune diseases including SLE, Sjogren's syndrome, Rheumatoid Arthritis, Polymyositis and Systemic Sclerosis[307]; The properties of interferons are used to treat a number of viral infections (e.g., hepatitis B and hepatitis C), inflammatory diseases (interferon beta for multiple sclerosis and interferon gamma for systemic sclerosis), and malignancies [308].

5.2.1.1 Type II interferons

There is only one type II interferon, IFN-γ. It is produced by dendritic cells during the innate immune response, under the influence of IFN Is and IL-12. It is produced by NK cells, NK T cells and cytotoxic Th1 cells that are stimulated via their respective activating receptors (e.g. IL-12R – JAK2/TYK2-STAT4 pathway) [309]. The IFN-γ protein is composed of 140 amino acids and shares no homology with type I IFNs.

5.2.1.2 JAK –STAT signalling

The IFN-γ receptor is distinct from the type I receptor. It is a heterodimer of 2 membrane spanning proteins, IFN-γ R1+2, of which the first is constitutively expressed on all cell types and the second is tightly regulated and expressed on a narrower spectrum of cell types [310]. Conventional IFN-γ signalling follows the binding of IFN-γ to its cell surface receptors, resulting in oligomerization of the receptor. The Janus kinase (JAK) family of kinases JAK1 and JAK2 are pre-associated with IFNγR1 and IFNγR2 respectively, and receptor

oligomerization brings these kinases close together, allowing them to trans-phosphorylate each other and the cytoplasmic domains of the receptors. Tyrosine phosphorylation of IFNγR1 (on residue 440) provides a docking site for the SH2 domain of the STAT1 transcription factor. Docked STAT1 is phosphorylated in the C-terminus (on tyrosine Y701) [311], enabling a homodimer of tyrosine phosphorylated STAT1 to form and dissociate from the receptor. A second independent C-terminal phosphorylation on the serine residue S727 occurs following IFN-γ stimulation [312]. This phosphorylated STAT1 homodimer translocates to the nucleus and initiates transcription of genes containing a gamma activated sequence (GAS) [313] in their promoter region. STAT1 S727 phosphorylation has no impact on the formation of the STAT1 homodimer, nuclear translocation and DNA binding [314]. However, S727 phosphorylation is important for STAT1 function because STAT1-mediated transcription is markedly attenuated in its absence [312]. This serine phosphorylation occurs by a non-JAK mediated mechanism, and several kinases have been shown to be involved, such as MAPK, discussed below.

Further complex inhibition and enhancement of IFN generated signals occurs including through the MAPK, PI3Kinase, CaMKII and NF-kB pathways discussed below. Additionally perimembranous heparan binding influences the activation and local concentration of IFN-γ.

5.2.1.3 Non JAK1/2-STAT1 signalling

Other STATs have been shown to play a lesser role in IFN-γ signalling. STAT3 and STAT5 have been shown to be activated to some extent by IFN-γ [315, 316]. STAT2 has also been found to be very weakly activated by IFN-γ [317]. As mentioned above, STAT1 undergoes serine kinase (S727) phosphorylation in addition to the tyrosine kinase phosphorylation [318]. This may be mediated by phosphatidylinositol-3'kinase (PI3'K)-AKT [319], Protein kinase Cepsilon (PKC) [320] or MAPK [321] pathways. In addition to phosphorylating STAT1 on S727, these kinases activate other molecular pathways related to IFN-γ signalling [322].

5.2.1.4 The role of phosphatidylinositol (PI) 3'K-AKT-mTOR pathway in control of ISGs.

The PI3'K-AKT-mTOR pathway is an intracellular signalling pathway, typically activated by the Insulin-like growth factor – 1 (IGF-1), and has a number of downstream effects which either promote protein synthesis or inhibit protein breakdown. Both Type I and II IFNs activate the PI3'K-AKT-mTOR pathway [323]. IFN-γ activates PI3-K equally well in cells lacking JAK1 or 2 [319, 323]. The PI3'K kinase plays an important role in mediating gene transcription in response to both Type I and II IFNs and also regulates the translation of ISG15 and CXCL10 proteins, upon treatment of cells with IFN-α or IFN-γ, respectively [324].

Although AKT does not appear to play a role in mediating the IFN-dependent activation of STAT1, protein expression of CXCL10 by IFN-γ is attenuated in the absence of the Akt1 and Akt2 [323]. Both Type I and II IFN-mediated engagement of mTOR leads to hyperphosphorylation of translational regulation protein 4E-BP1 [325]. The non-phosphorylated form of 4E-BP1 binds to the initiation factor eIF4E and prevents its inclusion in the eIF4F initiation complex thereby inhibiting translation imitation. Phosphorylation of 4E-BP1 by mTOR prevents the interaction of the protein with eIF4E and thereby enhances mRNA translation [326]. 4E-BP1 deficient mouse embryonic fibroblasts have an enhanced protein expression of CXCL10 upon treatment with IFN-γ [327].

In addition to regulation of 4E-BP1 activity, IFN-signalling via mTOR leads to the activation of the p70 S6 kinase (S6K) and phosphorylation of the ribosomal protein S6 (RPS6) [326]. RPS6 may play a role in translation of terminal oligopyrimidine (TOP) mRNAs and may therefore play a role in regulating the translation of certain ISGs (TOP mRNAs are transcripts which encode for ribosomal proteins and eIF1α and eIF2) [328]. The mTOR/S6K pathway is also known to regulate the phosphorylation of the eukaryotic initiation factor 4B (eIF4B) [328] which can facilitate translation [329]. eIF4B is also known to be phosphorylated by the p90 ribosomal protein S6K (RSK), a downstream effector of the MEK/ERK pathway [322, 330].

5.2.1.5 The role of Mitogen-activated protein kinases (MAPK) in control of ISGs.

MAPK are serine and threonine kinases and are classified into three main categories: the extracellular regulated kinases (ERK1-7), the stress activated p38 family (p38a-d) and the c-Jun N-terminal kinases (JNK1-3). Type I IFNs and IFN-γ activates p38 MAPK [331, 332]. It is not known if engagement of the p38 MAPK pathway plays a role in mRNA translation of ISGs, its major role in the induction of IFN-biological responses appears to be related to regulation of transcriptional activation [322].

In addition to the p38 MAPKs, type I IFNs and IFN-γ are also known to activate the ERK1/2 MAPKs [333]. This pathway is classically activated by epidermal growth factor (EGF) binding to the EGF receptor (EGFR) in the cell membrane and the pathway consists of the Ras/Raf/MEK/ERK signalling cascade. The role of the MEK/ERK pathway in the regulation of the anti-proliferative effects of IFNs is variable in different systems. IFN-mediated activation of ERK1/2 plays an essential role in RSK-dependent engagement of the translation initiation factor eIF4B.

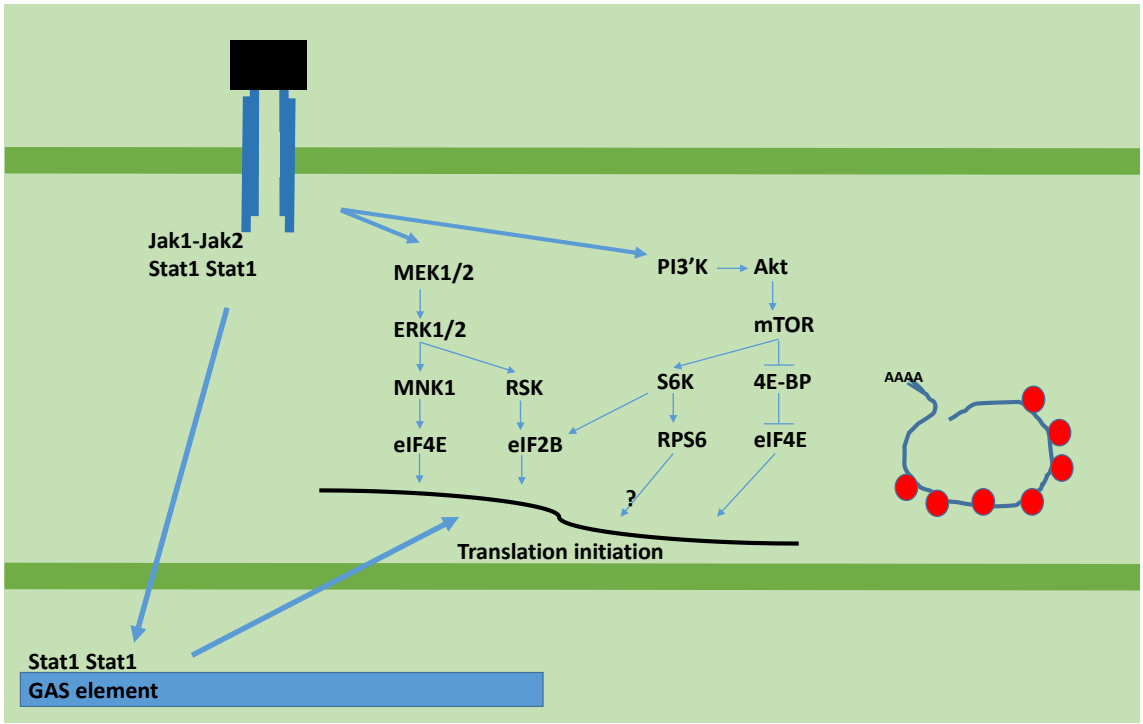


Figure 5-1 Proposed model for the control of mechanisms of mRNA translation of ISGs.

IFN-γ binds to its receptor leading to the formation of STAT1 dimers that translocate to the nucleus and initiate transcription via GAS elements. The expression of ISGs is then regulated at the level of mRNA translation. IFNs also activate the PI3'K pathway ultimately leading to the phosphorylation of 4E-BP1 and activation of S6K. Phosphorylated 4E-BP1 leads to reduced inhibition of translation. Activation of S6K leads to the phosphorylation of eIF4B which also positively regulates mRNA translation. IFN mediated engagement of the MEK-ERK pathway leads to the activation of Mnk1 and its downstream target eIF4E further promoting the translation of ISGs. Depending on the cell type, the MEK-ERK pathway also leads to the phosphorylation of eIF4B by RSK, again enhancing the translation of ISGs.

5.2.1.6 Heparan sulphate binding

IFN-γ binds directly to glycosaminoglycan heparan sulphate (HS) on cell surfaces. The binding of IFN-γ to HS limits the extent of its C-terminal domain degradation which strongly increases the cytokine activity, in contrast to free IFN-γ which is rapidly inactivated by extensive C-terminal processing. This mechanism means that IFN-γ is rapidly captured and activated by HS in the vicinity of the secreting cells, ensuring a localized action of the cytokine, while unbound IFN-γ is inactivated, thereby preventing unwanted cell stimulation by diffusing cytokines [334].

5.2.1.7 IFN-γ activates transcription factors other than STAT1

Although the STAT1 homodimer is the primary and best characterized route to IFN-γ induced transcription, IFN-γ additionally activates other transcription factors, that fall into three categories: (i) those transcription factors that are activated downstream of STAT1, i.e. they are transcriptional targets of STAT1 and therefore represent secondary responses (''STAT1 dependent''), e.g. IRF-1; (ii) those transcription factors that form a protein complex with STAT1 on gene promoters to augment IFN-γ mediated transcription (''STAT1 co-operative''), e.g. ISGF3, STAT1/IRF9 and STAT1/c-Jun; and (iii) those transcription factors that can be activated independently of STAT1 (''STAT1-independent''), e.g. NF-kB and AP-1[318]. The interferon regulatory factor (IRF) family of transcription factors are important IFN-γ factors, the most important being IRF-1 and IRF-8 which have the capacity to bind to ISRE and other

IFN regulated response elements in the promoter of genes and initiate the transcription of both ISGs and the IFNs themselves[335].

5.2.1.8 Inducers of IFN production

Viruses, some bacterial, bacterial lipopolysaccharide (LPS) (TLR4) can induce type I IFNs. The production of IFN-γ is stimulated by several lectins that are mitogenic for T cells, such as phytohemagglutinin (PHA), double stranded RNA (TLR3), single stranded viral RNA (TLR 7,9) and hypomethylated bacterial or viral DNA via TLR9. Other receptors that respond include helicases containing a caspase recruitment domain (CARD) and known as RIG-1 and MDA5 [336]. Transduction from the TLRs require either junction proteins such as MyD88 and TRAF6 or TRIF and transcription factors belonging to the IFN regulatory factor (IRF) family such as ITFR3 for TLR3/4 . Several studies point to involvement of specific allelic IRF5 variants in SLE where IFN-α has been implicated [337].

5.2.1.9 Immunological effects of IFNs

IFN signatures include genes encoding cytokines and chemokines, membrane receptors, signal transduction proteins, growth factors or apoptosis factors, antimicrobial proteins, adhesion molecules [338]. Th1 cell maturation is influenced by IFN-γ via the T-box transcription factor for TBX21 (T-Bet) [339], and this in turn increases IFN-γ by activating STAT4. IFN-γ increases the expression of Class II HLA molecules and promotes ThCD4+ response. It also increases the expression of Class I HLA molecules, antigen priming and antigen presentation. Other effects include effector cell recruitment to sites of inflammation via the production of chemokines and chemokine receptors, stimulation of the macrophage oxidative burst via enhanced expression of iNOS and NADPH oxidase [340, 341].

5.2.2 Chemokines

Chemokines are low molecular weight cytokines whose major biological activity is that of chemotaxis of both specific and overlapping subsets of leukocytes. They play a critical role in the directed movement of leukocytes from the bloodstream into tissue. They are almost

exclusively secreted and act as extracellular messengers for the immune system. However, emerging data also show that various members of the chemokine gene superfamily exert other biological effects outside the immune system [342].

CX3CL chemokines are subdivided into two categories, those with a motif of glutamic acid-leucine-arginine (ELR motif) immediately before the first cysteine of the CXC motit, and those without. ELR-positive CXC chemokines specifically induce the migration of neutrophils, and interact with chemokine receptors CXCR1 and CXCR2. They are pro-angiogenic. An example of an ELR-positive CXC chemokine is interleukin-8 (IL-8). CXC chemokines that lack the ELR motif include those induced by IFN-γ CXCL9, CXCL10 and CXCL11 tend to be chemoattractant for lymphocytes and are anti-angiogenic [343-345].

Members of the chemokine gene superfamily can be roughly divided into two main categories based on functional expression—the inducible and the constitutive chemokines. The inducible chemokines participate primarily in inflammation responses and are the bulk of the members of the supergene family. The constitutive chemokines are expressed primarily in secondary lymphoid organs and appear to play a major role in lymphocyte homing. All chemokines exert their effects via ligation of cell surface receptors, which are G protein-coupled seven transmembrane domain receptors. Few receptors that bind only one ligand have been identified. However, chemokine subfamily integrity is maintained, in that binding to receptors generally occurs for chemokines within one subfamily. An exception to this rule is the Duffy antigen receptor for chemokines (DARC), a receptor expressed on erythrocytes and endothelial cells that binds chemokines from both the CXC and the CC subfamilies and is also used as a binding site for the malarial parasite Plasmodium vivax [346]. Ligation of chemokines to their receptors leads to the generation of second messengers and activation of effector enzymes [342].

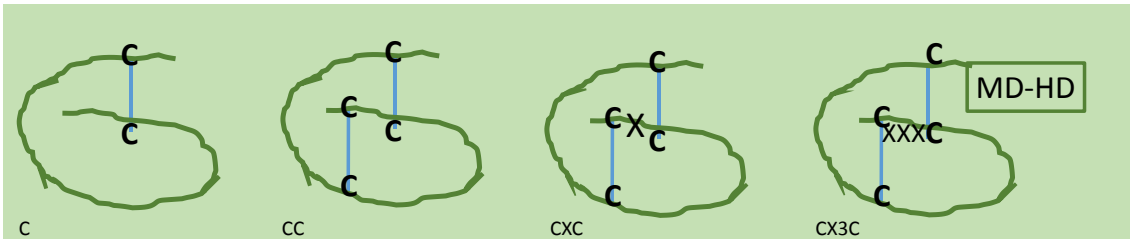


Figure 5-2. Members of the chemokine superfamily.

Schematic representation of the cysteine signature motifs with disulphide bonds. The X represents an amino acid separating the cysteine residues. The CX3C chemokine also contains a mucin domain (MD) and a hydrophobic domain (HD).

