Pathogenic immune responses in Spondyloarthritis

Mohammad Hussein Al-Mossawi

Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences

Botnar Research Centre

University of Oxford

Jesus College

Hilary Term 2016

Thesis submitted for the degree of DPhil

Supervisors: Professor Paul Bowness & Professor Fiona Powrie

Mohammad Hussein Al-Mossawi
Jesus College, University of Oxford
Thesis submitted for degree of Doctor of Philosophy (D.Phil), Hilary Term, 2016

Pathogenic immune responses in Spondyloarthritis

Abstract

Immune dysfunction in spondyloarthritis (SpA) causes significant morbidity to a large number of patients. An understanding of the underlying pathological processes involved in causing the disease is essential for the development and better targeting of therapies.

I have shown for the first time in patients with SpA an overall expansion of peripheral blood and synovial fluid T cells producing GM-CSF and an expansion of GM-CSF positive Th17 cells. In addition, I have shown GM-CSF to be a major effector cytokine of joint-derived innate lymphoid cells. Surface phenotyping and transcriptional analysis of the IL-17A/GM-CSF double positive cells showed that whilst they are related to classic Th17 cells, they also have a uniquely activated transcriptional profile.

Additionally, I have shown IL-7 to be a promoter of GM-CSF production by CD4 T lymphocytes in vitro. However, my data shows that polymorphisms of the IL7R, which have been shown to be associated with AS in genome-wide association studies, play a functional role through cell surface expression of the IL7R on CD14 monocytes. This genotypic expression of the IL7R on monocytes is only seen after activation with LPS or TNFα. I further show CD14 monocytes expressing IL7R to be present in SpA joints.

My data shows that RORγt, the master transcription factor of Th17 cells, can be therapeutically targeted using small molecule inhibitors in-vitro. These inhibitors lead to a specific suppression of both polyfunctional and IL-17A single positive Th17 cells.

The work in this thesis has highlighted several areas of novel immune biology with potential therapeutic applications in SpA and across the spectrum of related inflammatory diseases.

Declaration

I declare that the work presented in this D.Phil thesis entitled “Pathogenic immune responses in Spondyloarthritis” is entirely my own work, except for where the contributions of my collaborators have been clearly acknowledged. No part of my thesis has been submitted for any degree or other qualification in this University or elsewhere.

Mohammad Hussein Al-Mossawi

Hilary Term 2016

Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences

Botnar Research Centre

University of Oxford

Acknowledgements

I wish to thank my supervisors Prof Paul Bowness and Prof Fiona Powrie for their help and support over the last three years. In particular I wish to thank Paul Bowness for inspiring me to do research and mentoring me in the lab. I wish to thank the members of the Bowness lab for being so welcoming and accommodating, in particular Dr Anna Ridley who tutored me in the many of the laboratory techniques in the early period of the DPhil. I would also like to thank Dr Ben Fairfax for his support and his contribution to the IL7R chapter of this thesis. I am fortunate to have a very supportive family who have always inspired me. In particular, I would like to thank my wife Dr Clare Statham for her companionship and immense support over the years and for proofreading this thesis. I would also like to thank my examiners Professors Ogg and Wedderburn for the stimulating discussion at the viva. Finally I would like to thank my funding body the Wellcome Trust for their generous support and inspiration.

Botnar Research Centre
Dr Jelle de Wit
Dr Anna Ridley
Dr Kristy McHugh
Dr Ariane Hammitzsch
Dr Liye Chen
Prof Paul Bowness

Kennedy Institute/Translational Gastroenterology Unit
Dr Ahmed Hegazy
Dr Michael Huhn
Dr Ben Owens
Dr Anna-Lena Schaupp
Jonathan Webber
Prof Fiona Powrie

Wellcome Trust Centre for Human Genetics
Dr Ben Fairfax
Prof Julian Knight

Weatherall Inistitute of Molecular Medicine
Dr Maryam Salimi
Prof Graham Ogg

Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	Analysis of variance
APCs	Antigen presenting cells
APC	Allophycocyanin
AS	Ankylosing spondylitis
ASAS	Assessment of Spondyloarthritis International Society
BASDAI	Bath Ankylosing Spondylitis Disease Activity Index
BSA	Bovine serum albumin
BFA	Brefeldin-A
CCR	CC chemokine receptor
CHOP	C/EBP homologous protein
CRP	C-reactive protein
CSM	Cell staining medium
DC	Dendritic cell
DMARDs	Disease modifying anti-rheumatic drugs
DMSO	Dimethyl sulphoxide
EA	Enteropathic arthritis
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENAC	Epithelial sodium channel
eQTL	Expression quantitative trait loci
EULAR	European League Against Rheumatism
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDR	False discovery rate
FITC	Fluoresceinisothiocyanate

FSC	Forward scatter
GM-CSF	Granulocyte macrophage colony-stimulating factor
GM-CSFR	Granulocyte macrophage colony-stimulating factor receptor
GRP78	Glucose-regulated protein
GWAS	Genome wide association studies
HC	Healthy control
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICS	Intracellular staining
IFN	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IL7R	Interleukin-7 receptor
ILC	Innate lymphoid cell
IFIA	Immune function in inflammatory arthritis
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
IVIG	Intravenous immunoglobulin
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
KIR	Killer immunoglobulin-like receptor
LD	Linkage disequilibrium
LTBR	Lymphotoxin beta receptor
LTi	Lymphoid tissue inducer
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MoDC	Monocyte derived dendritic cell
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NET	Neutrophil extracellular traps
NICE	National institute for health and care excellence
NK	Natural killer
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
PAP	Pulmonary alveolar protreinosis
PB	Pacific blue
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR-SSP	Polymerase chain reaction sequence specific primers
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PMA	Phorbol-12-myristate-13-acetate
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
ReA	Reactive arthritis
ROR	Retinoic –related orphan receptor
RPM	Revolutions per minute
SCID	Severe combined immunodeficiency
SFMC	Synovial fluid mononuclear cells
SGK1	Serum glucocorticoid kinase 1
SpA	Spondyloarthritis
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation induced cytokine

TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cells
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UPR	Unfolded protein response

Chapter 1: Introduction

1.1 Spondyloarthritis

1.1.1 Clinical features

The term spondyloarthritis encompasses a group of inflammatory diseases with common pathological and genetic features. Classically this group of disorders was classified into distinct diseases including Ankylosing Spondyltis (AS) Reactive Arthritis (ReA), Psoriatic Arthritis (PsA) and Enteropathic Arthritis (EA). More recently this classification has been changed by international consensus and patients are diagnosed as having spondyloarthritis (SpA) with axial (spine) or peripheral involvement (M. Rudwaleit et al. 2010). As the disease progresses the phenotype can often change in the same individual and other organ systems can be involved. The prevalence of Spondyloarhtritis is estimated to be in the range of 0.5-2% in European populations (Andrianakos et al. 2003; Jürgen Braun et al. 1998; J. Braun, Listing, and Sieper 2005; Bruges-Armas et al. 2002; Saraux et al. 2005).

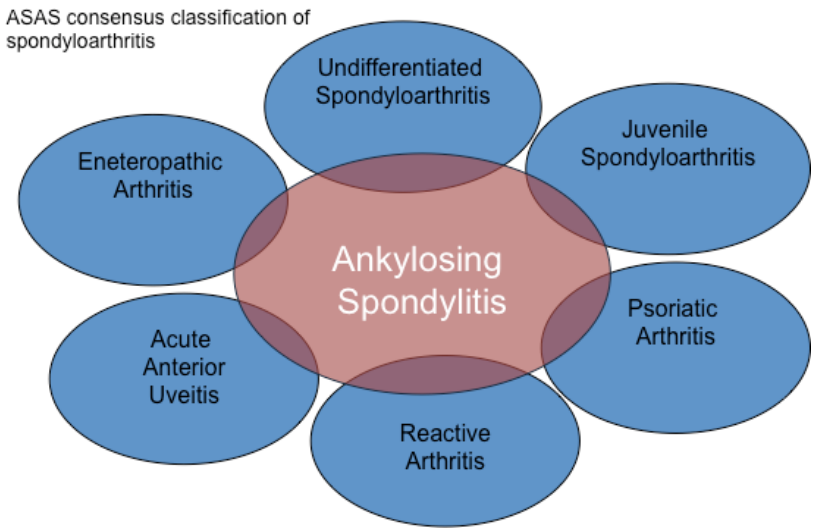


Figure 1.1. ASAS consensus classification of spondyloarthritis.
Adapted from www.asas-group.org

1.1.2 Prevalence and Diagnosis of Ankylosing Spondylitis

Ankylosing spondylitis (AS) is a common chronic inflammatory disease affecting approximately 0.5% of European populations (J Braun et al. 1998). The disease typically causes inflammation in the spine and sarco-illiac joints of patients and this can progress to new bone formation and eventual fusion of the spine in severe cases (Tam, Gu, and Yu 2010). The onset of disease is typically in the third decade of life (Feldtkeller et al. 2003) and patients suffer with inflammatory back pain, which is worse in the morning and associated with stiffness but improves as the day continues (M Rudwaleit et al. 2006). Diagnosis was traditionally made based on the modified NY criteria, 1984, which use radiographic changes at the sacroiliac joint as the key determinant plus either a history of inflammatory back pain or limitations in lumbar spine motion or chest expansion as supporting features (Linden, Valkenburg, and Cats 1984). However, it is widely accepted that sacoilliac changes are a late feature of the disease and do not occur in all individuals. The average age of symptom onset to formal diagnosis has been reported to be up to 10 years (Feldtkeller et al. 2003), partly because of the indolent nature of the symptoms and the importance of radiographic changes in the modified New York criteria.