CXCR3 is activated by the three IFN-γ inducible ligands CXCL9, CXCL10 and CXCL11 [347]. CXCR3 is rapidly induced on naıve T cells following activation, and preferentially remains highly expressed on Th1 CD4+ T cells, effector CD8+ T cells and innate-type lymphocytes, such as NK and NKT cells. Some studies have revealed that these ligands have redundant functions *in vivo*, other studies have demonstrated that the three CXCR3 ligands can also collaborate and even compete with each other. Differential regulation of the three ligands at specific times in defined anatomically restricted locations *in vivo* likely participates in the fine control of T-cell trafficking over the course of an immune response [348]. Unique promoters control the distinct pattern of expression of each CXCR3 ligand. The CXCL10 and CXCL11 promoters contain a functional IRSE and nuclear factor kappa B1 (NF-kB1) element, whereas the CXCL9 promoter contains an IRE-1 element and a weak NF-kB2 element [342]. For CXCL11 expression, a STAT3–STAT1 heterodimer, rather than the STAT1–STAT2 heterodimer, binds to the CXCL11 promoter following IFN stimulation. In addition, the NF-kB p65 and IRF-1 also bind to the CXCL11 promoter. As such, the CXCR3 ligands are differentially regulated. CXCL10 is strongly induced by IFN-γ as well as by the Type I interferons IFN, and weakly induced tumour necrosis factor, although tumour necrosis factor synergizes strongly with the IFNs for CXCL10 induction.

Chemokine	Stimulus	Promoter
CXCL9	IFN>>>>TNF	γ-IRE-1, NF-kB2
CXCL10	IFN-γ>IFN-α/β>TNF	IRSE, NF-kB1
CXCL11	IFN-γ=IFN-β>IFN-α>TNF	STAT3, NF-kB2

Table 5-1 CXCL9, CXCL10 and CXCL11 stimulus preference and promoter

5.2.3 BS and IFN-γ pathways

As mentioned in Chapter 1, STAT4 polymorphisms have been found to be associated with BS in the Turkish, Japanese, Han Chinese and Korean populations [98, 131, 132]. Furthermore the IL23R-IL12RB2 is a common variant SNP that was identified in the first two GWAS studies published in 2010 [6, 116]. In clinical studies, IL-12 was found to be elevated in peripheral blood of BS subjects and correlated with disease activity [133]. High levels IFN-γ were found in BS aqueous fluid versus other causes of uveitis [84]. High levels of CXCL9, 10 and 11 have been observed in BS associated uveitis versus other forms of uveitis, and high CXCL10 was found in CSF fluid of BS subjects [134, 135]. CCR5 and CXCR3 receptors have been shown to be highly expressed in BS biopsies from oral ulcers [154]. The pathology of pathergy reactions shows increased mRNA transcripts for the chemokines CCL20, CXCL10, CXCR3, and CXCL11 [61, 62].

5.2.4 mRNA Translation: from gene to protein

Translation initiation is the process of assembly of elongation-competent 80S ribosomes, in which the initiation codon is base-paired with the anticodon loop of initiator tRNA (Met-tRNAMeti) in the ribosomal P-site. It requires several eukaryotic initiation factors (eIFs) and comprises several steps which first lead to the formation of 48S initiation complex and then the joining of 48S complex with 60S subunit. On most mRNAs, 48S complexes form by a 'scanning' mechanism, whereby a 43S preinitiation complex (containing the eIF2–GTP–Met-tRNAMeti ternary complex) attaches to the capped 5′ proximal region of mRNAs in a step that involves the unwinding of the mRNA's 5′ terminal secondary structure by eIF4A, eIF4B and eIF4F. The 43S complex then scans the 5′ untranslated region (5′ UTR) in the 5′ to 3′ direction until it reaches the initiation codon. After initiation codon recognition and 48S complex formation, eIF5 and eIF5B promote the hydrolysis of eIF2-bound GTP, the displacement of eIFs and the joining of a 60S subunit. Although most mRNAs use the scanning mechanism, initiation on a few mRNAs is mediated by internal ribosome entry sites [349].

There is another protein associated with the eIF4F complex called the Poly(A)-binding protein (PABP), which binds the poly-A tail of most eukaryotic mRNA molecules. This protein has been implicated in playing a role in circularization of the mRNA during translation.[350]

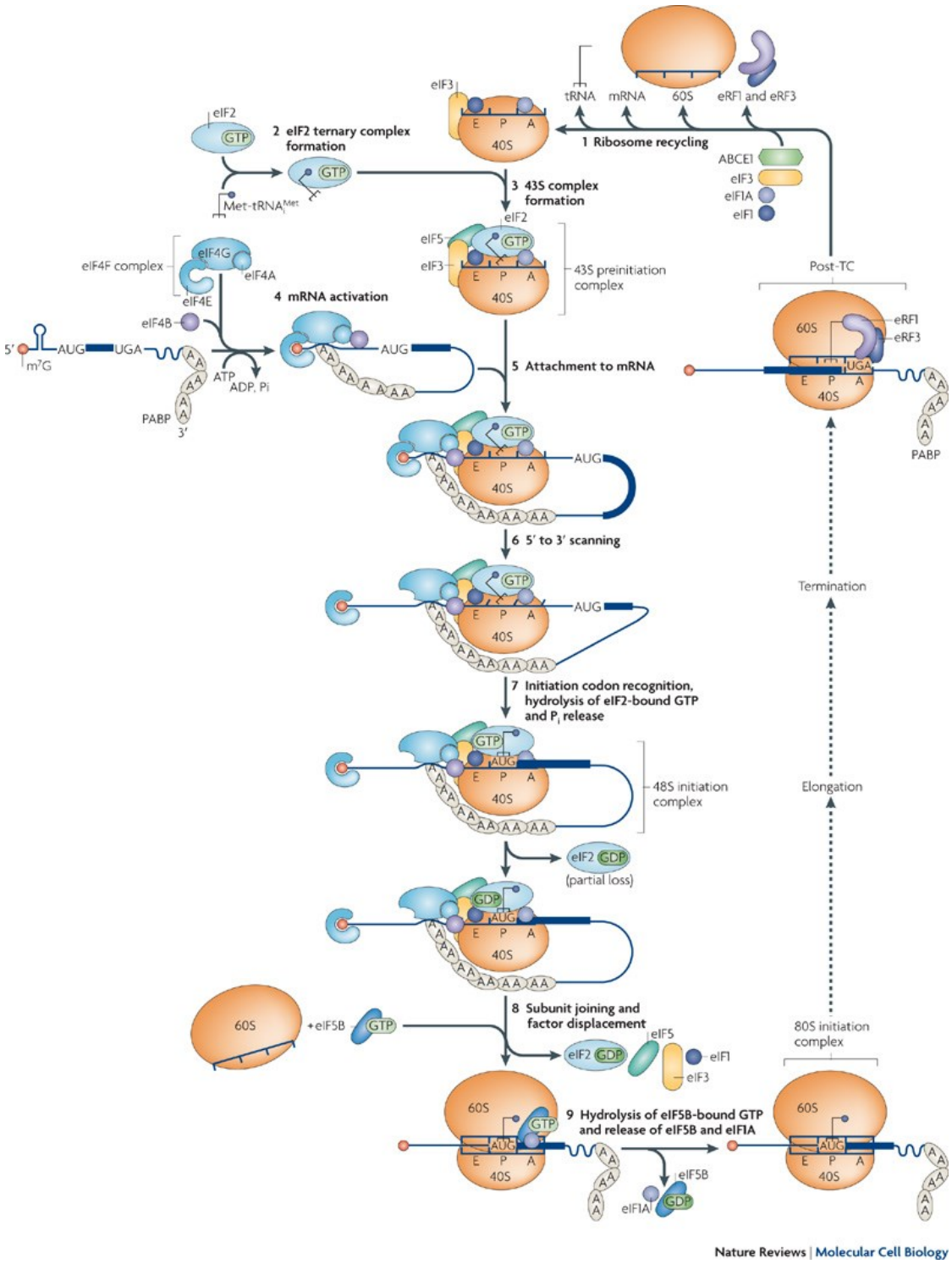


Figure 5-3. Eukaryotic translation. Reproduced from [349].

The pathway of eukaryotic translation initiation is divided into eight stages (2–9). These stages follow the recycling of post-termination complexes to yield separated 40S and 60S ribosomal

subunits, and result in the formation of an 80S ribosomal initiation complex, in which Met-tRNAMeti is base paired with the initiation codon in the ribosomal P-site and which is competent to start the translation elongation stage. These stages are: eukaryotic initiation factor 2 (eIF2)–GTP–Met-tRNAMeti ternary complex formation (2); formation of a 43S preinitiation complex comprising a 40S subunit, eIF1, eIF1A, eIF3, eIF2–GTP–Met-tRNAMeti and probably eIF5 (3); mRNA activation, during which the mRNA cap-proximal region is unwound in an ATP-dependent manner by eIF4F with eIF4B (4); attachment of the 43S complex to this mRNA region (5); scanning of the 5′ UTR in a 5′ to 3′ direction by 43S complexes (6); recognition of the initiation codon and 48S initiation complex formation, which switches the scanning complex to a 'closed' conformation and leads to displacement of eIF1 to allow eIF5-mediated hydrolysis of eIF2-bound GTP and Pi release (7); joining of 60S subunits to 48S complexes and concomitant displacement of eIF2–GDP and other factors (eIF1, eIF3, eIF4B, eIF4F and eIF5) mediated by eIF5B (8); and GTP hydrolysis by eIF5B and release of eIF1A and GDP-bound eIF5B from assembled elongation-competent 80S ribosomes (9). Translation is a cyclical process, in which termination follows elongation and leads to recycling (1), which generates separated ribosomal subunits.

5.2.4.1 Control of translation

Regulating protein production by control of mRNA translation offers advantages over transcriptional regulation. It provides a rapid on/off mechanism. Cytokines can be translated from RNA molecules stored in cytoplasmic granules more rapidly than from newly synthesized transcripts. Storage of mRNA also allows cells to be ‘primed’ by an initial stimulus, which allows a rapid response to a subsequent stimulus. Translational repression allows the rapid turning off of cytokine production.

Posttranscriptional control of cytokine production is achieved by the regulation of nuclear export, cytoplasmic localization, translation initiation and mRNA decay. Two general modes of control can be envisaged (1) global control, in which the translation of most mRNAs in the cell is regulated, for example in response to stress or infection; (2) and mRNA-specific control, whereby the translation of a defined group of mRNAs is modulated without affecting general

protein biosynthesis or the translational status of the cellular transcriptome as a whole. Global regulation mainly occurs by the modification of translation-initiation factors, whereas mRNA specific regulation may also occur through translation initiation factors or may occur by regulatory protein complexes that recognize particular elements that are usually present in the 5′ and/or 3′ untranslated regions (UTRs) of the target mRNA.

5.2.4.2 Regulation of initiation: Global control: eIF4E–4E-BPs and eIF2α kinases

Global control of protein synthesis is generally achieved by reversible changes in the phosphorylation state of initiation factors or the regulators that interact with them, or sometimes irreversible proteolysis of these factors. Two well-characterized examples are the eIF2α kinases and the eIF4E–4E-BPs.

5.2.4.3 eIF2α kinases

eIF2 is part of the complex that associates with the 40S ribosomal subunit in its GTP-bound form. This GTP is hydrolysed when the initiator AUG is recognized during translation initiation, producing eIF2 in the GDP-bound state. Exchange of GDP for GTP again on the eIF2 is catalysed by eIF2B. eIF2 is made of three subunits (α, β and γ) and phosphorylation of the α subunit at residue Ser51 blocks the GTP-exchange reaction by reducing the dissociation rate of eIF2 from eIF2B [351]. In effect, this sequesters eIF2B and, as a consequence, GDP–GTP exchange no longer occurs and global mRNA translation is inhibited.

A number of kinases can phosphorylate eIF2α at Ser51 including the haem-regulated inhibitor which is stimulated by haem depletion; the general control non-repressed 2 (GCN2) which is activated by amino-acid starvation; Protein kinase R (PKR) which is stimulated by viral infection and protein kinase RNA-like endoplasmic reticulum kinase (PERK) which is activated under circumstances of endoplasmic reticulum (ER) stress [352, 353]. Although phosphorylation of eIF2α by these kinases decreases global translation, this modification can also result in the translational activation of specific mRNAs.

5.2.4.4 eIF4E

The availability of the cap-binding protein eIF4E also regulates general translation rates. eIF4E interacts with the scaffold protein eIF4G to recruit the 43S ribosomal complex to the mRNA during translation initiation. Association between eIF4E and eIF4G requires a small domain in eIF4G that is shared by proteins that are known as the 4E-binding proteins (4E-BPs). Hypophosphorylated 4E-BPs bind to eIF4E and competitively displace eIF4G, resulting in the inhibition of the association of the 43S complex with the mRNA and hence, translational repression[354]. In addition to the phosphorylation-mediated reversible global regulation, proteolytic cleavage of translation factors can inhibit cellular protein synthesis. For example, the apoptotic protein caspase-3 cleaves eIF4G and Poly-A binding protein (PABP) [354]

5.2.4.5 Specific translation regulation

Specific regulation occurs by many mechanisms. Translation inhibition by message-specific 4E-BPs target steps of the translation-initiation pathway. Canonical end modifications of mRNA molecules may occur and the cap structure and the poly(a) tail are strong promoters of translation initiation; internal ribosome-entry sequences (IRESs) mediate cap-independent translation initiation; upstream open reading frames (uORFs) which normally reduce translation from the main ORF; secondary or tertiary RNA structures play important roles for example hairpins and pseudoknots which may block initiation, but can also be part of IRES elements and therefore promote cap-independent translation; and, specific binding sites for regulatory complexes, are all crucial determinants of mRNA translation [354].

5.2.4.6 Regulation of elongation or termination

Translation can also be controlled at late-initiation and post-initiation steps. For example, the RNA-binding proteins heterogeneous nuclear ribonucleoprotein (hnRNP) K and E1 inhibit the translation of 15-lipoxygenase (LOX) mRNA during early erythroid differentiation. They bind to a repeated CU-rich element, which is known as the differentiation-control element (DICE) that is located in the LOX 3′ UTR. Translational repression of LOX is occurs at a late step in

initiation [355]. Sucrose-gradient analysis showed that 48S-complex formation occurred, but formation of the 80S ribosome was inhibited by the hnRNP-K–hnRNP-E1 complex [356].

5.2.4.7 mRNA stability or decay: ARE binding motifs

Adenine and uridine–rich elements (AREs) are found in the 3′ untranslated regions (UTRs) of newly cloned mRNA molecules. The ARE recruit several different ARE-binding proteins (ARE-BPs) that can positively or negatively regulate mRNA stability and or translation. ARE-BPs shuttle between the nucleus and the cytoplasm so binding to specific mRNA molecules may occur in either compartment. Several protein domains specifically bind AREs, including the RNA recognition motif (e.g. HuR), the zinc-finger domain (e.g. TTP) and the K-homology domain (e.g. KSRP). These domains may also bind to stemloop–containing elements. Each of these proteins can individually affect the translation and/or decay of ARE-containing mRNA molecules: Usually TTP promotes decay[357] whereas HuR inhibits decay [358]. IFN-γ mRNA contains an ARE that HuR may act on leading to mRNA stability. Once HuR stabilises, it may either promotes or inhibits translation depending on the context [359]. p38–MAPK-activated kinase 2 pathway can lessen ARE-dependent destabilization and translational silencing leading to more stable mRNA and promoting translation [359].

In T cells, costimulatory molecules such as CD28 and the integrin LFA-1 can influence the stabilization of mRNA molecules encoding cytokines [356]. Ligation of CD28 stabilizes IL-2 transcripts, suggesting that CD28 engages an unidentified general signalling pathway to stabilize mRNA [360].

5.2.4.8 mRNA decay initiation

In eukaryotes, degradation of mRNA is usually initiated by shortening of the 3’ polyA tail (deadenylation) which generally leads to the removal of the 5’cap structure by Dcp1/Dcp2 decapping enzyme followed by 5’-3’ exonucleolytic digestion by Xrn1. The competition between mRNPs engaged in translation and those assembled with the decapping machinery correlates with the location of the mRNA. Translating mRNAs are distributed throughout the

cytosol but mRNAs complexed with the decapping machinery can concentrate in P bodies (regulation by sequestration).