Modern imaging modalities especially MRI have allowed earlier diagnosis of axial inflammation in patients who suffer from inflammatory back pain (M. Rudwaleit, Jurik, et al. 2009). This has led to the development of new classification criteria by the assessment of spondyloarthritis international society, which are more focused on inflammatory back pain and take into account MRI imaging and genetic risk (M. Rudwaleit et al. 2010). Based on

these new criteria patients can be classified as having peripheral or axial SpA. Within axial SpA there is now a new subgroup called non-radiographic axial SpA where there is spinal inflammation on MRI but no new bone formation on x-ray (M. Rudwaleit, Landewé, et al. 2009). In reality these patients are a mix of early AS, some of whom will go on to develop new bone formation, together with a distinct subset of disease who may never develop new bone formation, and were previously overlooked by the New York criteria. Unfortunately current MRI imaging is unable to distinguish these two subgroups within the non-radiographic axial SpA.

1.1.3 Overlap with inflammatory bowel disease & the role of the microbiome

A particular feature of SpA is the clinical overlap with inflammatory bowel disease. Up to 30% of patients with inflammatory bowel disease develop either axial or peripheral arthritis, where the severity often correlates with the amount of bowel inflammation (Orchard et al. 2000). In addition, up to 60% of SpA patients who do not have gut symptoms show evidence of macro or microscopic inflammation on endoscopy (De Vos et al. 1989; Eliakim et al. 2005). This overlap has led many in the field to suggest a link between gut and joint inflammation in SpA via the “joint-gut axis” where immune response priming and loss of tolerance is initiated in the gut and subsequently spreads to the joints (Brakenhoff et al. 2010; Praet et al. 2012; Jacques and Elewaut 2008). However the precise mechanisms for this theory remain very poorly understood.

Recent advances in gut microbial phenotyping using bacterial 16S ribosomal sequencing have suggested that alterations in the microbial populations of the gut may play an importnant role in the loss of self-tolerance observed in autoimmunity (Proal, Albert, and Marshall 2013). The use of sequencing techniques has allowed the study of microbial gut populations that are often difficult to identify using more traditional culture based techniques (Grice and Segre 2012; Drancourt et al. 2000).

The importance of the gut microbiome in SpA has long been suspected because of the observation that B27-transgenic rat models of AS do not develop the disease in germ-free conditions (Rath et al. 1996). Moreover a recent study using the SKG mouse model of SpA where the T cell receptor signal strength is altered due to a mutation in the signalling molecule ZAP-70 (Sakaguchi et al. 2003) found the arthritis to be significantly attenuated under germ-free conditions (Rehaume et al. 2014). The only published study in human SpA used 16S microbial sequencing to interrogate the microbiome in terminal ileal biopsies from 9 patients with AS compared to healthy volunteers. The authors found significant increases in bacterial families in AS (Lachnospiraceae, Ruminococcaceae, Rikenellaceae, Porphyromonadaceae, Bacteroidaceae) and a decrease in abundance of two families (Veilonellaceae and Prevotellaceae) (Costello et al. 2015). Other studies have suggested a microbiome alteration in early rheumatoid arthritis based on 16S sequencing of stool derived gut microbiome with an abundance of Prevotella corpi in rheumatoid arthritis (RA) (Scher et al. 2013). Collectively these observations provide more tangible evidence for the “gut-joint axis” but the precise

mechanisms that drive microbiome-mediated loss of tolerance in the gut and how this might lead to a manifestation of the disease in the joint still remain unsolved.

1.1.4 Overlap with uveitis

Apart from the gut, other extra-articular manifestations of the spondyloarthritides include inflammation in the eyes. Approximately one third of patients with ankylosing spondylitis will suffer attacks of anterior uveitis. Individuals who are HLA-B27 positive have a particularly increased risk of developing this complication with an odds ratio of 4.2 compared to HLA-B27 negative AS patients (Zeboulon, Dougados, and Gossec 2008). Increasing evidence seems to suggest an important role for type-17 immunity in driving the pathogensis of uveitis (R. W. J. Lee and Dick 2012). However clinical trials of the anti-IL-17A antibody failed to show benefit in this disease group (Dick et al. 2013). It is still not clear why some patients develop certain extra-articular manifestations while others do not, and why therapies targeting IL-17A do not show the same efficacy in the different affected organ systems.

1.1.5 Overlap with psoriasis

Up to 10% of patients who have a primary diagnosis of ankylosing spondylitis develop skin psoriasis at some point during the course of their illness (Stolwijk et al. 2015). On the other hand, the prevalence of axial disease in patients with a primary diagnosis of psoriatic arthritis been reported to be as high as 70% in some studies based on sacroiliac joint radiograph analysis (Battistone

et al. 1999). However, other studies which look at the pattern of symptoms in PsA patients report much lower rates of axial involvement at around 5% (Lindqvist et al. 2008). One intriguing observation has been the development of psoriasis (Collamer and Battafarano 2010) and other autoimmune disease such as uveitis (Wendling et al. 2011) in SpA patients triggered by treatment with anti-TNFα antibodies. A particular association with Etanercept was observed for the triggering of uveitis with 23/31 (74%) reported cases associated this agent rather than the other two agents Adalimumab and Infliximab. Interestingly a randomised controlled trail of Etnercept treatment for uveitis in JIA failed to show any benefit in this patient group (Smith et al. 2005) but the analysis of uveitis rates in randomised controlled trials of Etanercept in AS did not show an increase incidence of uveitis (Sieper et al. 2010).

The only prospective study of new onset psoriasis in anti-TNFα (Infliximab and Adalimumab) treated individuals with IBD showed 5% of patients develop this paradoxical phenomenon and treatment with Ustekinumab can be used successfully in these patients (Tillack et al. 2014). The mechanism of these paradoxical immune phenomenona is not understood and so far these types of anti-TNFα triggered autoimmune disease have not been reported with agents targeting IL-17A (although the experience in using these agents is significantly shorter compared to anti-TNFα agents).

1.2 Genetics of AS

AS has long been associated with inheritance of the Human Leukocyte Antigen (HLA) allele B27 (Schlosstein et al. 1973). In addition, more recent genome wide association (GWAS) studies have highlighted several other important genes involved in the interleukin-17 (IL-17) and interleukin-23 (IL-23) inflammatory axis (D. M. Evans et al. 2011). The most recent GWAS study conducted by the International Genetics of AS (IGAS) consortium has now implicated over 40 genes in AS (Cortes et al. 2013).

The HLA-B27 association with AS is one of the strongest genetic associations of any common human disease. Heterozygote HLA-B27 carriage results in an increased odds ratio for AS of around 50 while homozygosity has an odds ratio of around 100. The concordance in monozygotic twins is 63% and the risk in first degree relative is 8.2%. Of the 136 reported subtypes of B27, B*2702, B*2703, B*2704, B*2705, and B*2710 are reported to significantly increase risk, whereas B*2706 and B*2709 are not associated with disease. Even though up to 94% of patients with AS are HLA-B27 positive, this only accounts for 30% of the total heritability of the disease which is estimated to be in the region of 90% (Bowness 2015).

Other important genetic loci which have been identified encode two endoplasmic reticulum aminopeptidases (ERAP-1 and ERAP-2) on chromosome 5, which are involved in peptide trimming for presentation on MHC class I. Disease associated ERAP-1 variants only increase risk of AS in the context of HLA-B27, suggesting that the effects of ERAP-1 disease-

associated polymorphisms are likely to play out through altering the interaction of HLA-B27 with peptides. This was confirmed in a study which showed silencing of ERAP-1 in cell lines altered the length of peptides presented by HLA-B27 (Chen et al. 2014).

Another important pathway that has been genetically linked with AS is the IL-23/IL-17 pathway. IL-23R signalling on CD4 T-helper cells is required for the differentiation of Th17 cells (McGeachy et al. 2009). Polymorphisms of this receptor have been strongly associated with the risk of developing AS (Burton et al. 2007; Reveille et al. 2010) and psoriasis (Nair et al. 2009). It was later shown that the protective allele coded for a loss of function mutation resulting in defective signalling and decreased percentage of Th17 cells (Laggner et al. 2011). The IL-23R receptor signals via downstream signalling cascades including STAT3 and TYK2 (Cho et al. 2006, 3; Parham 2002). Polymorphisms in these molecules have also shown disease susceptibility in AS (Danoy et al. 2010; Cortes et al. 2013). In particular rare variants of TYK2 show an odds ratio of 7.7 for the disease. This is one of the strongest non-MHC genetic effects observed in AS (Robinson and Brown 2014).

Another important cytokine involved in Th17 differentiation is IL-6 (Zhou et al. 2007). Polymorphisms in the IL6R gene were shown to be associated with AS (Cortes et al. 2013) and Asthma (Dehghan et al. 2011). The importance of IL-6 signalling in Th17 biology and the success of monoclonal antibodies targeting IL-6 in RA (Maini et al. 2006) led to similar trials in AS and SpA. However two trials in AS failed to show sufficient efficacy in AS (Henes et al.

2010; Sieper et al. 2014). Interestingly this same polymorphism is associated with RA but in the opposite direction (Eyre et al. 2012). This highlights the importance of a good understanding of the immunobiology of these pathways before embarking on costly randomised controlled trials.

Various T cell transcription factors and pathways involved in T cell proliferation and survival have also been shown to be associated with AS. Some of these the key transcription factors include EOMES, RUNX3 and TBX21 (Reveille et al. 2010; Cortes et al. 2013). RUNX3 is a key transcription factor for the development of CD8 cells in the thymus (J.-H. Park et al. 2010) and AS patients carrying the disease-associated polymorphism have been shown to have lower numbers of circulating CD8 cells (D. M. Evans et al. 2011). One of the key activating cytokines for RUNX3 in CD8 cells is IL-7. Polymorphisms in the IL7Rα gene have been associated with AS (Cortes et al. 2013; D. M. Evans et al. 2011) and with several other inflammatory diseases including multiple sclerosis (Gregory et al. 2007) and primary biliary sclerosis (Mells et al. 2011). It is not clear how the interactions between IL-7 signalling and RUNX3 play out at a functional level in AS and other diseases.