5.2.4.9 Regulation by sequestration

As mRNA exists the nucleus, the cap binding protein 20 (CBP20)-CBP80 complex initiates a round of translation that strips off exon-junction complexes (multiprotein complexes that are deposited at exon exon junctions during splicing) and the CBP20-CPB80 is replaced by the cytoplasmic cap binding protein eukaryotic translation initiation factor 4E (eIF4E), which recruits the translation initiation complex as described above [361]. Recruitment of this initiation complex leads towards translation, whereas recruitment of the CCR5-NOT complex promotes deadenylation and eventually mRNA decay [362]. In many cases, newly formed transcripts are packaged into distinct classes of RNA granules that help control translation and decay. Disassembly of translating polysomes can drive the assembly of either processing bodies (P bodies) [363] or stress granules (SGs) [364]. SGs contain components of the translation initiation machinery whereas PBs contain components of the mRNA decay machinery [365]. Both SGs and PBs contain Argonaute proteins and associated miRNAs [365]. The components of most RNA granules appear to be in dynamic equilibrium with the actively translating pool allowing rapid shifts between translation storage and decay [365].

5.2.4.10 Regulation by microRNAs

MicroRNAs (miRNAs) are being increasingly recognised as important players in translational control. miRNAs are transcribed as primary transcripts that are processed in the nucleus by Drosha, a member of the RNase III superfamily to yield precursors of ~70 nucleotides (pre-miRNAs) that have the capacity to form stem–loop structures. The pre-miRNAs are further processed into mature miRNAs in the cytoplasm by another RNase-III-like enzyme that is known as Dicer. miRNAs hybridize by incomplete base-pairing, usually to several sites in the 3′ UTR of target mRNAs. Because the target mRNA remains intact after miRNA binding, the miRNAs are believed to repress translation, rather than prevent translation by degrading the mRNA. miRNA is biochemically indistinguishable from another small RNA species that is

known as small interfering RNA (siRNA). siRNAs are double-stranded RNA molecules of 21–23 nucleotides in length, and they mediate the degradation of mRNAs that show perfect complementarity to either of the siRNA strands [366].

The miRNA are associated with multiple proteins to form micro ribonuclear proteins (miRNPs) and these proteins include the Argonaute (AGO) family [354, 367]. AGOs are intimately associated with the paired miRNA–mRNA interaction, and many other proteins are present more peripherally. miRNAs appear to act as adaptors that confer sequence-specific mRNA binding on AGO [349]. Repression seems to have end results: repression of mRNA translation, and an accelerated rate of mRNA degradation through the normal deadenylation-dependent pathways. The relative importance of these two components seems to vary between different miRNA–mRNA pairs [349, 368].

The mechanism of translational repression by miRNAs is largely unknown. Some investigators find the repressed mRNA is displaced from large polysomes into small polysomes or sub-polysomal particles, which is indicative of inhibited initiation. Others find the repressed mRNA in polysomes that are a similar size to those present when the reporter mRNA is not repressed, implying inhibition at a post-initiation stage. For example, translational repression of lin-14 mRNA by lin-4 miRNA has been shown not to alter its association with polysomes [354, 369]. It has been suggested in the later model, where polysomes continue to be associated with the mRNA that specific co-translational degradation of the nascent protein is the mechanism of control [370]. Two opposing theories are of premature ribosome drop-off (which would have to be infrequent to maintain polysome size) and a reduced rate of elongation (which would cause polysome size to increase unless it was coupled with a quantitatively similar reduction in initiation frequency) [349]. Those who do not find polysome association, feel that the mechanism of inhibition is that of inhibition of initiation but more exact theories remain controversial [371]. Those who observe inhibition of initiation are generally agreed that strong repression is only seen if the mRNA has the normal Gppp cap, and not if this is replaced by Appp, nor if the mRNA has a viral IRES, suggesting that it may be the cap–eIF4F interaction that is the proximal target of the repression mechanism [349, 372].

5.2.4.11 IFN-γ mediated translational control

IFN-γ mediated translational control is incompletely understood. One well described example is that of IFN-γ mediated translational control involving the large ribosomal subunit protein L13A. Ceruloplasmin synthesis is inhibited 24 hours after treatment with interferon-γ by the action of the ribosomal cellular factor L13a, that binds to a stem–loop structure in the 3′ UTR of the mRNA [373-375]. Phosphorylation of L13a after interferon-γ treatment causes its release from the 60S ribosomal subunit and promotes binding to the 3′ UTR the mRNA [375]

5.2.5 miRNAs with IFN-γ and CXCL10

Recent evidence suggests that miRNAs may play an important role in IFN-signaling. Using a microarray based approach, a study by Pedersen et al. demonstrated that both Type I and II IFNs can up regulate or decrease the expression of several cellular miRNAs [376] . Also, it was recently shown that IFN-γ induced ICAM-1 mRNA translation is mediated by IFN-γ dependent suppression of miRNA-221 in a STAT1 dependent manner in cholangiocytes [322, 377].

5.2.6 Polysome analysis

Polysome analysis is a technique used to investigate differential translation regulation[378]. RNA from cells is layered on top of a tube containing a sucrose density gradient (sucrose 50% at the bottom to 20% at the top of the tube). It is then spun at very high speeds which results in 'heavy' polysomes travelling further down the tube then 80S or 40S subunits. Free RNA will travel the least far. A needle pierces the bottom of the tube and very even denser sucrose is introduced slowly (60%) to push the contents of the tube into a collecting tube and past a laser (which feeds to a graph tracer giving an image of passing fluid contents), and finally the sucrose is fed into collecting ependorfs, which allows the sample to be collected in fractions and analysed. Usually polysomal fractions reflect actively translating mRNA and free RNA fractions reflect untranscribing mRNA. The main exception to this is that mRNA may be arrested in both free and polysomal states by miRNAs [369, 370].

5.2.7 BS and miRNA work to date

Work has been undertaken by a Chinese group studying miRNAs in BS. They genotyped over 800 BS as well as HC and disease controls, for specific miRNA-146 polymorphisms and found they were associated with ocular BS and Vogt-Koyanagi-Harada syndrome (VKH). miRNA-146a has been identified as a negative feedback regulator in TLR signalling by targeting TRAF6 and IRAK. Carriers of the disease associated SNP (GG) was associated with higher TNF-α, IL1-β and IL-17 production. They did not assess CXCL10 or IFN-γ but STAT1 has been shown to be a target for this miRNA [158]. A second study examined miRNA-155 and found decreased expression in ocular BS. Overexpression of this miRNA has been shown to inhibit IL-6 and Il-1β and to promote IL-10.This experiment used miRNA-155 mimics and inhibitors (single stranded chemically modified oligonucleotides) [157]. A third study using genotyping, found a functional variant of pre-miRNA-196a2 confers risk for BS but not VKH, specifically the arthritis phenotype. PBMCs from subjects with the disease associated genotype produced more IL-1β and MCP-1 protein following LPS stimulation, but not more IFN-γ [379]. Published miRNA work to date in BS has been undertaken in a Chinese cohort.

5.2.8 The choice of SLE as a disease control

Type I interferons have been thought of as central players in SLE for many years, and IFN-γ has also been implicated [380]. A study examining the IFN-γ signalling pathway in peripheral blood mononuclear cells and purified monocytes in SLE found an increased expression of STAT1, and also increased expression of CXCL9 and CXCL10 mRNA at 6 hours (cells had been previously primed with IFN-α/γ two days prior to the start of the experiment) [381].

5.2.9 The choice of RA as a disease control

We chose RA as a disease control for our experiments as it is a disease that this pathway has been studied in detail. CXCL10 have been found to be upregulated in RA Synovial fluid (SF) compared with osteoarthritis(OA) SF [382]. CXCL10 serum levels in rheumatoid factor (RF)–positive individuals have been found to be higher than those in control (OA or trauma) subjects

[382]. Serum CXCL10 levels were reported as being similar to normal in one study (40) but increased in another study [382]. RA synovial tissue (ST) was found to express greater amounts of CXCL10 messenger RNA (mRNA) than OA ST [383]. Overall, CXCL10 is felt to be a key player in recruitment of Th1 cells into RA ST [384, 385]. An *in vitro* study of PBMCs and CD14+ monocytes stimulated with IFN-γ for 48 hours, in RA and HC, found higher STAT1 mRNA expression in RA versus HCs, There was also increased CXCL10 in the RA group at this time point, although mRNA levels did not show a significant difference between the groups. MDX-1100, a fully human anti-CXCL10 monoclonal antibody (Bristol Myers Squibb) bind to CXCL10 with high affinity. A randomized, double blind, placebo controlled phase II proof of concept study was undertaken in RA to determine the clinical efficacy and safety of this therapy. Although most subjects improved by 20% (ACR20), almost none (2.9%) improved by 70% (ACR70).

5.3 Hypothesis

That the IFN-γ inducible chemokine CXCL10 may be an important player in the abnormal inflammatory response observed in BS.

5.4 Aims of *in vitro* work

1. To compare the CXCL10 chemokine response in CD14+ monocytes following stimulation with IFN-γ in BS and HCs, at a protein and mRNA level.

2. To assess specificity of any of observed differences. Specificity has three main prongs: (i) IFN-γ chemokine specificity; (ii) Stimulus specificity (e.g. observing if any differences are global observations that may also be observed following stimulation with other cytokines and (iii) disease specificity (we recruited Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) as disease control groups).

3. To assess if any significant *in vitro* observations correlated clinical findings such as: (i) having a large MSU skin response; (ii) the disease activity score of BS subjects; (iii) medications that BS subjects were taking.

4. To dissect any significant observations to try and understand underlying mechanistic processes.

5.5 Methods

Chapter 2 provides a detailed description of *in vitro* tissue culture methods. Peripheral blood derived CD14+ monocyte cultures were used for all experiments. Cells were allowed rest for one hour after isolation, following which they were stimulated with IFN-γ or other stimuli.

5.6 Results

5.6.1 Demographics

In total, 27 BS subjects, 25 HC, 15 RA and 15 SLE subjects donated samples for *in vitro* work, following written consent. There were three rounds of subject recruitment over an 18 month period to answer consecutive questions relating to the aims of this chapter. The demographic data and clinical characteristics (including medication use) of these 3 cohorts of subjects is listed in Tables 1-4. Many subjects donated samples during each round of recruitment, and where results are pooled, the first sample received from a subject is the one that was included. In addition, we also had serum samples stored at minus 80 degrees Celsius on numerous HC, BS and SLE subjects who had previously participated in research at Hammersmith Hospital. These samples were used to gather preliminary data and ELISA results are included in the first results section.

Cohort 1 Demographics	Healthy Controls		Behçet Syndrome	
Cohort size	9		9	
Median age (Interquartile range)	34	(32-36)	35	(26-53)
Male sex (%)	6	66.7%	5	55.6%
Clinical Characteristics				
Mucocutaneous	0		9	100%
Erythema nodosum			5	56%
Pathergy or poor wound healing			6	67%
Musculoskeletal - arthralgias	0		8	89%
Arthritis			1	11%
Cardiovascular	0		3	33%
Aneurysm			2	22%
Thrombosis			2	22%
Ocular	0		3	33%
Posterior/panuveitis			2	22%
Gastrointestinal inflammation	0		1	11%
Neurological	0		2	22%
Disease Activity Score *	0		3	
Current medications				
Colchicine	0		2	22%
Azathioprine	0		1	11%
Mycophenolate	0		1	11%
Anti-TNF therapy	0		5	56%
Cyclosporine	0		0	
Methotrexate	0		2	22%
Steroids **	0		2	(5.5mg)

*** Behçet: Leeds Behçet's disease activity form (2002 version) score of 0-12 (>4 indicates active disease).**

**** Number of subjects taking steroids, and the median prednisolone dose taken by those subjects**

Table 5-2. Demographics of cohort 1 HC and BS subjects recruited for *in vitro* experiments.

Cohort 2 Demographics	Healthy Controls		Behçet Syndrome	
Cohort size	13		13	
Median age (Interquartile range)	34	(24-39)	46	(27-58)
Male sex (%)	7	53.8%	5	38.5%
Clinical Characteristics				
Mucocutaneous	0		13	100%
Erythema nodosum			6	46%
Pathergy or poor wound healing			10	77%
Musculoskeletal - arthralgias	0		13	100%
Arthritis			9	69%
Cardiovascular	0		1	8%
Aneurysm				
Thrombosis			1	8%
Ocular	0		9	69%
Posterior/panuveitis			5	38%
Gastrointestinal inflammation	0		3	23%
Neurological	0		2	15%
Disease Activity Score *	0		3	
Current medications				
Colchicine	0		1	8%
Azathioprine	0		4	31%
Mycophenolate	0		0	
Anti-TNF therapy	0		4	31%
Cyclosporine	0		0	
Methotrexate	0		0	
Steroids **	0		7	(6mg)

*** Behçet: Leeds Behçet's disease activity form (2002 version) score of 0-12 (>4 indicates active disease).**

**** Number of subjects taking steroids, and the median prednisolone dose taken by those subjects**

Table 5-3. Demographics of cohort 2 HC and BS subjects recruited for *in vitro* experiments.

Cohort 3 demographics	HC	BS	RA	SLE
Cohort size	15	16	15	15
Median age (IQR)	37 (35-42)	46 (37-64)	59 (52-67)	52 (36-53)
Male sex (%)	8 53.3%	6 37.5%	2 13.3%	3 20.0%
Clinical Characteristics				
Mucocutaneous	0	16 100%	0	7 47%
Erythema nodosum		7 44%	0	0
Pathergy		14 88%	0	0
Arthralgias	0	14 88%	15 100%	14 93%
Arthritis		7 44%	15 100%	4 27%
Cardiovascular	0	3 19%	0	3 20%
Aneurysm		2 13%	0	0
Thrombosis		1 6%	0	3 20%
Ocular	0	8 50%	0	1 7%
Posterior/panuveitis		5 31%	0	0
Gastrointestinal	0	4 25%	0	1 7%
Neurological	0	3 19%	0	2 13%
Renal	0	0	0	11 73%
Median DAS *	0	2.6	4.3	5.2
Current medications				
Colchicine	0	3 19%	0	0
Azathioprine	0	1 6%	0	1 7%
Mycophenolate	0	5 31%	0	9 60%
Anti-TNF therapy	0	5 31%	3 20%	0
Cyclosporine	0	1 6%	0	0
Methotrexate	0	2 13%	11 73%	0
Tacolimus	0	0	0	3 20%
Plaquenil	0	0	4 27%	9 60%
Toculizumab	0	0	1 7%	0
Rituximab	0	0	0	1 7%
Steroids **	0	8 (10mg)	6 (5mg)	6 (5mg)

***Behçet: Leeds Behçet's disease activity form (2002 version) score of 0-12 (>4 indicates active disease). SLE: Sledai score 0-105 (>6 indicates active disease). RA: Disease Activity Score 1-10 (>5.1 indicates active disease). IQR=interquartile range.**

**** Number of subjects taking steroids, and the median prednisolone dose taken by those subjects**

Table 5-4. Demographics of cohort 3 HC, BS, RA and SLE subjects recruited for *in vitro* experiments.

General Demographics	Healthy Controls		Behçet Syndrome	
Cohort size	25		27	
Median age (Interquartile range)	35	(30-38)	45	(33-51)
Male sex (%)	13	52.0%	12	44.4%
Clinical Characteristics				
Mucocutaneous	0		27	100%
Erythema nodosum			12	44%
Pathergy or poor wound healing			19	70%
Musculoskeletal - arthralgias	0		24	89%
Arthritis			13	48%
Cardiovascular	0		6	22%
Aneurysm			3	11%
Thrombosis			4	15%
Ocular	0		13	48%
Posterior/panuveitis	0		9	33%
Gastrointestinal inflammation	0		6	22%
Neurological	0		9	33%
Disease Activity Score *	0		3	
Current medications				
Colchicine	0		3	11%
Azathioprine	0		6	22%
Mycophenolate	0		5	19%
Anti-TNF therapy	0		10	37%
Cyclosporine	0		1	4%
Methotrexate	0		4	15%
Steroids **	0		15	(9mg)

*** Behçet: Leeds Behçet's disease activity form (2002 version) score of 0-12 (>4 indicates active disease).**

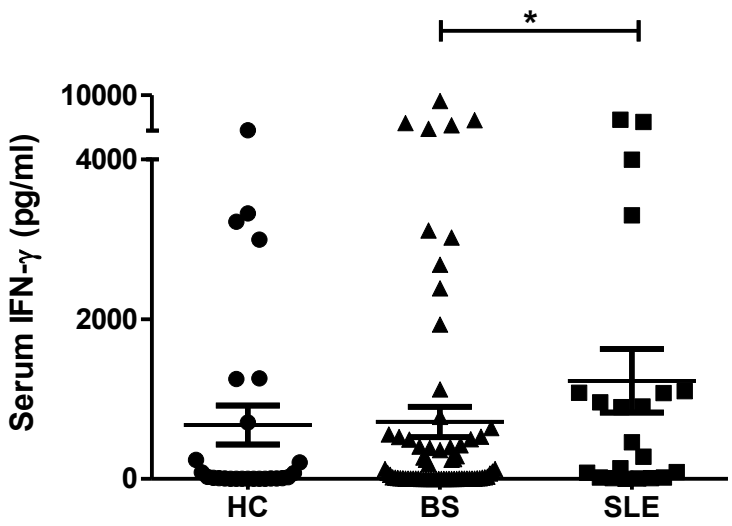
**** Number of subjects taking steroids, and the median prednisolone dose taken by those subjects**

Table 5-5. Demographics of pooled cohort of HC and BS subjects recruited for *in vitro* experiments.

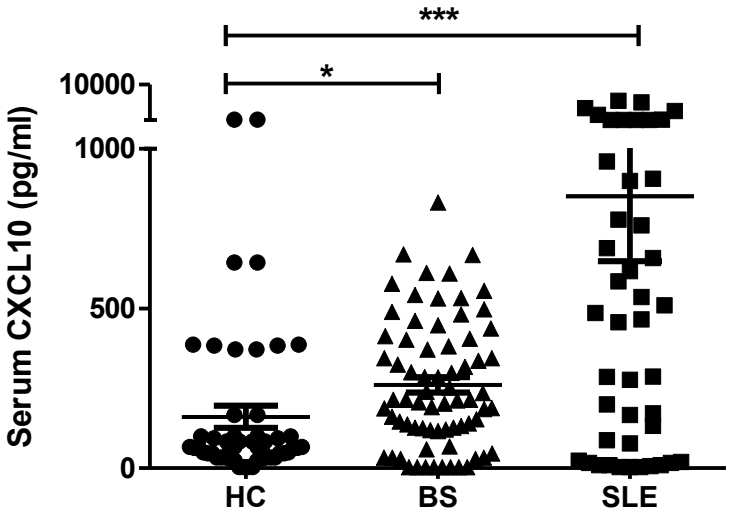
5.6.2 Pilot data

5.6.2.1 Assessment of cytokines in serum from HC, BS and SLE subjects

We had noted that there was a difference in CXCL10 concentrations in blister fluid of BS versus HC in Chapter 1. We had serum stored from 69 HCs, 72 BS and 65 SLE subjects from previous work undertaken in our laboratory. We decided to undertake ELISAs to look at random serum IFN-γ and CXCL10 protein concentrations in these samples. We did not have clinical details on this cohort and therefore the aim of this experiment was simply to gather preliminary data and to help form a hypothesis for *in vitro* work. We found that serum from SLE subjects contained significantly more IFN-γ protein than serum from BS or from HC. Serum from SLE and BS subjects contained significantly more CXCL10 protein than serum from HC.



(a).



(b).

Figure 5-4. Serum protein levels of IFN-γ and CXCL10 in samples from HC (n=69), BS (n= 72) and SLE (n=65).

(a).There was significant variance between the three groups (P=0.03) with more IFN-γ protein in SLE samples than in either HC or BS samples. Dunn's Multiple comparison test revealed a significant difference between the BS and SLE groups (P<0.05). There was no significant difference in IFN-γ protein concentrations between BS and HC (b). Despite no significant difference between HC and BS in IFN-γ concentrations, significantly more CXCL10 protein was observed in BS samples versus HC (Dunn's multiple comparison testing p<0.05). There was also a marked increase in CXCL10 protein in SLE samples versus HC (Dunn's multiple comparison testing p<0.05). ANOVA Kruskal Wallis test showed an overall significant variance between groups (P=0.0001). Analysis: ANOVA Kruskal Wallis test with Dunn's multiple comparison testing.

This preliminary data suggested that there may be a difference in the IFN-γ –CXCL10 pathway in BS versus either HC or BS. This was preliminary data and clinical correlation relating to disease activity or medications was not available. However, the data was interesting in that it suggested that the IFN-γ - CXCL10 pathway may be worth formally investigating *in vitro*. BS subjects had more CXCL10 protein than HCs but only a moderate (non-significant) increase in IFN-γ concentration. In contrast, in the SLE samples there was a straightforward increase in both IFN-γ and CXCL10. Possibilities for the observation in BS were numerous but included

that other cytokines such as TNF-α may be stimulating the CXCL10 pathway or that BS may have and increased transcriptional or translational response to modest levels of IFN-γ.

5.6.3 Comparison of CXCL10 protein response in BS and HCs

In order to evaluate the IFN-γ –CXCL10 pathway formally, we recruited 9 HC and BS subjects (Table 5-2.) They were similar in age (median 34 yr in HC, 35 yr in BS), 6 of the HC were male versus 5 of the BS subjects. The BS subjects were on a range of medications, and 5 were receiving anti-TNF therapy. Only 2 were taking prednisolone (median dose 5.5mg daily). CD14+ monocytes were isolated from peripheral blood and plated. Cells were allowed to rest for an hour, following which, they were stimulated with IFN-γ, or left unstimulated. At 2 or 4 hours, supernatants were aspirated for ELISA of CXCL10 protein levels and cells were scraped and stored for later mRNA extraction.

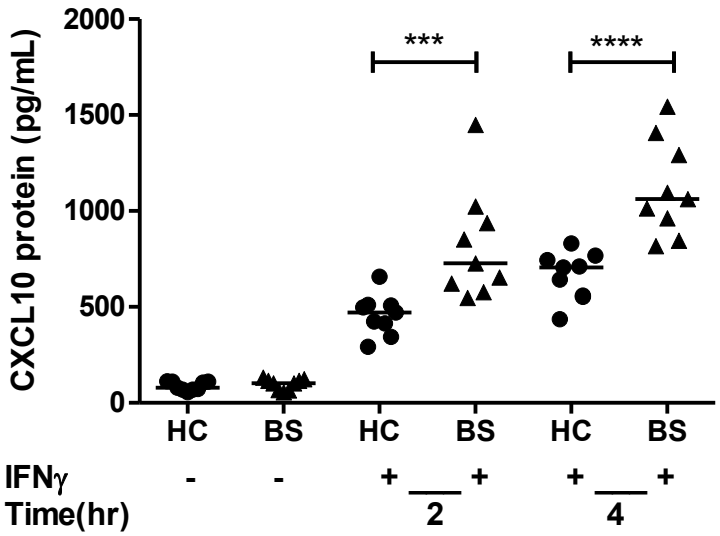


Figure 5-5. CXCL10 protein concentration. CD14+ monocytes from HC and BS subjects. Monocytes were stimulated with 20ng/mL IFN-γ for either 2 or 4 hours, or else were left unstimulated. Supernatants from unstimulated cells contained low concentrations of CXCL10 in both HC and BS, with no difference between the two groups. In contrast, supernatants from BS samples had a significantly higher concentration of CXCL10 protein at both 2 hours (P=0.005) and 4 hours (P<0.001) despite being stimulated with the same concentration of IFN-γ. Analysis: Two tailed Mann Whitney test. Line represents median values. Subject cohort 1.

We found that at both the 2 and 4 hour time point more CXCL10 protein was present in the BS samples. There was very little CXCL10 in unstimulated samples from either group. In order to evaluate if there was a pool of preformed protein in monocytes from BS accounting for the observation, the experiment was repeated, this time samples were collected every 30 minutes from time 0 to 2 hours. For this experiment 13 HC and 13 BS subjects were recruited. Again, the groups were well matched for age and sex (Table 5-3). Seven of the 13 BS subjects were receiving prednisolone (median 6mg), and 4 were on anti-TNF therapy. We found that CD14+ monocytes from BS subjects began to produce more CXCL10 protein than those from HC from 2 hours

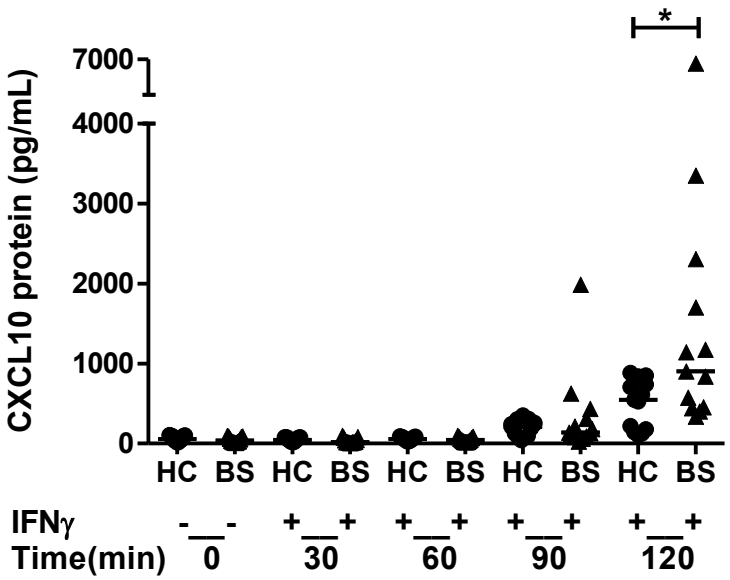


Figure 5-6. CXCL10 protein concentration. CD14+ monocytes from HC and BS subjects. Monocytes were stimulated with 20ng/mL IFN-γ for periods from 30 minutes to 2 hours, or else were left unstimulated. Supernatants from unstimulated cells contained low concentrations of CXCL10 in both HC and BS, with no difference between the two groups. Supernatants from BS samples had a significantly higher concentration of CXCL10 protein from 2 hours (P=0.027). Analysis: Two tailed Mann Whitney test. Line represents median values. Subject cohort 2.

These results inferred that our observations were not due to the release of preformed protein. The two hour time point was used for all further experiments, as this was the earliest time that a significant difference in protein production was observed between BS and HC.

5.6.3.1 Evaluation of CXCL10 mRNA in response to IFN-γ

During the experiments detailed above, cells had been scraped and stored allowing later mRNA extraction. mRNA was extracted from the samples from the above 2 experiments (cohort 1 and 2, Tables 5-2 and 5-3), and RT PCR was undertaken to assess the relationship between CXCL10 mRNA and protein levels in monocytes following IFN-γ stimulation.

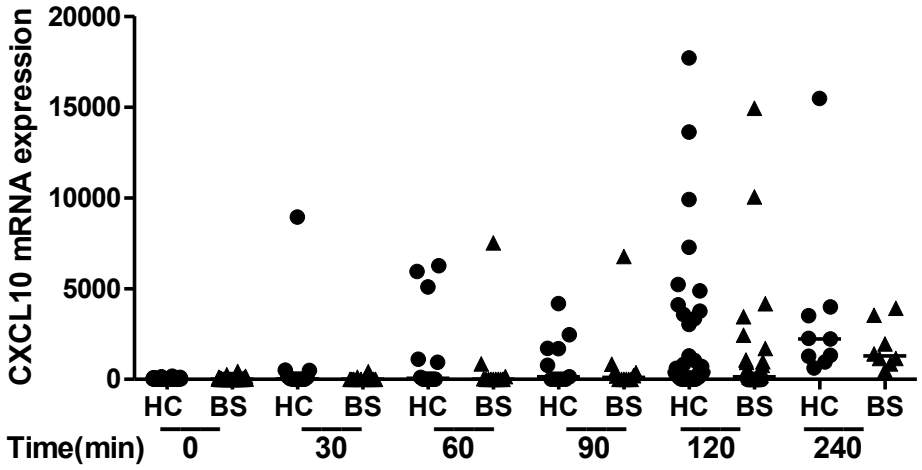


Figure 5-7. CXCL10 mRNA expression. CD14+ monocytes from HC and BS subjects. Monocytes were stimulated with 20ng/mL IFN-γ for periods from 30 minutes to 4 hours, or else were left unstimulated. mRNA was semi-quantitatively investigated by Reverse Transcriptase Real Time Polymerase Chain Reaction and results expressed relative to the expression of the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). mRNA expression of CXCL10 in both HC and BS showed no significant differences at any time point. Indeed, contrary to expectations, there tended to be lower values of mRNA expression in the BS samples although these differences were not statistically significant. Analysis: Mann Whitney t test at each time point. Line represents median values. Subject cohort 1 and 2 pooled.

During preliminary experiments other housekeeping genes (Actin and GAPDH) had also been assessed, but results indicated that HPRT remained the most reliably constant following IFN-γ stimulation (results not shown).

These results indicated that although there was no difference in the mRNA expression (or if anything, there was lower mRNA expression of CXCL10 in the BS cohort), there was an increased protein concentration in BS samples following IFN-γ stimulation. This protein was likely to be newly formed as results were only apparent from 2 hours. This indicated an uncoupling between transcription and translation. We expressed this by plotting CXCL10 protein against mRNA (below), and by dividing protein by mRNA to establish the ratio, and this confirmed the observation of an uncoupling between the two processes.

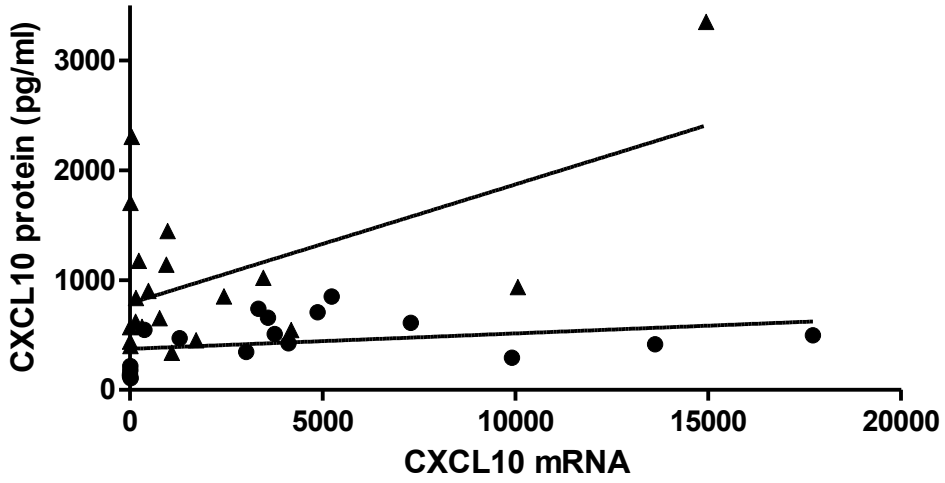


Figure 5-8. The relationship of CXCL10 protein to mRNA. CD14+ monocytes from HC and BS subjects.

Protein values (y axis) were plotted against mRNA expression (relative to HPRT) (x axis) from monocytes stimulated with IFN-γ (20nL/mL) at the 2 hour time point. It was found that BS (▲) values clustered along the y axis and HC (●) clustered along the x axis, suggesting more efficient translation in the BS samples. Linear regression of the groups showed that the slopes were significantly different (F = 6.55706. DFn=1, DFd=34, P=0.015). Subject cohort 1 and 2 pooled.

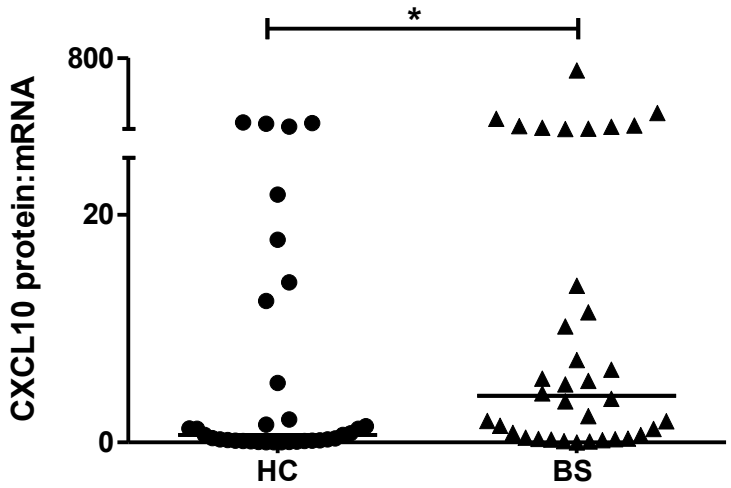


Figure 5-9. CXCL10 protein:mRNA ratio. CD14+ monocytes from HC and BS subjects. Monocytes were stimulated with IFN-γ (20nL/mL) for 2 hours. The ratio of protein production to mRNA expression at 2 hour time point was calculated. BS were found to have a significantly increased ratio of protein: mRNA production versus HC, again indicating more efficient translation. For this analysis results pooled from subject cohort 1, 2 and 3 are shown. P= 0.01. Analysis: Two tailed Mann Whitney test. Line represents median values. Results were also statistically significant when cohorts were analysed individually.