1.3 Pathogenesis of SpA

A main pathological feature of SpA is inflammation at the enthesis (the site of attachment of bone into tendon or ligament) (McGonagle, Gibbon, and Emery 1998). This distinct anatomical localisation of the disease remains poorly understood but it has been suggested that the enthesis is a site of high stress and that inflammation is due to disregulation of normal homeostatic repair

mechanisms at this site. More recently murine studies have suggested that the enthesis has a distinct resident immune cell population which can become over-activated in the context of chronic inflammation and elevated levels of systemic cytokines (Sherlock et al. 2012). The existence of these cell populations in humans has not yet been established, but if shown this would be of huge significance as it would provide a pathological basis of inflammation at the enthesis.

The role of HLA-B27 in disease pathogenesis remains elusive 40 years after the initial discovery of its association. The most obvious lines of enquiry looked to identify specific self or environmental peptides presented by HLA-B27 that would activate CD8 cells via the MHC class I pathway. A particularly encouraging observation was the discovery of antigen-specific CD8 cells using HLA-B27 tetramers in Chlamydia-triggered reactive arthritis (Appel et al. 2004). Unfortunately no arthrogenic peptides have been identified to date in AS and furthermore, disease was seen to develop in the B27 transgenic rat models in the absence CD8 cells (Taurog et al. 2009).

A second theory for the pathogensis of HLA-B27 in AS stems from the observation that HLA-B27 can misfold in the endoplasmic reticulum (Mear et al. 1999). This misfolding leads to ER stress which activates the transcription factor C/EBP homologous protein (CHOP), leading to the upregulation of IL-23 in dendritic cells (Goodall et al. 2010). In the transgenic B27 rat model, LPS stimulation of bone-marrow derived macrophages in the context of HLA-B27 misfolding leads to upregulation of IL-23 (DeLay et al. 2009). However, in

AS patients, studies of gut biopsies did not show evidence of upregulated unfolded protein response gene transcription (Ciccia et al. 2014). Meanwhile, one study of macrophages from the inflamed joints of patients with AS showed increased expression of 78-kDa glucose-regulated protein (GRP78), a marker for the activated unfolded protein response. However this study did not demonstrate HLA-B27 as the driver of the unfolded protein response in these patients. The role of the unfolded protein response in HLA-B27 driven inflammation is therefore interesting but largely unproven.

The third major theory for the pathogenesis of HLA-B27 in AS revolves around the ability of HLA-B27 to aberrantly fold to form homodimers (Allen et al. 1999) or beta-2-microglobulin free heavy chains on the cell surface (Bird et al. 2003). Expression of these aberrant forms of HLA-B27 have been shown on the cells of AS patients, and aberrant HLA-B27 can be recognised in vitro by Killer-Immunoglobulin-like receptors (KIRs) (Kollnberger et al. 2002). These receptors are primarily expressed on NK cells (Lanier 2005) but have also been shown to be expressed on circulating CD4 T cells (Remtoula, Bensussan, and Marie-Cardine 2008). HLA-B27-positive Individuals with AS and HLA-B27 healthy donors have been shown to have a higher frequency of T cells expressing this receptor and these cells are polarised towards a Th17 phenotype (Bowness et al. 2011). In further work it was shown this receptor is expressed on CD4 cells after activation and induces transcription of the Th17 transcription factor ROR-γt (Ridley et al. 2015). This body of work links the Th17 immune pathway in AS with HLA-B27 for the first time. However, it is

quite possible that all three theories of HLA-B27 driven pathogenesis contribute to disease in the same individuals.

In terms of other pathogenic pathways identified by GWAS, the best studied pathway in AS is the IL-23/IL-17 axis. Evidence for the expansion of this immune pathway has come from several studies showing increased numbers of circulating T-helper 17 CD4 cells (Th17s) in the peripheral blood of patients with AS (Shen, Goodall, and Hill Gaston 2009; Sarkar, Cooney, and Fox 2010). More importantly, manipulating this axis using monoclonal antibodies to block the main effector cytokine IL-17A has proved to be successful in human trials for AS (Baeten et al. 2013) but intriguingly not in Crohn's disease which shares many of the genetic and pathological features of AS (Hueber et al. 2012).

1.4 The role of T cells

1.4.1 T cell differentiation

Both GWAS and functional data point towards CD4 T cells as important participants in the pathogenesis of AS and other inflammatory disorders such as PsA. CD4 T cells derive from common lymphoid progenitors and their receptor diversity is generated by random RAG-mediated recombination and junctional diversity. They undergo positive and negative selection in the thymus before being released as naïve CD4 cells into the periphery. A full discussion of these complex process is outside the scope of this thesis. RAG recombination is reviewed in (Gellert 2002) and thymic selection in (Timothy

K. Starr, Stephen C. Jameson, and Hogquist 2003). For the purposes of this thesis I will focus on the role of IL-7 in some of these processes.

1.4.2 The role of IL-7 and IL-7R signalling

IL-7 is a survival factor for lymphocytes and it is predominantly produced by stromal cells (Fry and Mackall 2005), with some reports suggesting DCs have a limited ability to also make IL-7 (Guimond et al. 2009). IL-7 signals through the IL7R, which is a heterodimer composed of the IL7Rα chain (also known as CD127) and the common γ chain. Downstream signalling of the IL7R is mediated by PIK3 and STAT5 (Pallard et al. 1999), and also antagonises the effects of TGF-β signalling through the induction of SMAD ubiquination regulatory factor 2 (SMURF2) (J.-H. Park et al. 2004). At rest the IL7R is expressed on most resting T cells and is downregulated following T cell activation or IL-7 signalling (J.-H. Park et al. 2004; Fry et al. 2003).

IL-7 signalling is crucial for the development of T lymphocytes and mutations in the IL7R lead to a severe combined immunodeficiency phenotype (SCID) in humans and mice (Puel et al. 1998). That is because IL7 signalling is tightly controlled in the thymus during T cell positive and negative selection and is required for VDJ recombination of the T cell receptor (Durum et al. 1998; Plum et al. 1996). In human thymic in-vitro cultures, the addition of anti-IL7 antibody prevents the production of T cells (Yeoman, Clark, and DeLuca 1996).

IL-7 signalling is also required for the development of γδ T cells (He and Malek 1996) and CD8 cells (Brugnera et al. 2000). In mice it is required for B cell differentiation (Namen et al. 1988) but in humans with mutations in the IL-7 pathway B cells are present (Puel et al. 1998). IL-7 is not required for the development of NK cells (He and Malek 1996). In addition to the role of IL-7 for the development of lymphocyte populations, it has also been shown to induce the proliferation of T cells in the periphery (W. Q. Li et al. 2006). IL-7 does not seem to be essential for myeloid development. However, expression of the receptor has been reported at mRNA level in both CD14 monocytes (Martinez et al. 2006) and DCs (Guimond et al. 2009).

Thymic stromal lymphopoietin (TSLP) has also been shown to signal through the IL-7Rα chain when it forms a heterodimer with TSLPR (Levin et al. 1999). The expression of human TSLPR is quite broad and mRNA expression has been reported in heart, muscle, kidney and liver (Tonozuka et al. 2001). In contrast to IL-7, the effect of TSLP signalling in human myeloid cells is seen most strongly in CD11c DCs, where an induction of thymus and activation-regulated cytokine (TARC) is seen. TSLP does not seem to have a significant effect on human lymphoid cells (Soumelis et al. 2002). The overlap between TSLP and IL-7 signalling may explain why the phenotype of IL7R-deficient mice appears to be more severe than that of IL-7-deficient mice (Leonard 2001). However IL-7 seems to be the dominant signalling molecule for lymphocytes, since mice lacking the TSLPR gene show normal lymphoid compartment development (Carpino et al. 2004; Al-Shami et al. 2004).

In the periphery, naïve CD4 and CD8 cells both express IL7R but this is downregulated following TCR triggering (Schluns et al. 2000; Franchimont et al. 2002). In addition both effector memory and central memory T cells retain their IL7R expression (Huster et al. 2004) and require IL-7 for survival (Seddon, Tomlinson, and Zamoyska 2003; Schluns et al. 2000). The production of IL-7 by stromal cells in the lymph nodes is thought to be independent of the concentration of IL-7, leading to some to develop the "altruistic hypothesis" where systemic IL-7 concentrations are controlled by T cell consumption rather than production (J.-H. Park et al. 2004). However this has not been clearly shown and in inflammatory arthritis there are several reports to suggest increased concentrations of IL-7 in inflamed joints compared to osteoarthritis (van Roon et al. 2005; Hartgring et al. 2009).

1.4.3 T Helper subsets

The existence of two functionally different subsets of T cells was first described in 1986 (Mosmann et al. 1986). Since then it has been shown that naïve CD4 cells are activated in lymph nodes by antigen presenting cells and differentiate into distinct effector lineages (Fig1) based on the signals in their environment. These T helper subsets are defined by their cytokine production and key master transcription factors have been identified. Th1 cells require the master transcription factor T-bet and produce IFN-γ, while Th2 cells produce IL-4 and IL-5 and require the transcription factor GATA-3. Th17 cells produce the signature cytokine IL-17A and require the key transcription factor ROR-γt. Lastly, regulatory T cells (Tregs) express the transcription factor FOXP3 and broadly suppress inflammation (reviewed in Zhu and Paul 2008).

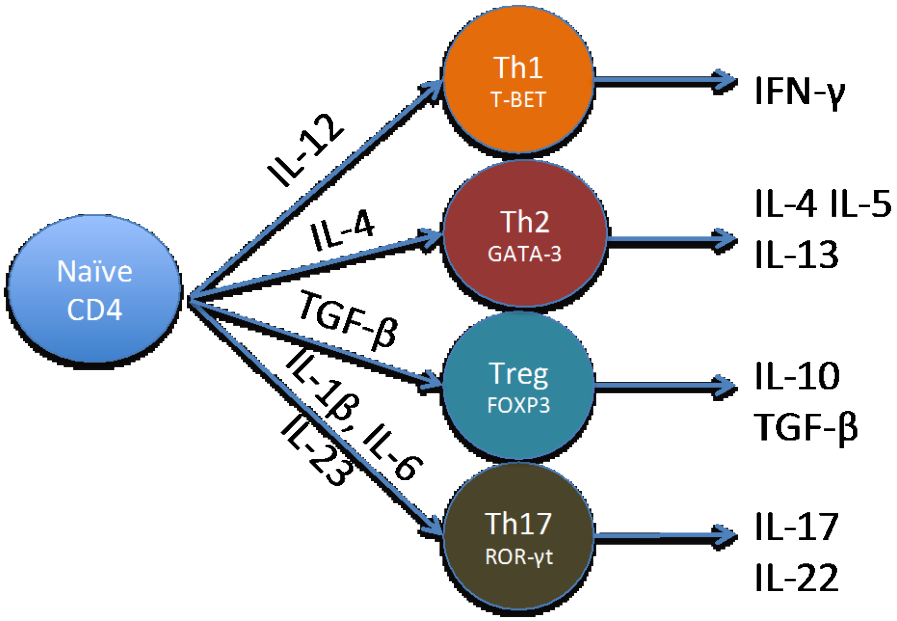


Figure 1.2. Model for differentiation of T helper effector subsets based on cytokine environment and transcription factor activation

1.4.4 Type 17 immunity and Th17 cells

Th17 cells are a relatively new addition to the T helper subset paradigm and were first described around 2005 (H. Park 2005; Harrington et al. 2005; Aggarwal et al. 2003). The key effector function of these cells is thought to be immunity against extracellular bacteria and fungal infections, with patients lacking Th17 cells suffering from recurrent staph aureus and candida infections (Ma et al. 2008; Milner et al. 2008). These functions are mediated through the release of cytokines such as IL-17A and IL-17F in addition to IL-22. One of the key cytokines driving the differentiation of this subset of T cells is IL-23, which shares a subunit (p40) with IL-12 (Oppmann 2000). The importance of this subset of T cells was highlighted in mouse models of autoimmunity where it was shown that knocking out IL-23 but not IL-12 made

mice highly resistant to auto-inflammatory diseases (Cua et al. 2003; Murphy et al. 2003).

The signals involved in the differentiation of naïve CD4 cells towards a Th17 programme are still not clearly elucidated with key areas of confusion arising from differences between mice and human cells. In mice three studies have independently reported the requirement for IL-6 and TGF-β as key cytokines driving a Th17 programme (Mangan 2006; Veldhoen et al. 2006; Bettelli 2006). This shared role for TGF-β, which also is involved in the differentiation of regulatory T cells suggests a key role for IL-6 in the determination of the balance between Tregs and Th17s (Bettelli 2006). The interplay between Th17 and Tregs is intriguing and two studies have reported human Th17 to preferentially differentiate from Tregs (Ayyoub, Raffin, and Valmori 2012; Valmori et al. 2010).

In human T helper cell development the role TGF-β is less clear. Two studies have shown the combination of IL-6 and IL-1β to be sufficient in promoting human Th17 cells with TGF-β seemingly playing a inhibitory role (Eva V. Acosta-Rodriguez et al. 2007; N. J. Wilson 2007). Critiques of these two studies suggested that TGF-β in serum used for cultures was not controlled. In two studies using serum free medium, it was shown that TGF-β is required, in addition to a pro-inflammatory cytokine such as IL-21 (Li Yang et al. 2008) or IL-6 plus IL-23 (Nicolas Manel, Unutmaz, and Littman 2008).

In addition to the inflammatory cytokine milieu, other factors that may influence the differentiation of Th17 cells include the metabolic environment. In two independent studies it was shown that raised extracellular sodium concentration enhances the differentiation of Th17 cells (Wu et al. 2013; Kleinewietfeld et al. 2013). The mechanisms of the effects of sodium ions on T cell differentiation are poorly understood. Wu and colleagues showed that serum glucocorticoid kinase 1 (SGK1) was upregulated downstream of IL-23R signalling. They cited evidence in epithelial cells where SGK1 is involved in regulating the epithelial sodium channel (ENAC) for renal sodium reabsorption, but did not show the presence of this channel in T cells. Furthermore, it would be difficult to see why a T cell might want to allow further sodium to enter intracellular space in response to high extracellular sodium when it is already expending so much energy via the sodium-potassium ATPase to keep its intracellular sodium low (Skou 1957). The true mechanism may well involve the cell sensing metabolic stress via ATP metabolites as secondary phenomenon in response to overactivation of the ATPase.

The evolutionary role for Th17 differentiation in response to raised extracellular sodium concentration is more apparent. The main physiological role of Th17 cells is to combat extracellular infections, in particular Staph aureus, which preferentially grows in high salt conditions (Kateete et al. 2010), hence colonising sites in the human body that have an abundance of sweat glands (Eriksen et al. 1995).

The strength of TCR signalling may also be an additional factor influencing the differentiation of Th17 cells. In one study whole human CD4 T cells were stimulated with TCR activating beads (CD3/CD28 coated) and it was shown a one bead to 50 cell ratio yielded significantly more Th17 cells compared to a one to one ratio (Purvis et al. 2010). Antigen dosing has previously been shown to alter the Th1/Th2 balance of naïve CD4 cells (Rogers and Croft 1999). In addition signalling strength is important during thymic selection for the deletion of auto-reactive T cells (Hogquist 2001). This concept is intriguing because it may be a potential mechanism for how KIR3DL2 interactions might influence the TCR signalling strength, especially as they are associated with an intracellular phosphatase associated inhibitory motif (Döhring et al. 1996).

As with the differentiation of human Th17 cells, the surface markers that define these cells are also a matter for debate. Some studies stress the importance of CCR6 co-expression with CCR4 in defining Th17 cells (E. V. Acosta-Rodriguez 2007) while other studies suggest CD161 to be an important surface marker induced by RORγt (Maggi et al. 2010). There is also evidence to suggest that CD161 is expressed in cord blood-naïve CD4 cells and that these cells are pre-programmed to becoming Th17 upon antigen stimulation (Cosmi et al. 2008). Human peripheral CD4 Cells co-expressing CD161 and CCR6 have shown be highly enriched for cells producing IL-17A (Francesco Annunziato et al. 2008).

1.4.5 Th17 Plasticity

Within the CD4 T-cell compartment, Th17 cells show considerable plasticity (Y. Lee et al. 2012), and IL-17A cytokine production is often coupled with other inflammatory cytokines such as IL-22 (Bluestone et al. 2009), IFN-γ (Ghoreschi et al. 2010) and GM-CSF (El-Behi et al. 2011) in the same cell. In mouse models it has also been shown that Tregs can convert to a Th17 phenotype upon adoptive transfer into a mouse model of inflammatory arthritis (Komatsu et al. 2014). Conversely it has been shown that Th17 can transdifferentiate into regulatory T cells under the influence of TGFβ (Gagliani et al. 2015).

In human studies, it has been shown that the majority of Th17 cells found in the inflammed joints of children with juvenile arthritis show an intermediate phenotype between Th1 and Th17 and express both RORγ and TBET (Nistala et al. 2010). In this study Th17 cells were able to take on the Th1 characteristic of IFN-γ secretion under the influence of IL-12, but cells sorted on Th1 differentiation markers were not able to make IL-17A. It is thought that the surface expression of CD161 remains stable and that this can be used to identify “ex-Th17” cells (Nistala et al. 2010). The expression of CD161 is also observed in FOXP3 positive Treg cells capable of producing pro-inflammatory cytokines (Pesenacker et al. 2013).

Understanding the precise phenotype of a pathogenic Th17 cell remains elusive. In a mouse model of autoimmunity it was shown that Th17 cells (generated with TGFβ1 and IL-6) were able to produce IL-17A but were not

pathogenic upon adoptive transfer. Meanwhile TGF-β3, IL-23 and IL-6 were able to generate pathogenic Th17s. Transcriptional analysis of the "pathogenic Th17s" showed a number of genes to be upregulated including CXCL3, IL22 and CSF2 (gene encoding GM-CSF) (Y. Lee et al. 2012). Recent advances in single cell sequencing have also shown heterogeneity within the mouse Th17 pool. Analysis of Th17 cells from the draining lymph nodes of mice with EAE revealed CSF2 to once again be a marker of pathogenic cells. In this study four genes found to be highly ranking by gene clustering analysis were GPR65, PLZP, TOSO and CD5L (Gaublomme et al. 2015). Knock out of these genes in mouse models was shown to restrain Th17 mediated inflammation in EAE (Gaublomme et al. 2015; Wang et al. 2015).

Other independent studies of EAE have also shown that that co-expression of IL-17A and GM-CSF by CD4 cells marks out a pathogenic subset of Th17 cells (Shiomi et al. 2014; El-Behi et al. 2011). Understanding the biology of and regulation of GM-CSF within the human inflammatory disease setting is crucial for better targeting of future therapies.

1.4.6 RORγt

Retinoid-related orphan receptors (ROR) are a group of intracellular transcription factors first cloned in the 1980s. The human ROR gene encodes four α isoforms, one β isoform and two γ isoforms γ1 and γ2 (commonly referred to as RORγt). The ROR proteins have a nuclear hormone receptor structure with a DNA binding domain and C-terminal ligand-binding domain. It

is thought that RORs regulate gene transcription by translocating to the nucleus following ligand binding interactions, and subsequently bind to specific DNA response elements in the regulatory region of target genes (Huang, Chandra, and Rastinejad 2010). The natural ligands of some of the RORs have been identified and include cholesterol or cholesterol derivatives for RORα. For RORγ all-trans retinoic acid and the synthetic retinoid ALRT 1550 have been shown to bind and act as antagonists (Stehlin-Gaon et al. 2003). Until recently the natural agonists of RORγt were not known, but two recent reports have suggested that sterol lipids (Santori et al. 2015) and oxysterols (Soroosh et al. 2014) are able to act as agonists. Furthermore, it was shown that Th17 cells can produce oxysterols under the control of the enzyme CYP27A1 (Soroosh et al. 2014) suggesting a self-reinforcing feedback loop.