5.6.4 Assessing the specificity of observed differences in CXCL10 protein and mRNA

We wished to evaluate if this was a specific observation for just this one cytokine/chemokine pathway, just in this one inflammatory condition, or whether it was a response that would be seen in BS in response to any stimulus, or indeed one that would be observed in other inflammatory conditions. As discussed in the introduction, we choose RA and SLE as suitable disease controls (DC). For this experiment 15 HC, 16 BS, 15 RA and 15 SLE subjects were recruited. The RA and SLE subjects were slightly older and had a higher proportion of females. We felt we may have recruited a rather atypical cohort of SLE /RA subjects and we matched exactly for sex. Most BS, RA and SLE subjects were taking disease modifying anti-rheumatic drugs (DMARDs), with SLE subjects more likely to be taking hydroxychloroquine (plaquenil), RA more likely to be taking methotrexate, and slightly more BS subjects taking azathioprine or

anti-TNF therapy. The BS group were taking slightly more prednisolone. The disease activity score for all groups showed mild to moderately active disease (Cohort 3, table 5-4).

We divided specificity questions into three main prongs: (i) IFN-γ chemokine specificity; (ii) Stimulus specificity (iii) BS specificity. For IFN-γ chemokine specificity we analysed samples from all BS and HCs (Cohorts 1-3).

In order to assess the first prong of the specificity question, we decided to look at CD14+ monocytes production of other chemokines following IFN-γ stimulation. We choose to study CXCL9, CXCL11 (both are documented to be IFN-γ inducible) and CCL2. To this end we evaluated these chemokine protein concentrations in samples from Cohorts 1-3 of HC and BS subjects. Results showed that CXCL9 protein was significantly higher in BS versus HC. There was a trend towards higher CCL2 but this was not significant. Very little CXCL11 was present in any samples.

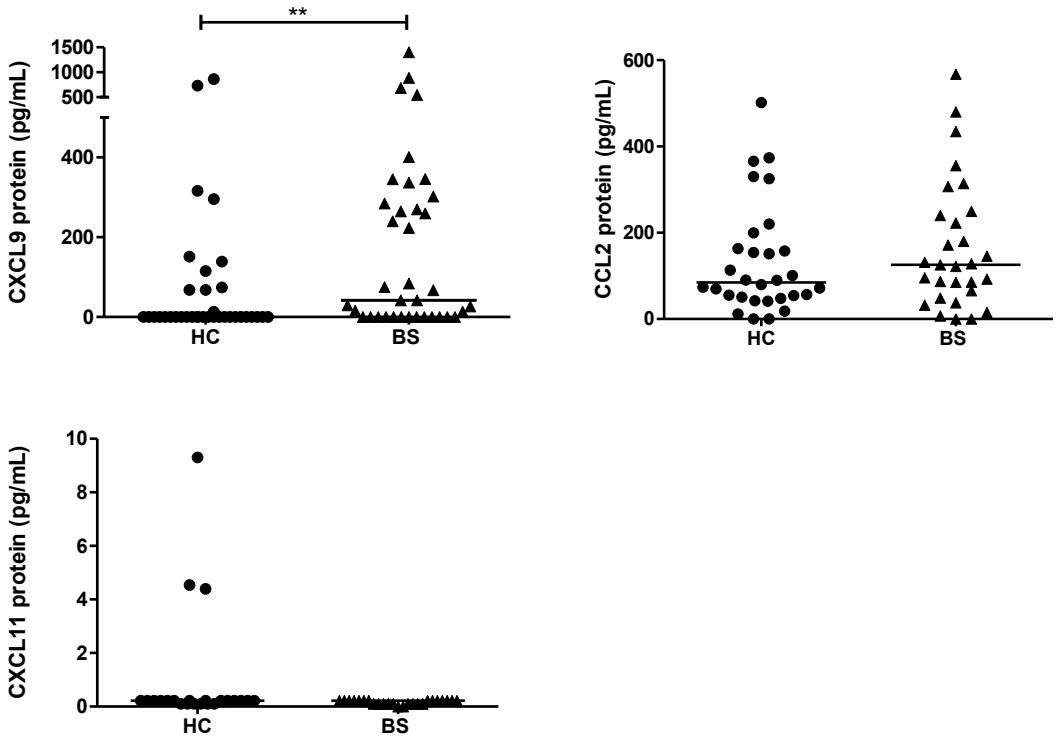


Figure 5-10. CXCL9, CXCL11 and CCL2 protein concentration. CD14+ monocytes from HC and BS subjects.

Monocytes were stimulated with 20ng/mL IFN-γ for 2 hours. Supernatants from BS cells contained significantly more CXCL9 protein than HC (P=0.001), similar concentrations of

CCL2 (P=0.34) and very low concentrations of CXCL11. Analysis: Two tailed Mann Whitney test. Line represents median values. Subject cohort 1-3.

As CXCL9 protein was found to be significantly higher in the BS group compared to HC, we next assessed the CXCL9 mRNA expression and calculated the ratio of CXCL9 protein:mRNA. In contrast to the CXCL10 mRNA, there was a trend towards the BS cohort having more rather than less CXCL9 mRNA expression, and there was no significant difference between the groups when the ratio of protein:mRNA expression was calculated. This indicated that for CXCL9 there was a more direct relationship between transcription and translation.

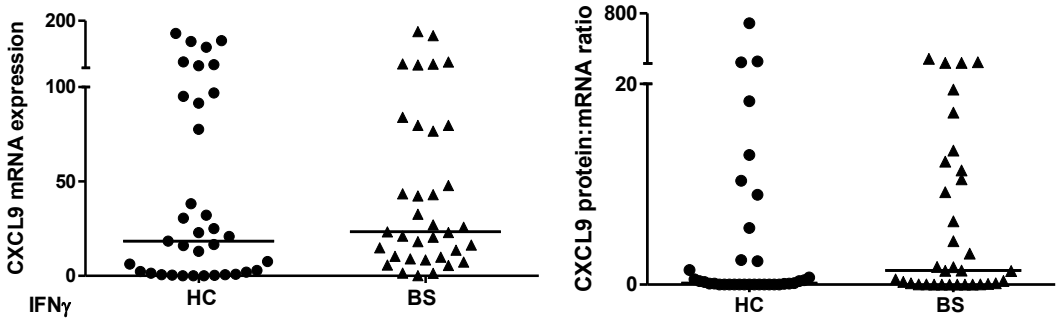


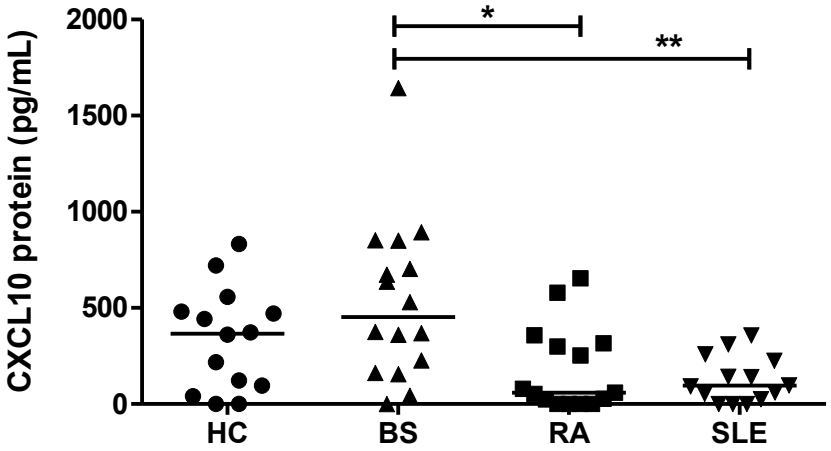
Figure 5-11. CXCL9 mRNA and protein:mRNA ratio. CD14+ monocytes from HC and BS subjects.

Monocytes were stimulated with 20ng/mL IFN-γ for 2 hours. CXCL9 mRNA was semi-quantitatively investigated by Reverse Transcriptase Real Time Polymerase Chain Reaction and results expressed relative to the expression of the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). mRNA expression of CXCL9 showed the opposite pattern to that of CXCL10, with a non-significant trend towards the BS cohort having a higher CXCL9 mRNA expression. The protein to mRNA ratio showed no statistically significant difference between HCs and BS. Analysis: Two tailed Mann Whitney test. Line represents median values. Subject cohort 1-3.

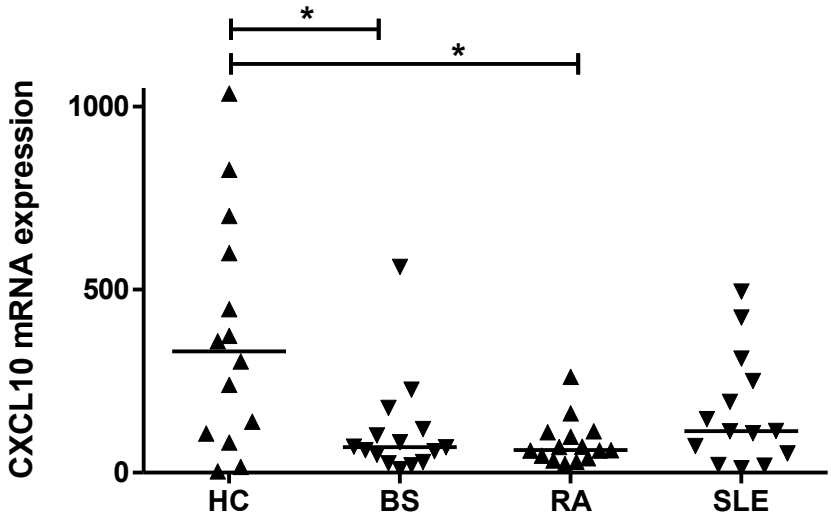
5.6.4.1 Assessing CXCL10 protein and mRNA in disease controls

We next wished to assess if this observation would be found in other inflammatory conditions. We therefore isolated CD14+ monocytes from HC, BS and disease controls (RA and SLE) (cohort 3, Table 5-4). As before, cells were isolated, plated, rested then stimulated with IFN-γ

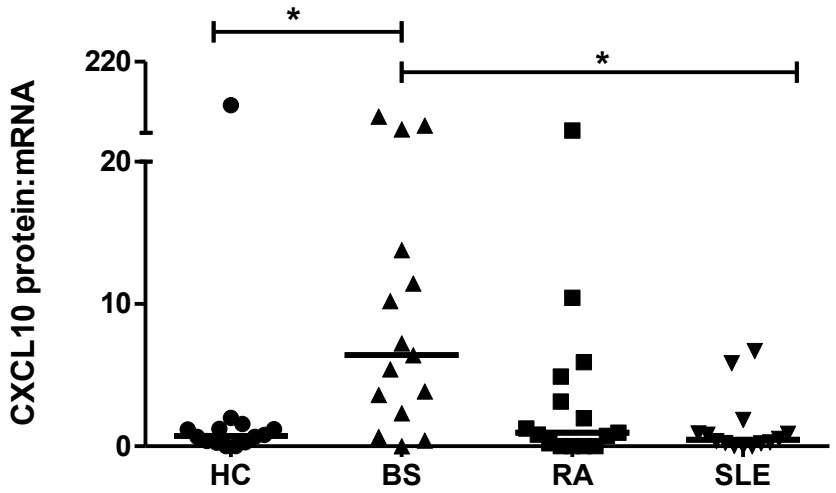
for 2 hours, following which supernatants were collected for ELISA protein measurement, and cells were scraped and stored for mRNA extraction.



(a).



(b).



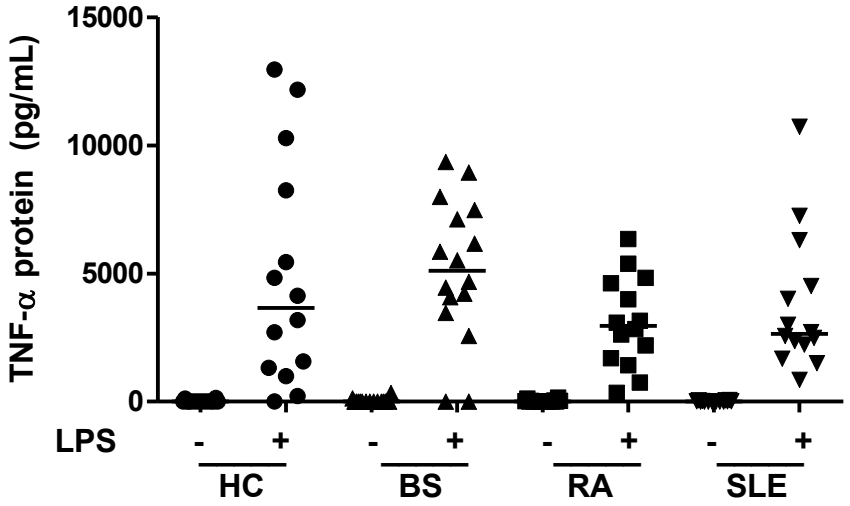
(c).

Figure 5-12. CXCL10 evaluation in Disease controls. CD14+ monocytes from HC, BS, RA and SLE subjects.

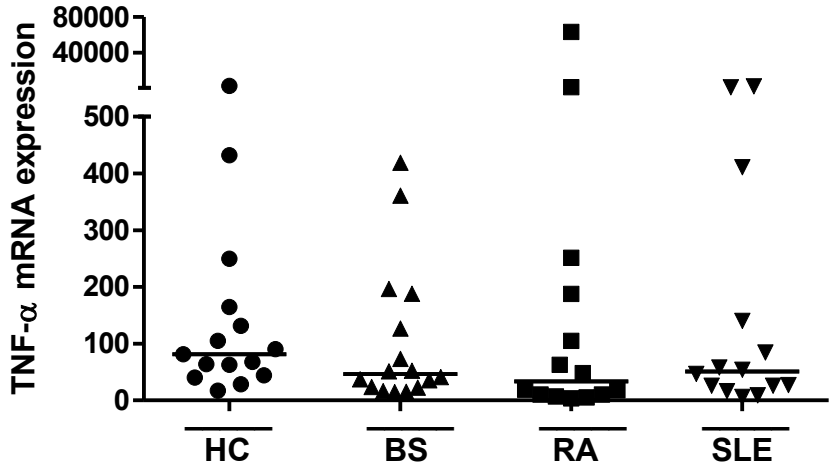
Monocytes were stimulated with 20ng/mL IFN-γ for 2 hours. (a). CXCL10 protein concentration was higher in the supernatant of samples from BS than those of RA ($P<0.05$) or SLE ($P<0.05$) subjects. Analysis of variance between the groups was highly significant ($P=0.0024$). While this cohort did not show a statistically significant difference in protein concentrations between HC and BS ($p=0.17$), pooled results from all cohorts (not shown) continued to show a significant difference. (b). CXCL10 mRNA was semi-quantitatively investigated by Reverse Transcriptase Real Time Polymerase Chain Reaction and results expressed relative to the expression of the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). There was a significant difference between groups ($P=0.02$), with significantly greater mRNA expression in HCs compared to BS or RA subjects ($P<0.05$). (c). The protein to mRNA expression of CXCL10 was highest in the BS cohort. Analysis of variance showed significant variance between the groups ($P=0.008$) with a significant difference between the BS and HC and SLE subjects ($P<0.05$) implying that post-transcriptional regulation may be more important in BS than in the other groups. Analysis: ANOVA to look for variance between all groups and Dunn's multiple correction. Line represents median values. Subject cohort 3.

These results showed that the protein:mRNA pattern observed in the BS cohort was not observed for CXCL10 in the disease control cohorts, indicating that this observation is specific for BS.