In humans the expression of RORγt occurs exclusively in a subset of immune cells (Jetten 2009). RORγt knock-out mice exhibit a defective development of thymocytes, lymphoid organs and Th17 cells. The effect on lymphoid organs is due to the loss of lymphoid tissue inducer cells which are required during lymphoid organogenesis (Kurebayashi et al. 2000). The specificity of RORγt to immune cells makes this transcription factor an ideal target for the manipulation of type 17 immunity in adults, in whom secondary lymphoid organ structures have completed their development. Several inhibitors have shown efficacy in mice (Huh and Littman 2012), but these compounds are yet to enter clinical trials in patients with type 17 driven immune diseases.

1.5 Other Type 17 cells

In addition to Th17s, CD161-expressing CD8 T cells (Walker et al. 2012), γδ T-cells (Kenna et al. 2011) and innate lymphoid cells (Cua and Tato 2010) have also been shown to make IL-17A. These various IL-17A-producing cell types share expression of the ROR-γt transcription factor (Manel, Unutmaz, and Littman 2008). Therefore targeting RORγt therapeutically will have an impact across the whole range of cells involved in the type 17 immune axis. To what extent non-CD4 cells making IL-17A contribute to disease pathogenesis in AS remains unclear. In one study it was suggested that γδ T cells are the main source of IL-17A in AS but these findings have not been replicated by others (Kenna et al. 2011).

1.6 Innate Lymphoid cells

Innate lymphoid cells (ILCs) are a part of the innate immune system that has only been discovered in the last 5-6 years. They are distributed across tissue sites and are thought to be early initiators of inflammation in response to tissue stress signals. Interestingly they share the same intracellular transcription factors as the main T-helper subsets and thus mirror these subsets within the innate arm of the immune system (Artis and Spits 2015).

Members of the ILC family have lymphoid cell morphology but lack lineage markers that define the classic B and T cells and, in addition, lack a RAG recombined antigen receptor (Spits and Cupedo 2012). Thus the ILC family

includes cytotoxic NK cells, which were discovered in 1975 (Kiessling, Klein, and Wigzell 1975), and lymphoid tissue inducer cells (LTi) which were discovered in 1997 (Mebius, Rennert, and Weissman 1997). For the purposes of this project and the remainder of this section, I will concentrate on the role of non-cytotoxic ILCs.

Non-cytotoxic ILCs are defined by their expression of the IL7R alpha subunit (CD127) on the cell surface but also have receptors for other stimuli and cytokines (Spits et al. 2013). They can be divided into three distinct groups. ILC1 require the intracellular transcription factor T-bet and are capable of making the signature Th1 cytokine IFN-γ in addition to TNFα, and are involved in immunity against intracellular bacteria (Fuchs et al. 2013). ILC2 cells require GATA-3 and make the Th2 associated cytokines IL-4, IL-5 and IL-13 in addition to amphiregulin (Mjösberg et al. 2011; Monticelli et al. 2011). The ILC3 subset, which incorporates LTis, express RORγt and make type 17 cytokines IL-17A and IL-22 and have been shown to be involved in mucosal immunity (Cupedo et al. 2009; Cella et al. 2008). It remains unclear to what extent these ILC subsets represent distinct stable lineages and plasticity within these cells is under investigation.

Innate lymphoid cells have been described in the gut (Geremia et al. 2011), lungs (Monticelli et al. 2011) and skin (Salimi et al. 2013) and they are thought to play a role in maintaining homeostasis at their anatomical sites. ILC2s for example have been shown to be important in immunity against helminth infections in response to IL-25 and IL-33 (Price et al. 2010). ILC3 cells have

been shown to be crucial for the maintenance of the gut epithelial barrier and their depletion was shown to lead to dissemination of commensal bacteria (Sonnenberg et al. 2012). In addition, ILC3s have also been shown to regulate the proliferation of marginal zone B cells (Magri et al. 2014).

In addition to the role of ILCs in tissue homeostasis, several groups have shown a role in chronic inflammatory disorders. In mice intraepithelial ILC1 and ILC3 have been shown to contribute to colitis in mouse models (Buonocore et al. 2010; Fuchs et al. 2013). ILC2s have been shown to contribute to lung inflammation in mouse models (Y.-J. Chang et al. 2011) and have been shown to be expanded in the nasal polyps of patients suffering from chronic rhinitis (Mjösberg et al. 2011). They have also been shown to contribute to type 2 inflammation in the skin of patients with atopic dermatitis (Salimi et al. 2013).

Only one report has so far described the presence of ILCs in human inflamed joints in psoriatic arthritis (Leijten et al. 2015). These were shown to be ILC3s and were expanded as a percentage of the ILC compartment compared to blood, but their precise role in driving inflammation is not clear. One report claiming the expansion of ILC3 subsets in the blood, synovial fluid and bone marrow of patients with AS (Ciccia et al. 2015) did not define these cells based on the expression of the IL7R and thus the cells that are described do not represent the internationally agreed definition of ILCs (Spits et al. 2013). Therefore, the interpretation of these data are difficult.

1.7 GM-CSF Biology

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a pro-inflammatory haematopoietic growth factor which is essential for the differentiation of myeloid cell subsets including macrophages and granulocytes (Metcalf 2008). Many cell types including fibroblasts, smooth muscle cells, endothelial cells and monocytes are capable of producing GM-CSF in-vitro in response to innate inflammatory signals such as LPS or IL-1β (reviewed by Hamilton 2002). More recently GM-CSF production from CD4 T cells has been reported particularly in the context of pathogenic Th17 cells (Shiomi et al. 2014; El-Behi et al. 2011). The main in-vivo cellular source of GM-CSF in humans remains poorly defined; in mouse models of neuroinflamation T cell-specific GM-CSF knockouts were protected from disease, suggesting a crucial role for CD4 T cell-derived GM-CSF in inflammatory diseases (Codarri et al. 2011).

More recently several reports have shown the presence of GM-CSF-producing CD4 cells at the site of inflammation in human inflammatory disease. In one study of patients with juvenile inflammatory arthritis (JIA) GM-CSF-producing T cells were shown to have higher expression of CD161 and to be enriched in the inflammed joint (Piper et al. 2014). The authors also showed that capture sorted Th17 cells were able to produce GM-CSF under culture conditions containing IL-2, IL-12 and IL-23. In untreated multiple sclerosis (MS) the percentage of CD4 cells producing GM-CSF in the periphery was shown to be elevated compared to healthy controls and MS patients treated with IFN-β (Rasouli et al. 2015). The authors also showed

expression of GM-CSF in CD4 and CD8 T cells in MS brain biopsy samples by fluorescence microscopy where co-staining with IL-17A was also shown (Rasouli et al. 2015). A second independent study similarly showed the presence of GM-CSF producing CD4 cells in the CSF of patients with MS (Noster et al. 2014). In this study GM-CSF T cells were shown to express higher levels of T-bet than RORγt and once again a role for IL-12 priming was shown.

To what extent GM-CSF producing CD4 cells represent a distinct effector lineage rather than a state of activation remains an area for debate. In the mouse models of disease GM-CSF expression has been suggested to be downstream of RORγt and under the influence of IL-23 rather than IL-12, with mice lacking RORγt showing a diminished ability to produce GM-CSF (Codarri et al. 2011). A more recent study has suggested IL-23 acts via the transcription factor Blimp-1, which co-localises with RORγt and STAT3 to promote IL-17A and GM-CSF production (Jain et al. 2016). These two studies are supported by human data suggesting that GM-CSF is produced predominantly from CD161-positive cells, which have been shown to express higher levels of RORγt compared to Th1 cells (Piper et al. 2014). This is in contrast to the findings by Noster and colleagues who show high T-bet and low RORγt in ex-vivo human CD4 cells and suggest GM-CSF production to be part of a Th1 programme (Noster et al. 2014). In a study using STAT5 knock out mice it is suggested that GM-CSF is under the control of STAT5 signalling, with in-vitro generated GM-CSF-producing cells shown to express low levels of T-bet and RORγt (Sheng et al. 2014). The confusion arises

because all of these studies are looking at CD4 GM-CSF production in different disease and differentiation contexts. What is clear is that GM-CSF producing T cells seem to have an important pathogenic role in mice and humans, therefore understanding the downstream effects of GM-CSF are crucial.

GM-CSF binds to a heterodimeric receptor (GM-CSFR) which comprises a specific low affinity α-chain (GM-CSFRα or CD116) and a signal transducing common β-chain (GM-CSFRβ or CD131) which is shared with the IL-3 and IL-5 receptors (Broughton et al. 2015). Binding of GM-CSF to its receptor leads to a signalling cascade involving JAK2 and STAT5 (Matsuguchi, Lilly, and Kraft 1998). This leads to the activation of downstream inflammatory signalling molecules including MAPK, PI3K and NFκB (Guthridge and Lopez 2007).

Myeloid cells express high levels of GM-CSFR and this signalling is thought to be crucial for the differentiation and survival of myeloid cells in the bone marrow and periphery (Metcalf 2008; Cowburn et al. 2011; Wright et al. 2013; Griseri et al. 2015). In the context of inflammation, neutrophil expression of the adhesion receptor CD11b is upregulated to enhance trafficking (Yong et al. 1992). In addition, GM-CSF extends the survival of neutrophils and enhances the oxidative burst and neutrophil extracellular trap formation (NETs) (Yousefi et al. 2009). GM-CSF also enhances trafficking (Curran and Bertics 2012; Liu et al. 2015) and survival (Wong et al. 2013) of eosinophils.

The best characterised effects of GM-CSF are on the maturation of monocytes and macrophages (Reviewed in Hamilton and Achuthan 2013). In particular multiple in-vitro studies have reported GM-CSF to drive a pro-inflammatory (M1) macrophage subset (Akagawa et al. 2006; Joshi et al. 2014; Martinez et al. 2006; Shibata et al. 2001; Fleetwood et al. 2009). However, in GM-CSF (CSF2) knockout mice the myeloid compartment is relatively intact (Stanley et al. 1994) while M-CSF knockout leads to major myeloid deficiencies (Dai et al. 2002). This suggests that GM-CSF may have a more specific role in peripheral myeloid activation in response to stress rather than driving the development of the myeloid compartment.

CSF2 knockout studies show a deficiency of MoDCs (a monocyte derived subset of DCs defined by expression of CD103 and CD207) found in the skin (King, Kroenke, and Segal 2010) and gut lamina propria (Hirata et al. 2010). MoDCs are rare at baseline but increase during inflammation (Campbell et al. 2011; Naik et al. 2006; Segura and Amigorena 2013). MoDCs have been shown to activate T cells in vitro and produce pro-inflammatory cytokines (Zhan, Xu, and Lew 2012). Several in-vitro studies have shown a clear role for GM-CSF for the in-vitro differentiation of MoDCs from CD14 monocytes and these in-vitro generated DCs share many phenotypical features with in-vivo MoDCs (Xu et al. 2007; Caux et al. 1996; Fleetwood et al. 2009). In particular, human MoDCs isolated from sites of inflammation have been shown to drive autologous naive CD4 cells towards Th17 differentiation (Segura et al. 2013).

Overexpression of GM-CSF in transgenic mouse models leads to a phenotype characterised by the accumulation of DCs and macrophages at several tissue sites leading to tissue destruction (Lang et al. 1987). In one particular study it was shown that local GM-CSF overexpression in the stomach was sufficient to induce autoimmune gastritis (Biondo et al. 2001). Van Nieuwenhuije and colleagues mention in their review in 2013 unpublished findings where T-cell specific overexpression of GM-CSF was sufficient to induce a chronic inflammatory phenotype affecting multiple tissue sites (van Nieuwenhuijze et al. 2013). This once again suggests T cells to be an important source of GM-CSF in the pro-inflammatory peripheral setting.

The phenotype of the CSF2 knockout mouse shows an accumulation of surfactant in the lungs (Stanley et al. 1994), similar to a rare human phenotype of pulmonary alveolar proteinosis (PAP) which is associated with high circulating levels of anti-GM-CSF antibodies (Kitamura et al. 1999). Anti-GM-CSF antibodies are also reported in healthy individuals and IVIG blood products but at around 100 fold lower concentrations compared to individuals with PAP (Uchida et al. 2009). Therefore any therapies targeting this pathway will have to take into account this potential adverse effect.

1.8 Treatment of Ankylosing Spondylitis

Ankylosing spondylitis limited to the spine is treated initially with a combination of non-steroidal anti-inflammatory drugs (NSAIDs) and specialist physiotherapy (Braun et al. 2011). Unlike many other autoimmune inflammatory diseases the spinal inflammation does not typically respond to glucocorticoid therapy (Braun and Sieper 2002).

Response to treatment is often assessed using the Bath Ankylosing Spondylitis Disease Activity Index (Garrett et al. 1994) (BASDAI). This is a validated questionnaire that takes into account features of the disease affecting the spine using a patient reported visual analogue score. In particular pain and stiffness are key parameters which are included in this questionnaire. BASDAI does not take into account systemic measures of inflammation such as CRP and has been criticised for failing to discriminate between active inflammation and chronic non-inflammatory damage, e.g. due to new bone formation in AS. Other measures such as ASDAS have now been developed which incorporate serum CRP levels, but their correlation to active inflammation based on MRI evidence of inflammation on STIR sequences is still debatable. Part of the problem is that not all patients with AS develop a systemic inflammatory response manifesting as a raised CRP, therefore CRP in itself is only a useful marker of systemic inflammation in around one third of patients (Ruof and Stucki 1999).

Patients who fail to respond to exercise and NSAIDs are considered for a trial of anti-TNF therapy. Active disease is defined as a BASDAI score and visual

analogue score of pain both at equal to or greater than 4/10 on two occasions at least three months apart. These criteria are specified by the National Institute for health and Care Excellence (NICE), who also stipulate patients must trial at least two NSAIDs before qualifying for anti-TNF therapy. Unlike rheumatoid arthritis and psoriatic arthritis, synthetic disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate, have failed to show any benefit for the spinal pain and inflammation in AS (Dougados et al. 1995; Clegg, Reda, and Abdellatif 1999).

Five different monoclonal antibodies which neutralise TNFα, Infliximab, Etanercept, Adalimumab, Golimumab and Certolizumab, have now been shown to be effective in AS (Smolen et al. 2014). The rationale for using these agents was largely based on the successful trials in rheumatoid arthritis and the finding of TNFα mRNA in sacroiliac joint biopsies in AS (J Braun et al. 1995) but their success has led to a huge step-change in the treatment of AS. Although there are no head-to-head trials of the different agents, the responses seen are similar across the five agents. 60% of all patients will show at least a 20% improvement (ASAS20) compared to placebo and up to one third of those will show a greater than 70% improvement (ASAS70) compared to placebo. This still leaves 30% of patients failing to show any improvement on anti-TNFα agents and until very recently there was no other option for these patients. Moreover, it is still not clear if anti-TNFα therapies alter the new bone formation component of AS, or just act to relieve inflammation and pain (Machado 2013).

In the last five years a number of studies have reported positive outcomes for monoclonal antibodies targeting IL-17A and the IL-23 p40 subunit (shared with IL-12). Secukinumab, a monoclonal antibody targeting IL-17A, has shown efficacy in two phase three studies in ankylosing spondylitis. The data shows an ASAS20 response rate of 58-64% at 24 weeks. 40% of the patients included in this trial had previously failed to respond to anti-TNFα therapy, but the authors do not clarify the response rate in this sub-group (Baeten et al. 2015). Ustekinumab, a monoclonal antibody against the shared p40 subunit of IL-23 and IL-12, has shown an impressive 75% ASAS 20 response at 24 weeks. However this study had an open-label design rather than being a randomised controlled trial and non-responders to anti-TNFα therapy were excluded (Poddubnyy et al. 2014).

These two new therapies targeting the type-17 immune response are undergoing evaluation by NICE but have not yet received approval for use in the UK. This will lead to a second pathway that can effectively be targeted in AS, if and hopefully when these agents do receive approval, and therefore offers hope to the 30-40% of patients who fail to respond to anti-TNFα therapy. However the efficacy of these agents in anti-TNFα non-responders is yet to be clarified. One key challenge for the future as the number of therapeutic agents increases is being able to personalise treatments to patients from the outset.

Finally, there is still a lack of effective oral therapies in AS and this represents a problem in patients who find it difficult to inject using needles. One such oral

agent, Aprimelast, which is a phosphodiesterase-4 inhibitor, failed to meet its primary efficacy endpoint in phase 2 trials in AS but phase 3 studies are ongoing (Pathan et al. 2012). A second oral agent Tofactinib, which targets the JAK1 and JAK3 intracellular signalling cascades, has shown some efficacy in phase 2 trial reported in AS at a recent conference abstract (van der Heijde et al. 2016). However, this agent, which also showed efficacy in phase three trials in RA (E. B. Lee et al. 2014), has not been granted approval by the European Medicines Agency due to its toxicity. Therefore there is a need for effective and tolerable synthetic therapies that can be taken orally.

1.9 Project aims

Recent understanding of the biological processes involved in the pathogenesis of spondyloarthritides has translated into important treatments for patients. However, a significant number of patients are refractory to current available therapies and an unmet clinical need remains in this disease. This thesis seeks to answer some important questions about aspects of biology in the context of SpA in order to identify new potential targets.

1- What is the phenotype of GM-CSF producing cells in SpA and other inflammatory arthritidies?
    a. Which lymphocyte populations are making GM-CSF upon ex-vivo stimulation of PBMCs and which is the predominant GM-CSF producing cell type in the peripheral blood?
    b. What is the surface and functional phenotype of the GM-CSF producing lymphocyte population and which signals are controlling the expression of this cytokine?
    c. Which lymphocyte population is the predominant producer of GM-CSF in the inflamed joint and what it the role of innate lymphoid cells?
2- What is the functional role of polymorphisms in the IL7R in relation to inflammatory disease pathogenesis?
    a. What is the baseline IL7R expression in human PBMC subsets and does this change with stimulation?
    b. What are the effects of LPS stimulation on IL7R expression of CD14 monocytes?

c. What is the functional relevance of IL7R expression on monocytes?

3- Are small molecule RORγt inhibitors a viable therapeutic option in type 17 immune mediated human disease?

a. To develop an assay for the in-vitro expansion of type 17 cells from SpA patients.

b. To test the effects of small molecule RORγt-inhibiting compounds on the production of IL-17A and other T cell-derived cytokines by blood lymphocytes of SpA patients.

c. To test the effects small molecule RORγt-inhibiting compounds on SpA synovial fluid mononuclear cell Th17 responses.

Chapter 2: Materials and Methods

2.1 Patient and Control Recruitment

Peripheral blood and synovial fluid samples were recruited prospectively from the Oxford AS clinic as part of the immune function in inflammatory arthritis study (REC reference number 06/Q1606/139). Healthy donors were recruited under the same ethics. Synovial tissue samples were supplied by Mr Roger Gundle and Mr Ben Kendrick under the Oxford Musculoskeletal Biomedical Research Unit biobank ethics. Genotyped healthy donors for the IL7R study were recruited via the Oxford biobank with full ethical approval (www.oxfordbiobank.org.uk).