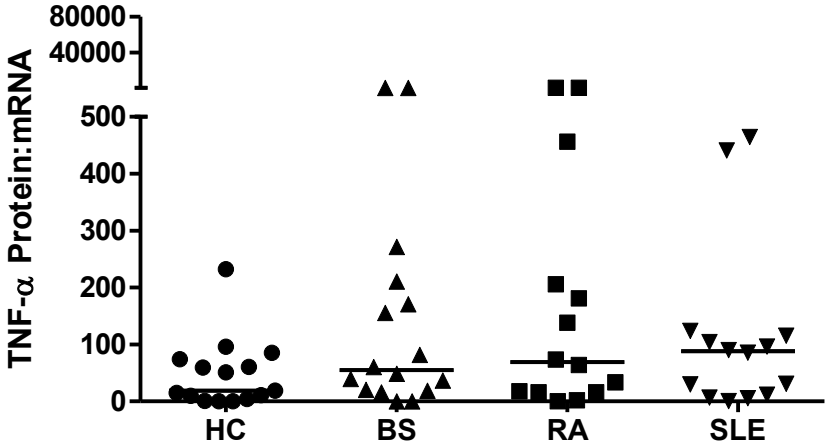
We wished to evaluate stimulus specificity for our observation, to decide if this was specific to the IFN-γ pathway. Monocytes from subjects in Cohort 3 were stimulated with LPS, and the TNF-α protein and mRNA response was evaluated. In the interest of consistency, the 2 hour time point continued to be used. We found that at 2 hours all groups produced a robust TNF-α response to LPS stimulation. Again we found a trend towards an increased protein:mRNA ratio in BS versus HC (non-significant), but the same trend was also observed in both the RA and SLE cohort, indeed the SLE group had the highest ratio. Therefore we concluded that there was no BS specific response regarding protein or mRNA for TNF-α following 20ng/mL LPS stimulation



(a).



(b).



(c).

Figure 5-13. LPS response in BS, HC and Disease Controls. CD14+ monocytes.

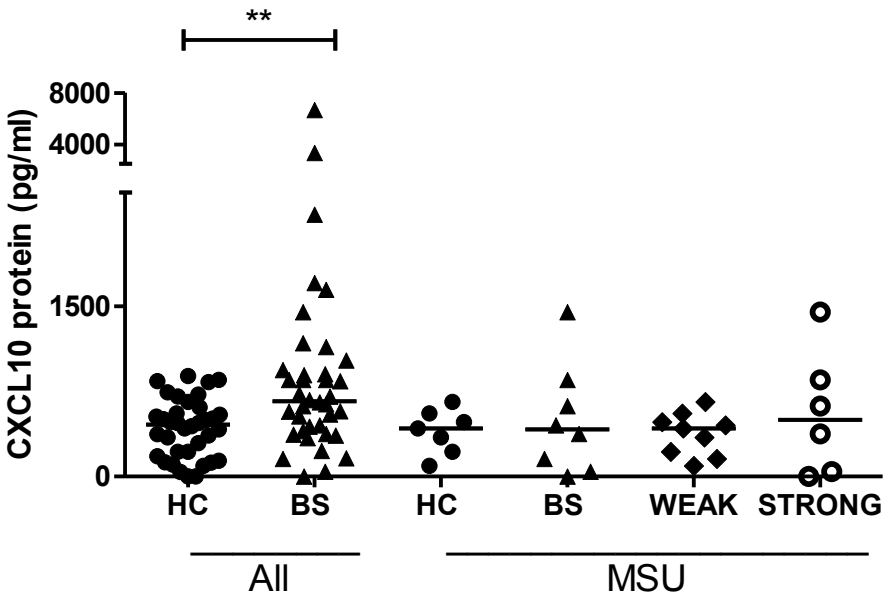
Monocytes were stimulated with 20ng/mL LPS for 2 hours. (a). All groups displayed a robust response with induction of TNF-α protein. There were no statistically significant differences between the groups. (b). TNF-α mRNA was semi-quantitatively investigated by Reverse Transcriptase Real Time Polymerase Chain Reaction and results expressed relative to the expression of the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). There were no significant differences between any of the groups, although there was a trend toward HC subjects having higher mRNA expression than other groups, which had also been seen with IFN-γ stimulation. (c). The protein to mRNA expression of TNF-α was no longer highest in the BS cohort, and there were no significant differences in protein:mRNA ratios

between any group. Analysis: ANOVA to look for variance between all groups Dunn's multiple testing. Line represents median values. Subject cohort 3.

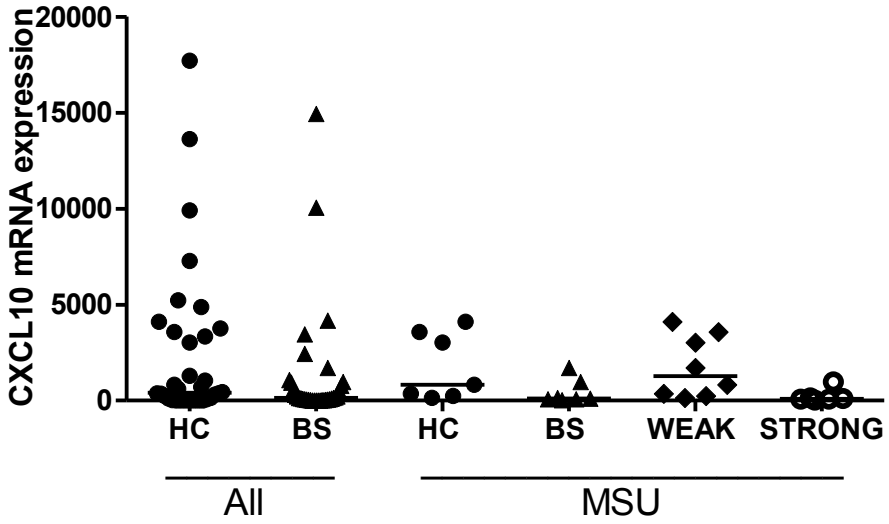
5.6.5 Correlation of observations to clinical features such as MSU response, disease activity and medications

We wished to investigate if the IFN-γ - CXCL10 observation would correlate with clinical features such as the MSU skin response, BS disease activity, BS subtype or medication use. We first evaluated the MSU response. A subgroup of *in vitro* volunteers had also volunteered for the skin blister experiment (Chapter 3), where a MSU skin prick had been undertaken and the size of the response had been recorded. We divided subjects either into HC and BS, or into 'weak' or 'strong' responders to the MSU injection regardless of their diagnosis, and then compared CXCL10 protein and mRNA results. We found no correlation between the *in vitro* IFN-γ-CXCL10 results and the MSU skin prick response, in the subgroup of subjects involved in both experiments.

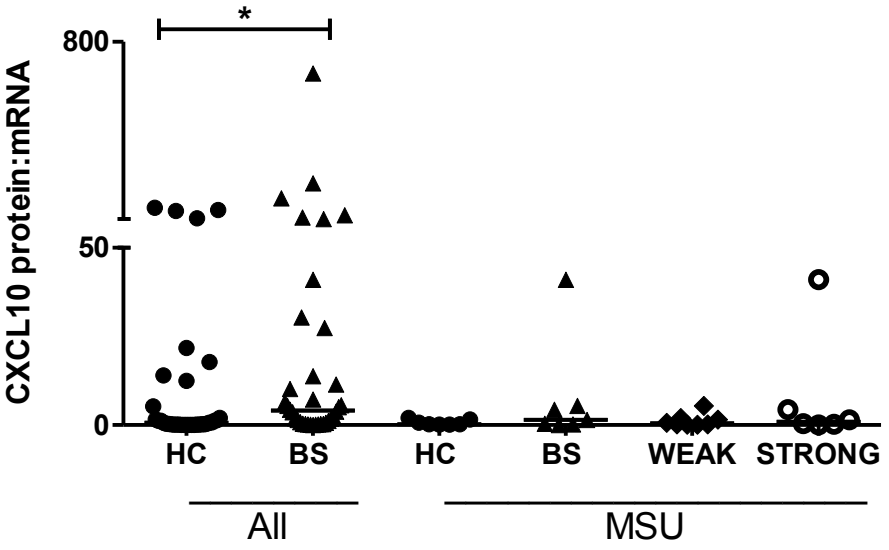
We found it interesting that most of the subjects who had volunteered for the skin blister experiment had quite low concentrations of CXCL10 protein relative to the entire BS cohort and this suggests that this subgroup may have had a milder disease phenotype (which perhaps explains the lack of positive results in Chapter 3).



(a).



(b).



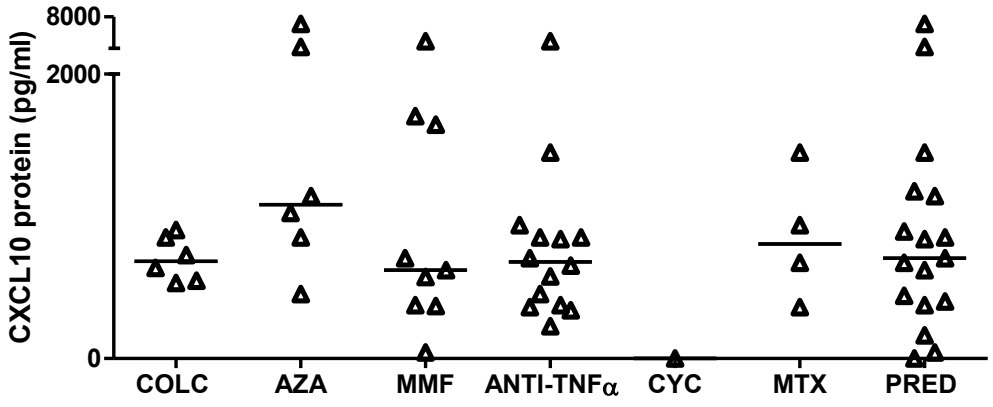
(c).

Figure 5-14. Comparison of *in vitro* IFN-γ – CXCL10 response with the *in vivo* MSU response.

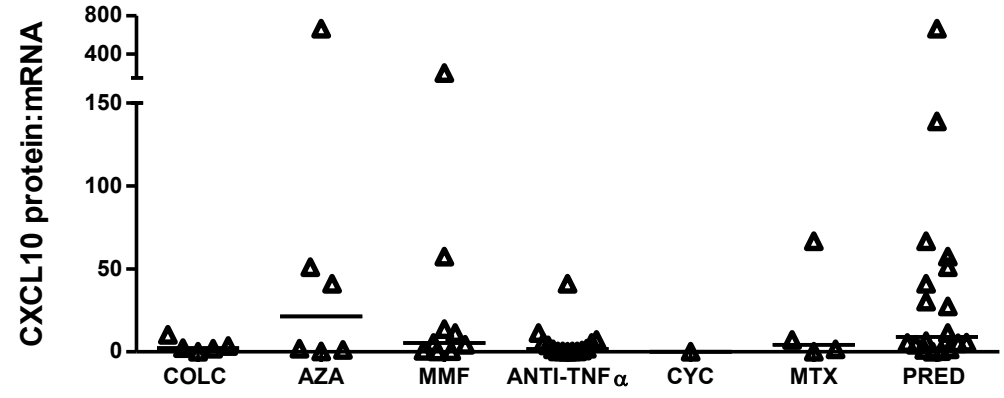
A subgroup of the *in vitro* volunteers had previously volunteered to have an MSU skin prick test as part of the cantharidin skin blister test. These figures pick out the subgroups either by their diagnosis (MSU HC and BS) or by the size of their MSU response regardless of underlying diagnosis (MSU Weak and Strong) and the CXCL10 (a) protein (b) mRNA and (c) protein:mRNA ratio in these subjects are shown. There does not appear to be a strong relationship between the skin response and the *in vitro* results, although numbers are small.

Another interpretation is that subjects chosen for the cantharidin skin blister test perhaps had milder disease phenotype than subjects later recruited for *in vitro* experiments, and this may possibly partially account for the disappointing lack of significant findings in the skin blister chapter.

We next wished to have a look for correlations between medication use and CXCL10 levels. While we were not sufficiently powered to out rule a correlation, we felt that if there were any very strong correlations, this may be a useful evaluation. We found no correlation between medications and CXCL10 levels in BS subjects.



(a)

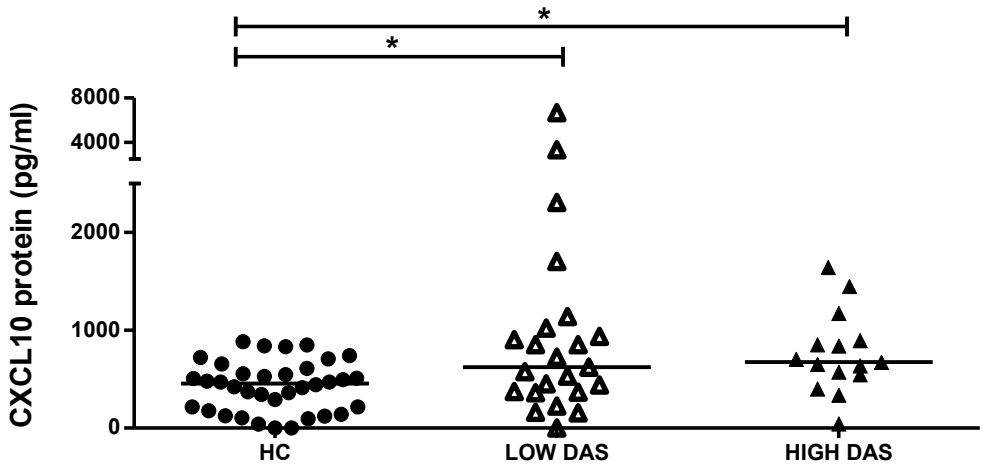


(b).

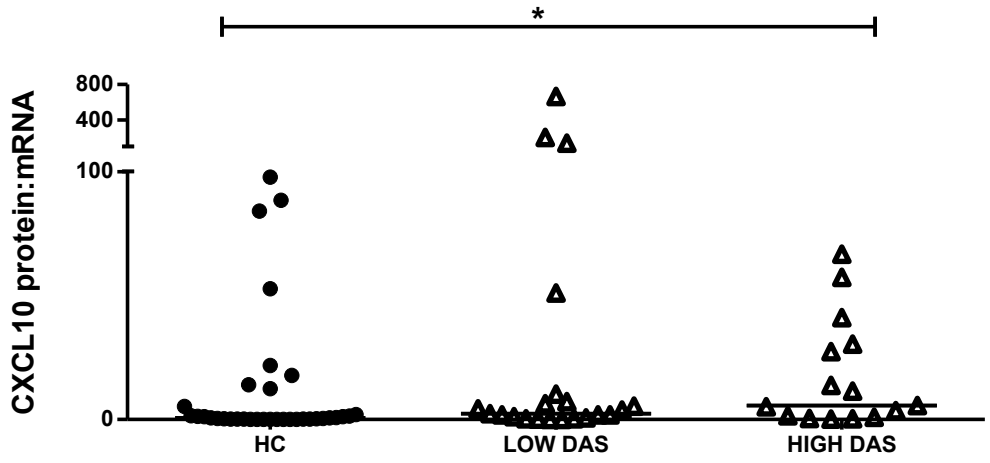
Figure 5-15. Comparison of CXCL10 (a) protein and (b) mRNA levels to medications that BS subjects were concurrently taking.

No significant differences were observed between the groups. The number of subjects recruited was not large enough for the influence of medications to be completely ruled out as partially responsible for our findings.

We next evaluated if CXCL10 protein and protein:mRNA would correlate with disease activity, and we found a positive correlation. We divided the BS cohort into those subjects that had a low or a high disease activity. While both these groups showed a significant difference from HCs at the protein level, when we evaluated the protein:mRNA ratio we found that only the high DAS groups results remained significant. This indicated a correlation in our observation with disease activity.



(a).