2.2 Laboratory Methods

Name of Media	**Components**
R0	RPMI-1640 (Sigma) + 2mM l-glutamine, 50µg/ml Penicillin and 50µg/ml streptomycin
R10	R0 + 10% sterile filtered heat inactivated fetal calf serum (Sigma)
R10-HS	R0 + 10% sterile filtered heat inactivated human AB serum from males (Sigma/NHS blood and transfusion services)
PBS	Phosphate buffered saline without calcium and magnesium (Sigma)
MACS Buffer	Phosphate buffered saline with 1% fetal bovine serum + 2mM EDTA
FACS wash	Phosphate buffered saline (Sigma) + 1% fetal calf serum (Sigma)
Fix/Perm buffer	BD biosciences, used according to manufacturer's instructions
Permwash® buffer	BD biosciences, diluted 1:10 in deionised water according to manufacturer's instructions
FACS Fix	FACS wash with 2% Formaldehyde (Sigma)

Table 2.1: Media used in experiments

2.2.1 Mononuclear cell separation from peripheral blood and synovial fluid

Venous blood and synovial fluid were collected in tubes containing sodium heparin (≥1U/ml BD vacutainer). Samples were diluted 1:1 in R0 medium (table 2.1) and separated by density centrifugation (Histopaque®, Sigma). A maximum of 35mls was layered over 15mls of Histopaque® in a 50ml falcon tube (BD Falcon). Tubes were centrifuged at 2000 rpm for 20 minutes without the brake. The cellular interface was collected with a Pasteur pipette and re-suspended in R10 medium (table 2.1). Cells were washed twice in R10 by centrifugation at 1800 rpm for 10 minutes, then at 1500rpm for 10 minutes. Cells were counted after a 1:1 dilution in Trypan blue (Sigma) using a haemocytometer under a light microscope.

2.2.2 Cryopreservation of peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC)

For storage PBMC and SFMC were pelleted by centrifugation at 1800 rpm for 10 minutes, the supernatant removed and cells re-suspended at 7-10x10^6 cells/ml in freezing medium (FCS containing 10% dimethyl sulphoxide, Sigma). Cells were frozen in 1ml aliquots in labeled cryovials (Alpha laboratories) and transferred to storage at -80°C in a freezing container (Nalgene). For long-term (>2 weeks) storage cells were transferred to liquid nitrogen.

2.2.3 Thawing of PBMC and SFMC

PBMC or SFMC were thawed by placing cryovials in a water bath at 37°C for rapid thawing, before transferring to a 15ml falcon. Cells were washed twice in R10, by centrifugation at 1500rpm for 10 minutes, before use.

2.2.4 Cell isolation using magnetic beads

2.2.4.1 CD4+ T cell isolation

Isolation was performed using the CD4+ T cell isolation kit II (Miltenyi Biotec) by a process of negative selection. This method uses a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and glycophorin A, leaving untouched CD4+ T cells. $1x10^7$cells/ml were suspended in 40μl MACS buffer. 10μl biotin-antibody cocktail was added per $1x10^7$cells/ml, mixed and incubated on ice for 10 minutes. Following incubation 30μl MACS buffer and 20μl anti-biotin microbeads were added per $1x10^7$cells/ml, mixed and incubated for a further 15 minutes on ice. Cells were washed by adding 10 times the labelling volume of MACS buffer and centrifuging at 300g for 10 minutes. After washing cells were re-suspended in MACS buffer, with up to $1x10^8$ cells/ml being resuspended in 500μl MACS buffer.

LS or MS columns were used for magnetic separation, depending on cell numbers, with MS columns being used for isolation with up to $1x10^7$ labelled cells and LS columns being used for isolation with up to $1x10^8$ labelled cells (Miltenyi Biotec). To prepare the columns 500μl MACS buffer was applied to a MS column or 3ml MACS buffer was applied to a LS column. The cell suspension was applied to a pre-separation filter (Miltenyi Biotec) to removed cell clumps and passed through the column. The column was washed 3 times in 500μl (MS) or 1ml (LS) MACS buffer. The eluted fraction, containing

the CD4+ T cells, was washed twice with MACS buffer. Using this method a mean purity of 95.5% (SD 1.87) was achieved.

2.2.4.2 CD14 cell isolation by positive selection

Isolation was performed using the Miltneyi™ CD14 microbead system. PBMCs were washed and re-suspended in 80µl of MACS buffer per 10^7 cells. 20µl of the CD14 microbeads were added and the mixture incubated at 4°C for 15 minutes. Cells were washed by adding 2ml of MACS buffer and spun at 300g and then resuspended in 500µl of ice cold buffer. Magnetic separation was carried out as above with the CD14 cells being positively labelled. Therefore the column was removed from the magnet and MACS buffer 1ml (MS) or 5ml (LS) was used to flush out the CD14 cells. Purity of >90% was regularly achieved.

2.2.4.3 Naïve CD4+ T cell isolation

Naïve CD45RO- CD4+ T cells were obtained from PBMC using the naïve CD4+ T Cell Isolation Kit II (Miltenyi Biotec) by a process of negative selection. This method uses a cocktail of biotin-conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR and glycophorin A, leaving untouched naïve CD4+ T cells. $1x10^7$cells/ml were suspended in 40µl MACS buffer. 10µl biotin-antibody cocktail was added per $1x10^7$cells/ml, mixed and incubated on ice. After 10 minutes cells were washed by adding 2mls of MACS buffer/$1x10^7$cells and centrifuging at 1800 rpm for 10 minutes. After removing

the supernatant 30μl MACS buffer and 20μl anti-biotin microbeads were added per $1x10^7$cells/ml, mixed, and incubated for a further 15 minutes on ice. Cells were washed by adding 10 times the labelling volume of MACS buffer and centrifuging at 1800 rpm for 10 minutes. After washing cells were re-suspended in buffer, with up to $1x10^8$ cells/ml in 500μl buffer.

To increase purity, labelled cells were first added to a LS column (Miltenyi Biotec) and then to a MS column (Miltenyi Biotec). Cells were passed through a pre-separation filter to remove cell clumps and then passed through the column. The column was washed three times in 500μl MACS buffer. The eluted fraction, containing the CD45RO-CD4+ T cells, was washed twice with MACS buffer. Using this method a mean purity of 96% (SD 1.17) was achieved.

2.2.5 Flow Cytometery

Flow cytometry was performed on a BD Fortessa instrument calibrated daily with calibration and tracking beads from BD biosciences. The instrument was compensated using single stained compensation beads (Onecomp® beads, Ebiosciences). Data were analysed using FlowJo software (Treestar®).

2.2.5.1 Fluorescence staining using antibodies

Table 2.2 shows the antibodies and staining reagents used during the project, which includes a live/dead exclusion marker (eFluor780, eBiosciences) in all samples, as well as appropriate isotype control antibodies, as detailed in

Table 2.3. Up to $5x10^5$ cells/well were stained in FACS wash in a total volume of 50μl per well. All staining was carried out for 20 minutes on ice and in the dark. After staining cells were washed twice in 200μl FACS wash and resuspended in 200μl FACS fix .

Antibody Target	**Fluorochrome**	**Clone**	**Supplier**	**Dilution**
CD3	BV605	OKT3	BioLegend	1:50
CD3	PE	UCHT1	BioLegend	1:50
CD3	PerCP-Cy5.5	UCHT1	BioLegend	1:50
CD3	AF700	UCHT1	BioLegend	1:50
CD4	APC	RPA-T4	BioLegend	1:25
CD4	BV421	RPA-T4	BioLegend	1:50
CD5	PE	UCHT2	BioLegend	1:50
CD8α	BV510	RPA-T8	BioLegend	1:50
CD11b	PE	M1/70.15.11.5	Miltenyi	1:50
CD11c	PE	MJ4-27G12	Miltenyi	1:50
CD14	PE	TÜK4	Miltenyi	1:50
CD19	PE	LNK16	Miltenyi	1:50
CD20	PE	2H7	BioLegend	1:50
CD34	PE	561	BioLegend	1:50
CD45	Pacific Blue	HI30	BioLegend	1:50
CD45RA	APC	HI100	BD	2:50
CD45RA	PerCP-Cy5.5	HI100	BioLegend	1:50
CD45RA	BV711	HI100	BioLegend	1:50
CD45RO	FITC	UCHL1	BioLegend	1:50
CD117 (C-Kit)	BV510	104D2	BioLegend	2:50
CD127 (IL-7Rα)	BV605	A019D5	BioLegend	2:50
CD161	eFluor450	HP-3G10	eBiosciences	2:50
CD161	BV421	HP-3G10	BioLegend	2:50
CD196 (CCR6)	PE-Cy7	11A9	BD	1:50
CD294 (CRTH2)	PE	BM16	Miltenyi	2:50
TCRδγ	PE	B1	BioLegend	2:50
KIR3DL2	AF647	DX31	In-house	2:50
Viability stain	eFluor780	N/A	eBiosciences	1:250
IL-17A	FITC	eBio64DEC17	eBiosciences	1:50
IFN-γ	AF700	B27	BioLegend	1:100
IL-22	Pe-Cy7	22URTI	eBiosciences	1:50
GM-CSF	PerCP-Cy5.5	BVD2-21C11	BioLegend	1:50
GM-CSF	eFluor660	GM2F3	eBiosciences	2:50
pSTAT5	AF647	47/Stat5(pY694)	BD	1:50
Steptavidin	APC	N/A	BioLegend	1:200
Fixable viability dye	eFluor780	N/A	eBiosciences	1:250
Table 2.2 Antibodies and dyes used in experiments				

Isotype	Fluorochrome	Clone	Supplier
IgG1	FITC	553443	BD
IgG1	BV605	MOPC-21	Biolegend
IgG2a	AF647	MOPC-173	Biolegend
IgG2a	FITC	IC003F	R & D Systems
IgG2b	FITC	MAB004	R & D Systems
IgG1	PE	550083	BD
IgG2a	PE	IM3046	Beckman Coulter
IgG2b	PE	559785	BD Biosciences

Table 2.3 Isotype controls used in experiments

2.2.5.2 Intracellular Cytokine Staining (ICS)

$5x10^5$ cells/well were stimulated with phorbol myristate acetate (100ng/ml; Sigma) and ionomycin (1µg/ml; Sigma), in the presence of Golgiplug® (Brefeldin A) and Golgistop® (Monensin) (both from BD biosciences diluted according to manufacturer's instructions).and incubated at 37°C in 5% CO_2. After 4 hours plates were centrifuged at 1500 rpm for 5 minutes to pellet cells and the supernatant removed. Staining was carried out in FACS wash in a total volume of 50µl per well. Cells were stained on ice and in the dark for 20 minutes. Following FACS staining plates were centrifuged at 1500 rpm for 5 minutes and washed twice in 200µl FACS wash. Cells were permeabilised by re-suspending in 100µl Cytofix/Cytoperm™ fixation and permeabilization solution (BD Bioscience) for 30 minutes at room temperature. Cells were washed once in 200µl Perm/Wash™ buffer (BD Bioscience). Cells were then stained for intracellular cytokines of interest in a total volume of 50µl per well of Perm/Wash™ buffer for 20 minutes at room temperature, and then washed

twice in 200µl Perm/Wash™ buffer. Cells were resuspended in 200µl FACS fix.