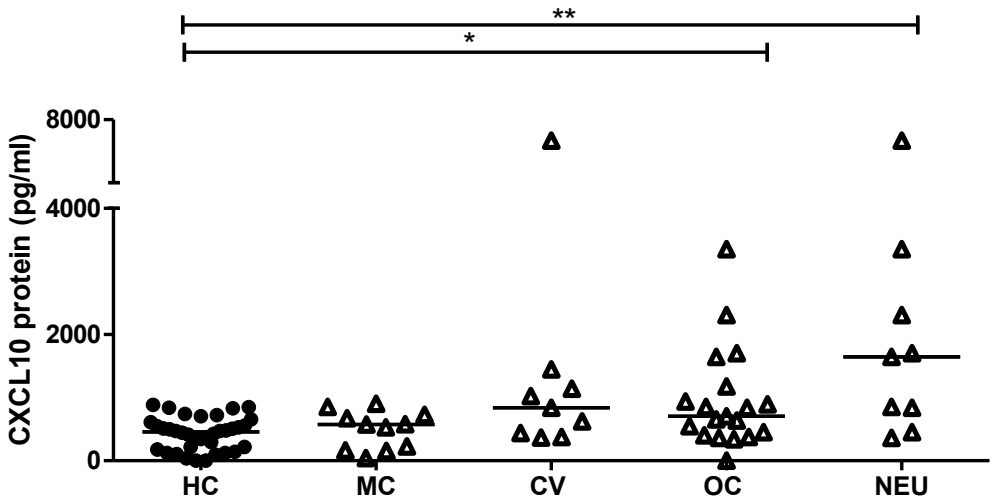
(b).

Figure 5-16. Correlation of CXCL10 (a) protein and (b) protein:mRNA with BS disease activity (DAS).

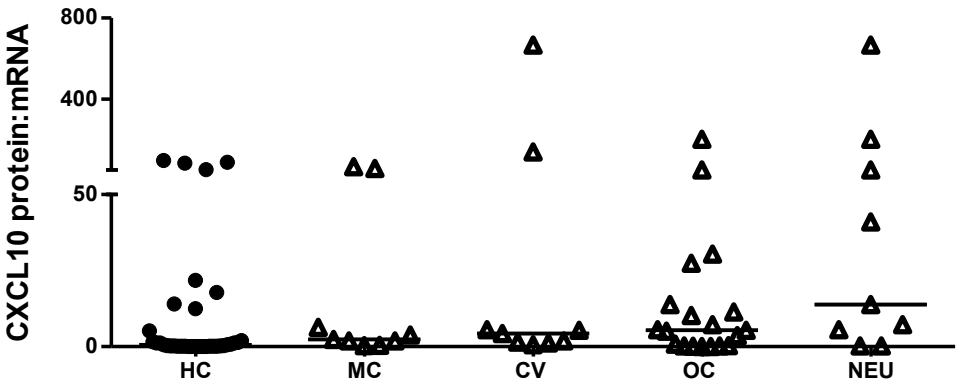
Disease activity was calculated using the Leeds Behçet's disease activity form (2002 version). The score ranges from 0-12 with values of 4 or greater indicating active disease (high DAS). (a) Analysis of variance of CXCL10 protein concentration showed a significant variance between the groups (Kruskal Wallis test of non-parametric analysis of variance P=0.005). Dunn's

multiple comparison test showed a significant difference between HC and low DAS ($P<0.05$) and also HC and High DAS ($P<0.05$). (b) Analysis of variance of CXCL10 protein:mRNA ratio also showed a significant variance between groups (Kruskal Wallis test of non-parametric analysis of variance $P=0.02$). Dunn's multiple comparison test showed a significant difference between the HC group and the high DAS group $P<0.05$, but no significant difference between HC and the low DAS group.

Lastly, we looked for correlations between clinical disease manifestations and CXCL10 protein and protein:mRNA levels. We divided the BS cohort into subgroups depending on clinical manifestations. Those in the mucocutaneous group (MC) had limited mucocutaneous disease with no cardiovascular (CV), ocular (OC) or neurological (NEU) involvement. We found no difference between the limited MC BS subjects and HCs regarding CXCL10 protein, or the protein:mRNA ratio. There was a significant difference in the ocular and neurological groups relative to HC at the protein level, but significance was lost in the protein:mRNA evaluation, once corrections had been made for multiple comparisons. These results indicate that, in particular, neurological and ocular BS show a stronger correlation to the CXCL10 observations than limited mucocutaneous subjects.



(a).



(b).

Figure 5-17. Comparison of CXCL10 (a) protein and (b) protein:mRNA with clinical subgroups of BS subjects.

MC= subjects with limited mucocutaneous manifestations; CV= subjects with a history of cardiovascular or thrombotic manifestations; OC = subjects with a history of ocular inflammation; NEU = subjects with a history of neurological involvement. (a) Analysis of variance of CXCL10 protein concentration showed a significant variance between the groups (Kruskal Wallis test of non-parametric analysis of variance P=0.0006). Dunn's multiple comparison test showed a significant difference between HC and 'ocular' subjects (P<0.05) and also HC and 'neurological' subjects (P<0.05). (b) Analysis of variance of CXCL10 protein:mRNA ratio also showed a significant variance between groups (Kruskal Wallis test of non-parametric analysis of variance P=0.03). Dunn's multiple comparison test showed no significant difference between the HC group and of the BS subgroups. Although differences were not statistically significant with multiple testing corrections, there is a trend towards having a higher protein:mRNA ratio with the ocular and neurological groups, and in contrast the HC and the limited mucocutaneous groups closely resemble each other.

5.6.6 Polysome profiling to assess post-transcriptional regulation of IFN-γ - CXCL-10

Results to this point indicated that BS subjects may translate CXCL10 protein more efficiently than HC or disease controls. To further investigate translational efficiency in BS and HC, sucrose density gradient analysis was undertaken. Sucrose density analysis or polysome analysis is a technique used to investigate differential translation

regulation. RNA from cells is layered on top of a tube containing a sucrose density gradient (sucrose 50% at the bottom to 20% at the top of the tube). It is then spun at high speeds resulting in 'heavy' polysomes travelling further down the tube then 80S or 40S subunits. Free RNA will travel the least far. Following high speed centrifugation, the tube is transferred to the analyser, where a needle pierces the bottom of the tube and 60 % sucrose is introduced slowly to push the contents of the tube into a collecting tube and past a laser (which feeds to a graph tracer giving an image of passing fluid contents), and finally the sucrose is fed into collecting ependorfs, allowing the sample to be collected in fractions and analysed. Usually polysomal fractions reflect actively translating mRNA and free RNA fractions reflect un-transcribing mRNA. The main exception to this is that mRNA may be arrested in both free and polysomal states by miRNAs.

As the sample flows past the laser, a graph voltage tracer produces an image of passing contents.

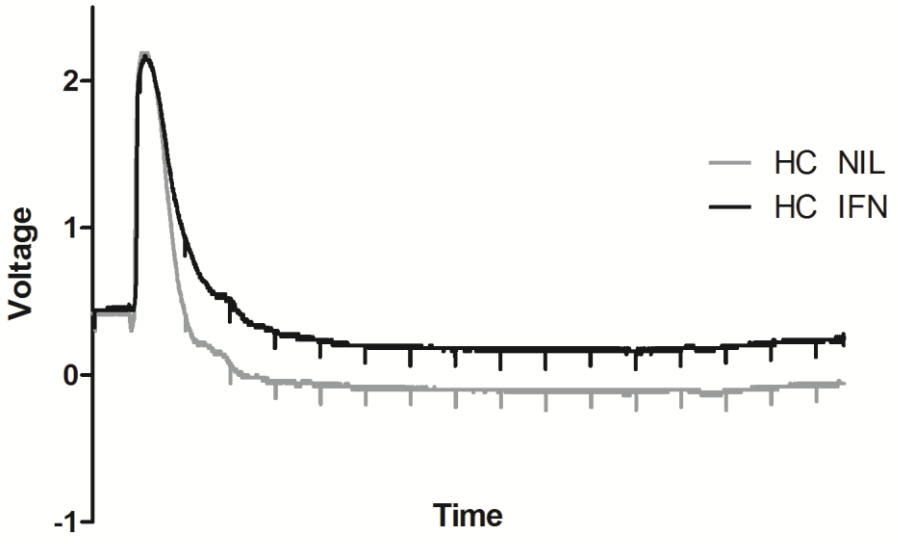


Figure 5-18. A representative voltage tracing of polysome factions within a sucrose density gradient passing though under laser over time.

The far left of the trace represented free RNA contained within the least dense upper faction of the gradient. The middle and later segments representws RNA associated with ribosomal

subunits and polysomes. Samples were collected and mRNA is extracted. A known quantity of GFP was spiked into each sample prior to processing. CXCL10 and GFP mRNA was then measured in each sample, and CXCL10 mRNA expressed relative to the GFP, thereby normalizing for any loss of mRNA that had occurred through the processing. Sample values were expressed as a percentage of the entire CXCL10 mRNA quantity (% occupancy).

For this experiment, we identified the 3 BS subjects with the highest CXCL10 protein:mRNA ratio and the 3 HCs with the lowest CXCL10 protein:mRNA ratio and asked them to attend to donate a further 100ml sample of blood.

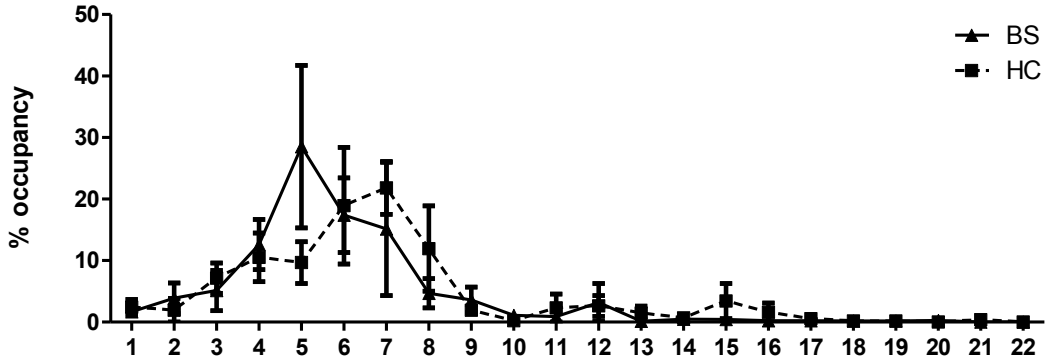
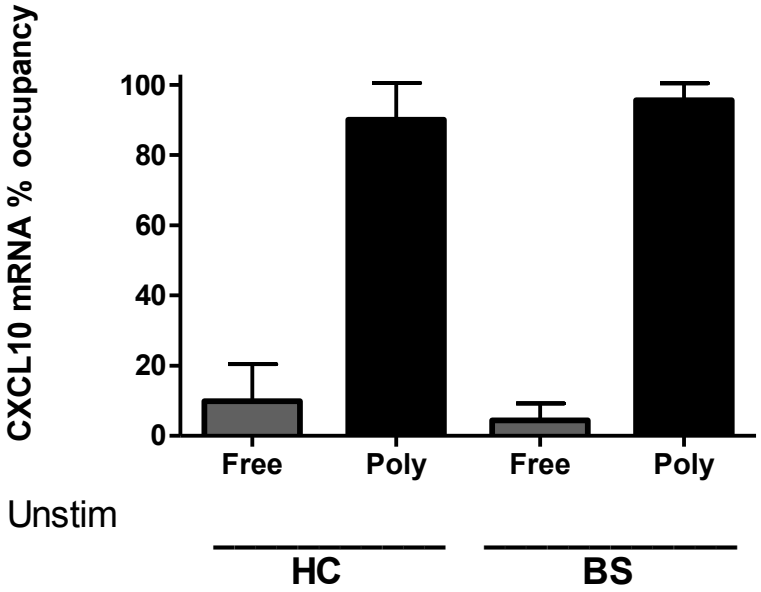
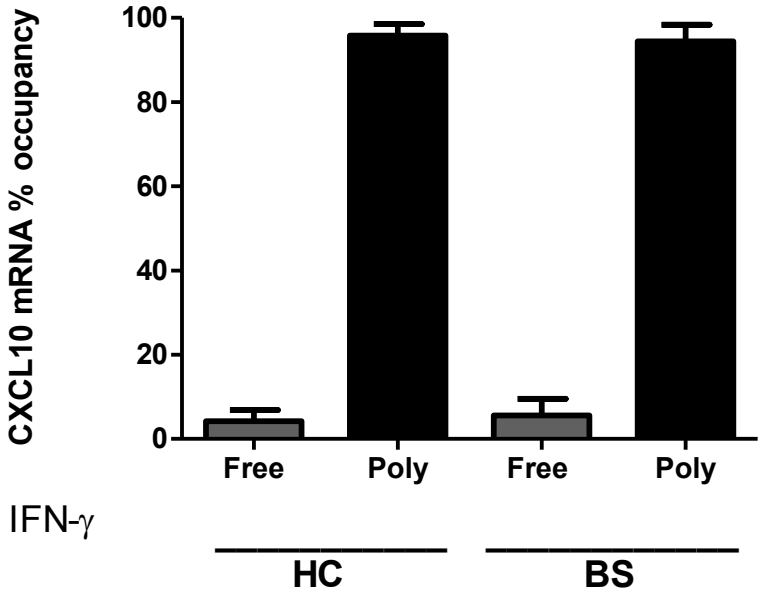


Figure 5-19. The percentage occupancy of CXCL10 mRNA.
CD14+ monocytes from BS (n=3) and HC (n=3) stimulated for 2 hours with IFN-γ (20μL/mL). The quantity of CXCL10 mRNA from each fraction collected (n=22) was expressed as a fraction of the entire CXCL10 mRNA from all the samples. Samples 1 and 2 represent free RNA. Samples from 3 onwards represent ribosomal and polysomal RNA. Samples to the far right represent heavier polysomes that had more ribosomes associated. We found high quantities of mRNA in both HC and BS. Collected samples had a known quantity of GFP RNA added before RNA extraction and RT PCR to normalise values. Values were expressed as mean and standard deviation. There was no statistically significant difference between HC and BS, suggesting that despite different quantities of protein being produced by the two groups, there was no change in polysomal association between the groups.

The laser trace indicated that the samples contained a large quantity of free RNA. However, the RT PCR CXCL10 values of samples collected showed very little of the CXCL10 mRNA existed in this unbound fraction. Most CXCL10 mRNA was found to be associated with polysomes in both BS and HC samples. Unstimulated samples had very low quantities of CXCL10mRNA, and this was true for both BS and HCs, but even in these unstimulated samples, the small quantity of mRNA present tended to be associated with polysomes.



(a).



(b).

Figure 5-20. The proportion of the mRNA present within the free RNA portion or the polysomal portion of each sample (represented as a percentage of the whole).

CD14+ monocytes from BS (n=3) and HC (n=3) (a) unstimulated or (b) stimulated for 2 hours with IFN-γ (20μL/mL). In unstimulated samples overall quantities of CXCL10 were very low, but appeared to be associated with polysomes (although not translating). In stimulated samples we found high quantities of CXCL10 mRNA in both HC and BS which was largely found to be associated with the polysomes. Collected samples had a known quantity of GFP RNA added before RNA extraction and RT PCR in order to normalise values for any loss of RNA during

processing. Values were expressed as mean and standard deviation. There was no statistically significant difference between HC and BS, supporting the finding that despite different quantities of protein being produced by the two groups, there was no change in polysomal association between the groups.

These findings indicate that the explanation for observed differences in protein expression are not related to production of more mRNA, but are more likely to be related to mechanisms involved in control of translation, for example miRNAs.

5.7 Discussion

The IFN-γ – CXCL10 pathway was of interest to us as IFN-γ appears to be an important cytokine in BS and CXCL10 has been found in excess in histological samples in BS subjects, as reviewed in Chapter 1. We observed that serum from BS did not have significantly more IFN-γ protein than HCs, in contrast to SLE samples. Despite this serum from BS subjects had greater quantities of CXCL10 protein. This chapter explored this observation in detail *in vitro*.

We initially stimulated peripheral blood derived CD14+ monocytes from HC and BS with IFN-γ and found that more CXCL10 protein was present in supernatants from the BS cohort at 2 and 4 hours. To assess if this was pre-formed protein, we brought the time point back to zero and found that the significant difference was only observed from 2 hours, indicating this was new protein being translated. Additionally others have shown that CXCL10 is not stored intracellularly, but rather, it is released on production [386].

When we evaluated the mRNA expression of CXCL10 relative to the housekeeping gene HPRT we found no difference between the groups, indeed there was a trend towards less CXCL10 mRNA in the BS cohort. This made transcriptional signalling less likely to account for our observations, and more likely that this as a post transcriptional process. When we plotted the CXCL10 protein to mRNA we found a significantly higher ratio in BS versus HCs, again suggesting post-transcriptional processes.

We next investigated the specificity of this observation. We found no significant difference between CCL2 protein in HC and BS following IFN-γ stimulation, and no significant induction

in either group of CXCL11. While we did find significantly more CXCL9 protein in the BS cohort, when we assessed the CXCL9 mRNA we found no significant differences between mRNA expression in HC and BS, and that the trend was in the opposite direction to CXCL10, with a trend towards more CXCL9 mRNA expression in the BS cohort, indicating that this control was likely to involve transcriptional +/- post transcriptional regulation. The protein:mRNA ratio was still marginally in favour of the BS cohort but differences were not significant, and we concluded that the observation of post-transcriptional regulation of the IFN-γ chemokine pathway in BS that we had observed was relatively specific for CXCL10.

We next wished to investigate if this observation was specific to BS or was observed in other inflammatory conditions. We chose RA and SLE as disease controls because the IFN-γ CXCL10 pathway has been implicated in these diseases. We found that at the 2 hour time point we did not observe an excess of CXCL10 production in these disease controls. We were surprised that these subjects did not produce more protein than HCs, and it is possible that medications played a role, or that the time point was too early. These experiments were also undertaken during winter and possibly our 'HC's had been exposed to more environmental viruses than the disease cohorts. When we assessed the mRNA expression and the CXCL10 protein:mRNA ratio, the RA and SLE cohorts did not show the same pattern of expression or the same elevated protein:mRNA ratio that had been found in the BS cohort.

We were interested in assessing if other stimuli would also induce an exaggerated response in BS. In order to address this, we stimulated CD14+ monocytes with LPS and assessed TNF-α protein production. All groups produced a robust response to LPS stimulation. Again HCs had the highest expression of TNF-α mRNA and it appeared that all the inflammatory cohorts had more efficient translation as compared to the HCs when the protein:mRNA ratio was assessed. However, these results were not statistically significant. Interestingly, for this pathway, it was the SLE cohort that had the highest protein:mRNA ratio.

We concluded from these experiments that only the IFN-γ– CXCL10 pathway showed differential translational control specific to BS.

The possible explanations for this observation include differential post-transcriptional regulation through specific regulation of initiation factors, mRNA stability (although mRNA was if anything reduced in BS, not supporting the notion of increased mRNA stability in BS), differential sequestration of mRNA into processing bodies, miRNAs being more highly (if pro-translational) or less expressed (if inhibitory) in BS, less protein being immediately degraded in BS, less protein being stored in BS (although CXC10 does not appear to be stored), or less degradation of secreted protein in BS. Transcriptional factors may also influence translation, in part by activating miRNA genes, and their role was not clear.

We undertook a sucrose density gradient experiment to assess if there was a difference in polysomal association between HCs and BS. We undertook these experiment in the 3 most 'extreme' responders in the HCs and BS, i.e. those BS subjects with the highest protein:mRNA ratio, and those HCs with the lowest. If differences were at the level of differential regulation of translational initiation we would have expected more polysomal association in the BS cohort than the HCs. If the difference was post-initiation we would not expect to find differences between the two groups. The sucrose density gradient experiments showed similar findings in both groups. Although the majority of the total mRNA in the samples was free mRNA (figure 22 shows the main peak in the free mRNA fraction), very little CXCL10 mRNA was free in either HCs or BS. Levels of CXCL10 mRNA expression were very low in the unstimulated samples, but even in these samples, most of that small amount of mRNA was associated with polysomes. It may be that there is a basal level of CXCL10 mRNA sequestered in association with polysomes and miRNAs, which upon stimulation may dissociate from miRNAs and translation may occur. The results of these experiments lead us to hypothesise that miRNAs may be responsible for the differential CXCL10 protein production in BS. In turn, miRNA expression is controlled by a number of factors, including transcription factors.

Intriguingly, Mycobacterium tuberculosis (MTB) has been shown to increase the production of CXCL10 and CXCL9 protein despite inhibition of CXCL10 and CXCL10 transcription and this mechanism has been explored *in vitro* [386]. IFN-γ is essential to the host defence against MTB, however, the ability of virulent MTB to survive within macrophages for extended periods of

time has led to the notion that it interferes with host signalling pathways activated by INF-γ. MTB has been shown in several studies to inhibit STAT1 [386, 387]. Bai et al studied the virulent strain of MTB Hc7Rv and its effect on IFN-γ signalling in differentiated THP-1 macrophages. They found that MTB infection inhibited IFN-γ stimulated induction of CXCL10 and CXCL9 mRNA, and observed that it unexpectedly enhanced the IFN-γ induced CXCL10 and CXCL9 protein production. They found they could replicate this finding in primary human monocyte derived macrophages. They found that both the p38 MAPK and NF-κB signalling pathways were involved in both inhibition of transcription and enhanced translation. They found MTB blocked STAT1 binding to its cis regulatory element. They found that the inhibition of mRNA was mediated by the NF-κB and p38 MAPK pathways, as this inhibition was no longer seen once highly specific NF-κB or p38MAPK inhibitors were added. This was not seen with the addition of an MEK1 inhibitor. Regarding protein production, stimulation of infected cells with IFN-γ was found to lead to an increase quantity of protein despite inhibited levels of mRNA. Lysed cells contained no CXCL9 or CXCL10 protein confirming that these proteins are not stored intracellularly. This increased protein response could be attenuated by pre-treatment with BAT 11-7082 (MAPK inhibitor) or SB203580 (NF-κB inhibitor) or both, again indicating that MTB via NF-κB and p38 MAPK signalling inhibited IFN-γ induced transcription but increased translation. The study did not assess if these transcription factors influenced miRNA expression, but commented that as no protein was stored intracellularly the mechanism was likely to be secondary to more efficient translation. [386]. The similarities to our study are obvious, and in future work it would be interesting to investigate if these signalling pathways are the link between the initial IFN-γ signal, and the downstream switch resulting in more efficient translation, possibly via regulation of miRNAs.

To our knowledge this approach to investigating protein regulation has not been undertaken in an inflammatory disease before. Our findings show an ‘uncoupling’ in the mRNA:protein ratio in BS. The marked, general uncoupling between transcriptome and translatome gene expression changes allow us to propose a biological model by which the exaggerated inflammatory response in BS may be operating.

5.7.1 Clinical relevance of the observation

The relevance of our findings was investigated by correlating findings with (i) the MSU response; (ii) clinical DAS and characteristics and (iii) medications that the BS subjects were taking. We did not find a correlation between the MSU skin prick response and the CXCL10 observation. The reason for this may be that the two are unrelated. As we found low levels rather than high levels of CXCL10 protein in skin blister fluid, again this suggests that our observation may be more relevant to non-skin related manifestations. However, another interpretation may be that we were unfortunate in choosing subjects with a mild disease phenotype for the earlier experiments, and this is supported by the fact that the CXCL10 protein was not all that elevated in any of these subjects, and they certainly were not the more extreme responders in the *in vitro* experiments. The BS Leeds questionnaire is only a crude measure of disease activity, and there are currently no good biomarkers to determine disease activity or severity reliably. We may have had more exciting results in our skin blister study had we chosen ten other subjects.

We found no significant differences in CXCL10 response in subjects taking different medications, although the experiment was not powered to look for this. The fact that subjects were on such a wide variety of medications, and that our findings were not reproduced in SLE or RA cohorts (who were on the same types of medications), makes it very unlikely that medications account for our observations. Additionally we would have expected a suppressed rather than exaggerated response in the medicated group if medications were influencing outcomes. Unfortunately there is no robust animal model for BS, and the limitation of studying patients who are taking medications is unavoidable, but we think it is not likely that our results can be explained by the effects of medications.

CXCL10 results, both at the protein level and the protein:mRNA ratio were higher in BS subjects with a high DAS than in subjects with a low DAS. While results between high and low DAS were not significantly different, there was a trend for higher CXCL10 with increasing DAS, and it is possible that this would have reached significance with higher numbers. There is currently no reliable biomarker for BS disease activity, and clinical measurement of CXCL10,

possibly as part of a panel, may be clinically useful to assess disease activity of patients. It may be of particular use in patients with ocular or neurological disease, in contrast to mucocutaneous or cardiovascular disease where levels are similar to those of HCs.

It is interesting that CXCL10 was highest in subjects with neurological disease, followed by ocular disease. No difference was seen between levels in HCs and those BS with limited mucocutaneous disease or cardiovascular disease. Where CXCL10 has been found to be implicated in BS, it has usually been related to those with neuro BS [388]. High level of CXCL10 have been found in CSF from BS [135]. CXCL10 has been implicated in neurological involvement of other inflammatory diseases also. For example in Multiple Sclerosis (MS) the both CXCL10 and the CXCR3 receptor has been described as being present on virtually all perivascular T cells and astrocytes associated with active lesions [389]. In addition there is an increased amount of CXCL10 protein in the CSF of patients with active attacks of MS [389]. Our study was not designed to examine the question of disease subtypes and a larger study to assess this would be interesting.

5.7.2 Correlation of elevated CXCL10 protein *in vitro* to low CXCL10 observed in skin blisters

There are several possible explanations for conflicting results between the skin blister model and the *in vitro* findings. Firstly, these are very different investigative tools. A skin blister contains an extensive milieu of cell types and proteins including metalloproteinases and other substances that degrade or take up CXC10. Therefore it is possible that CXCL10 is secreted to a greater extent in the blister environment in BS, but that there are also more CXCR3 receptors binding it, and more MMPs degrading it. It has been shown that biopsies from oral ulcers of BS subjects had increased numbers of CXCR3 receptors [154]. The other explanation is that CXCL10 is not found in high levels in the skin, and given that our findings correlated well to neurological and ocular disease but poorly skin or vascular disease this is an equally valid hypothesis.

5.8 Future work and potential clinical usefulness of this observation

Diagnosis of BS remains largely clinical. Furthermore there is no clinically useful biomarker for disease activity. The condition is poorly understood and treatments are suboptimal. As established from this work, the IFN-γ-CXCL10 inflammatory pathway in monocytes may behave fundamentally differently in BS, and could potentially be used as a tool for differentiating BS patients from other inflammatory conditions, or for assessing disease activity, or potentially to predict patients likely to develop neurological or ocular manifestations, or to identify potential candidates who may benefit from anti-CXCL10 monoclonal antibody therapy. An anti-CXCL10 monoclonal antibody has already been shown to be safe in RA patients, although it was only mildly effective in this disease. It may be that some patients, possibly those with neurological or ocular disease may benefit from this therapy. Additionally, future work aimed at dissecting the pathways involved, for example identifying miRNAs involved or transcriptional pathways that are also influencing translation, may provide us with new targets for treatments.

Future work will initially be directed towards gaining a deeper understanding the potential role miRNAs may play.

6. Concluding Remarks

BS is a multisystem disorder characterized by mucocutaneous lesions (oral ulcers, genital ulcers and skin pustules), arthritis and intraocular inflammation. The pathogenesis of BS is poorly understood but it is considered a complex polygenetic syndrome, with environmental triggers. A Th1 skew has been reported. This project aimed to dissect abnormal inflammatory responses in BS to gain an understanding as to which pathway(s) are responsible for the phenomena**.**

In Chapter 3, the hypothesis that the abnormal cutaneous inflammatory response to "non-specific" stimulation in BS is associated with a failure of deactivation of macrophages during the resolution phase of inflammation was explored using human *in vivo* models of inflammation (MSU injections and skin blisters). Secondary aims were to classify other cells present as well as relevant cytokines in blister fluid. We studied cantharidin-induced skin blisters in 12 BS and 12 Healthy controls (HC), analysing blister fluid cells and cytokines using flow cytometry and multiplex bead cytokine analysis respectively.

Generating suction blisters over inflammatory-responses using the MSU blister model did not turn out to be a useful approach for obtaining inflammatory tissue from BS subjects, although this technique may still have a use for protocols requiring fewer cells and offers the benefit of directly sampling cells and fluid from the site of the MSU reaction without the need for a biopsy. The cantharidin skin blister method was found to be a useful and robust model of skin inflammation, giving reliable volumes of fluid and adequate cell numbers for all analysis. We found trends but no significant differences between BS and HC with regard to our primary aim, assessing markers of macrophage activation/deactivation. In both HC and BS, subjects had low percentages of macrophages in early blisters (where neutrophils predominated) which increased in late blisters, and there was a non-significant trend towards BS having less deactivation in later blisters. However this finding was not statistically significant. Most subjects had low HLA DR in peripheral blood which increased in the early blister and begun to settle in the late blister. It is possible that with higher numbers of participants, and by excluding BS subjects taking corticosteroids we may have found greater differences. Both HC and BS had high percentages

of neutrophils in early blisters and fewer in late blisters. Low levels of lymphocytes and natural killer cells were observed in both groups at all time points which made it difficult to look for differences between the groups with our numbers. We were surprised that we found no differences in any cell type or cytokine level between the two groups apart from CXCL10 levels which were significantly different, but that significance was lost with multiple testing adjustments. *In vitro* also led us to wonder if we had chosen subjects with quite a mild disease phenotype for the skin blister experiment, as these subjects had among the least dramatic rise in CXCL10 protein.

The erythema surrounding blisters in BS subjects was a very interesting finding. This correlated with the size of erythematous responses to MSU crystals, but was not associated with any obvious differences in immune cells or cytokines. We reflected on what other mechanisms could be resulting in this erythematous response. Neuropeptides came to mind for several reasons, as agents that could be playing a role in this setting

In Chapter 4, the hypothesis that the abnormal cutaneous inflammatory response may involve neuropeptides was explored. We applied capsaicin topically to BS and HC, and measured the resultant change in dermal blood flow using laser doppler imaging to evaluate if neuropeptides such as CGRP may play a role in the abnormal cutaneous inflammatory response in BS. The primary question was to assess if there was a difference in capsaicin response between HC and BS. We also wished to assess if subjects with a large MSU skin reaction and subjects who had an erythematous response to the cantharidin blisters also had an abnormal capsaicin skin test, to see if this could offer the possibility of a quicker and simpler test than the MSU injection. Finding a simpler test to replace MSU /pathergy testing would be a useful advance. BS subjects who experience pathergy tend to describe an exaggerated response to any stimuli, from thorn pricks and nettle stings to surgical cuts and MSU injections. Therefore we were surprised when, contrary to our expectations, the BS cohort did not mount an exaggerated flare response to capsaicin. In fact the BS cohort displayed a smaller flare response than HCs. This experiment therefore did not find evidence of an exaggerated flare in BS subjects and did not find the capsaicin skin test to be of diagnostic use. Possible reasons for the smaller flare response found

in the BS cohort are either study limitation factors (a false negative) or else that BS subjects do release less CGRP upon stimulation (a true positive). The findings of this chapter may reflect a true representation of CGRP release in BS. CGRP differs from SP in that, in addition to vasodilation, there is evidence of it having anti-inflammatory properties. As discussed in detail in the introduction to Chapter 4, CGRP leads to less antigen presentation, inflammatory cell recruitment and also directly stimulates IL-10 release. A SNP in IL-10 which results in less IL-10 protein production in the disease-associated-allele was discovered in the first two GWAS studies of BS. Less IL-10 may slow down the resolution phase of inflammation, and therefore it is possible that if BS patients secrete less GCRP in response to insults such as MSU injections, then this may result in less activation of or slower activation of cutaneous anti-inflammatory pathways leading to a prolonged cutaneous inflammatory response, as seen following MSU injections.

Chapter 5 summarizes key *in vitro* experiments undertaken alongside *in vivo* experiments. The IFN-γ / chemokine pathways were explored in detail in CD14+ monocyte populations in BS, HC and disease control subjects. Significant differences were observed in the IFN-γ-CXCL10 pathway response in BS compared to HC and other disease controls. We found that following IFN-γ stimulation, monocytes from BS produced equivalent mRNA but more CXCL10 protein, indicating differential post-transcriptional regulation. Sucrose density gradient experiments suggested a role for miRNAs in this observation, as there was no difference in polysomal association of CXCL10 mRNA in BS and HCs. We have discovered that BS monocytes have dysfunctional post-transcriptional regulation of CXCL-10 mRNA, resulting in overexpression of CXCL-10 protein upon IFN-γ stimulation. There is an uncoupling of the transcriptome from the translatome. This may contribute to the exaggerated inflammatory responses that characterises BS.

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