2.2.5.3 Phospho-Flow

5 x 10^5 cells were placed in R0 medium in a 96 well plate for 4 hours at 37°C then spun down at 300g. Cells were re-suspended in R10 medium containing 50ng/ml of recombinant human GM-CSF (Peprotech™) for 15 minutes before bing placed on ice. Cell surface (CD4, CD8, CD14, CD56 and CD20) and live-dead staining was carried out on ice for 20 minutes. Cells were washed twice in FACS wash and resuspended in 100µl of warm (37°C) fixation buffer (BD Phosphoflow fix buffer I) diluted 1:1 in FACS wash and incubated at 37°C for 10 minutes. Cells were then washed once in FACS wash and 100µl pre cooled (-20°C) Phosphoflow perm buffer III (BD) was added whilst vortexing using a multi-channel pipette. Cells were then incubated for 30 minutes on ice. After incubation cells were washed twice in cold FACS wash and then re-suspended in FACS wash containing anti phophoSTAT-5 antibody and incubated on ice for 20 minutes. Cells were then washed twice in FACS wash and fixed with FACS fix before acquiring on flow cytometer on the same day.

2.2.5.4 Cytokine Capture

All steps were carried out in laminar-flow hood and all reagents used were sterile. Fresh lymphocyte cones were obtained from the NHS blood and transplant service. The cone was diluted into a final volume of 100ml in R0 medium and cells were separated by density centrifugation (section 2.2.1). CD4 cells were then isolated from the PBMCs by negative selection 2.2.4.1

on the same day and re-suspended in R10 medium. CD4 cells were rested overnight in a T25 flask at 37°C in an incubator at a concentration of 10^7 cells/ml.

The following day cells were washed and re-suspended in warm R10 medium at a concentration of 10^7 cells/ml and stimulated with phorbol myristate acetate (100ng/ml; Sigma) and ionomycin (1µg/ml; Sigma) for 2 hours. Cells were then spun down at 400g for 5 minutes and re-suspended in 80µl of cold (4°C) R10 medium per 10^7 cells. A mixture of capture antibodies was made at a ratio of 2:2:1 of IL-17A capture, GM-CSF capture and IFN-γ capture respectively (all Miltenyi biotech). 20µl of capture cocktail was added per 10^7 cells and the mixture was incubated on ice for 5 minutes.

After 5 minutes, cells were diluted to a final concentration of 10^6 cells/ml in 50ml falcon tubes and incubated at 37°C for 45 minutes. At 5 minute intervals the mixture was turned to stop settling. After 45 minutes cells were placed on ice to stop cytokine secretion and then spun down at 300g for 10 minutes in a cold centrifuge. Cells were re-suspended in cold MACS buffer at concentration of 10^7 cells/ml and stained with MACS detection antibodies (IL-17A-PE, IFN-γ-FITC, GM-CSF-Biotin) all at a concentration of 1:25µl. In addition, cells were also stained with CD45RA-PerCpCy5.5, CD3 BV605, CD4 BV421 and ef780 viability dye. Cells were incubated in antibody mixture for 10 minutes on ice then washed in 10ml of cold MACS buffer and spun at 300g for 10 minutes. Cells were then re-suspended in cold MACS buffer at a

concentration of 10^7 cells/ml and stained with anti-biotin APC at a concentration of 1:200 for 10 minutes on ice.

Finally cells were washed twice in 10ml of cold MACS buffer and re-suspended in sterile PBS containing 2μM EDTA (Sigma) at a concentration of 2 x10^7/ml and filtered with the BD cell strainer cap tubes. Cells were kept on ice until sort.

2.2.5.5 FACS sorting

Staining for FACS sorting was carried out in the dark and on ice, in a laminar flow hood. Compensation controls were set up for each fluorochrome used in the sort, including an unstained sample and compensation bead single colour stains. Sorting was carried out using an automated cell sorter BD Aria from the flow cytometry facility at the Kennedy Institute. Cells were sorted at 4°C into tubes containing sterile PBS.

2.2.6 CyTOF staining

Cryopreserved cells were defrosted (2.2.3) and rested overnight at 37°C in R10 medium. On the following day 8x10^6 cells were stimulated in R10 medium containing phorbol myristate acetate (100ng/ml; Sigma) and ionomycin (1μg/ml; Sigma) for 4 hours at 37°C. The concentration of the cells was 2x10^6/ml and staining was carried out in FACS tubes (BD).

After 4 hours 8µl of 103Rh intercalator (Fluidigm) was added to distinguish live cells from dead cells and cells were incubated for a further 15 minutes at 37°C. Cells were then spun for 5 minutes at 400g and washed twice in 2mls of cell staining medium (CSM) (Fluidigm). Cells were then re-suspended in 50µl of cell CSM containing surface antibody master mix of all surface antibodies in table 2.4 and incubated for 20 minutes at room temperature.

Tag	**Target**	**clone**	**concentration (µl/stain)**
170Er	CD3	UCHT1	1
145Nd	CD4	RPA-T4	2
168Er	CD8	SK1	1
144Nd	CD11b	ICRF44	1
148Nd	CD14	RMO52	1
147Sm	CD20	2H7	2
155Gd	CD27	L128	1
154Sm	CD45	HI30	1
153Eu	CD45RA	HI100	1
176Yb	CD56 (NCAM)	NCAM16.2	2
143Nd	CD117	104D2	1
149Sm	CD127	A0195D5	1
164Dy	CD161	HP-3G10	2
141Pr	CD196	G034E3	1
160Nd	TCRgd	B1	2
173Yb	CD11c	BU15	2
151Eu	CD16	3G8	2

171Yb	CD34	581	2
175Lu	CD23	EBVCS-5	2
174Yb	CD336	P44-8	2

Table 2.4

CyTOF surface antibodies used in experiments

Cells were then washed twice by adding 2ml of CSM and spinning at 400g for 5 minutes. Cells were then re-suspended in 500µl of fix buffer (Fluidigm-Maxpar fix buffer I diluted 1:5 in PBS) and incubated at room temperature for 20 minutes. 2ml of perm-S-buffer (Fluidigm-Maxpar) was then added the cells and they were spun down at 800g for 5 minutes. Cells were then re-suspended in 2ml of perm-S-buffer and spun down again at 800g for 5 minutes.

For intracellular staining, cells were re-suspended in 50µl of antibody mastermix made up in perm-S-buffer with the antibodies listed in table 2.5 and incubated for 30 minutes in the dark at room temperature.

Tag	Target	clone	concentration (µl/stain)
159Tb	GM-CSF	BVD2-21C11	1
165Ho	IFN-γ	B27	1
142Nd	IL-4	MP4-25D2	1
156Gd	IL-6	MQ2-13AS	1
169Tm	IL-17A	BL168	1
166Er	IL-17F	SHLR17	1
172Yb	IL-21	3A3-N2	1
150Nd	IL-22	22URTI	1
152Sm	TNF	MAB11	1
146Nd	IL-2	MQ1-17H12	1
158Gd	IL-10	JES3-9D7	1

Table 2.5 CyTOF intracellular antibodies used in experiments

Cells were then washed twice by adding 2ml of perm-S-buffer and spinning at 800g for 5 minutes. Cells were suspended in 500µl of the 191Ir/193Ir intercalator (made up by diluting stock at in 1:4000 Fluidigm-Maxpar Fix/Perm buffer) and incubated overnight at 4°C.

On the day of acquisition, cells were washed by adding 3ml of CSM and spinning at 800g for 5 minutes. Cells were then re-suspended in 1ml of milliQ-water for counting. Cells were then spun down for 5 minutes at 800g and the concentration was adjusted to 0.5 x 10^6 cells/ml. The sample was passed through a 70µm cell strainer. Equalisation beads (Fluidigm-Maxpar) were

added at a concentration of 1:10 after vigorous vortexing and the sample was acquired on a CyTOF-1 machine operated by the NDORMS CyTOF core facility. Analysis of data was carried out using FlowJo software (Treestar®) and Cytobank® software.

2.2.7 T cell activation and culture

2.2.7.1 Anti-CD2/3/28 stimulation

A T cell activation/expansion kit (Miltenyi Biotech) was used for T cell stimulation. MACSiBead particles were prepared according to the manufacturer's instructions. Briefly 100µl CD2-biotinylated antibody, 100µl CD3-biotinylated antibody and 100µl CD28-biotinylated antibody were added to 500µl of anti-biotin MACSiBead particles, MACS buffer was added to make the volume up to 1ml and the mix was gently rotated for 2 hours at 2-8°C. MACSiBead particles were used at a ratio of one MACSiBead particle to two cells unless otherwise stated.

2.2.7.2 Th17 polarization for ROR-γt inhibition assays

5 million freshly isolated PBMCs were re-suspeded in 5ml of R10 containing 100IU/ml IL-2 (peprotech) and anti-CD2/3/28 beads (Miltenyi biotech) at a concentration of 1 bead for every 20 cells. Cells were placed in a T25 flask which was kept upright in the cell incubator at 37°C. ROR-γt inhibitors (Merck) were added at this stage or DMSO (Sigma) added as a control. On day 3, 5ml of R10 with 200IU IL-2 was added to the flasks. On day 6 cells were counted and plated out at 500,000 cells per well in a 96 well plate for

activation with PMA/ionomycin and subsequent ICS staining protocol (2.2.5.2).

2.2.7.3 Tissue explant cultures

Freshly obtained surgical material was re-suspended in 25ml of sterile R10 immediately. The sample was placed in a large petri dish and cut into 3-4mm pieces by hand. 20-30 pieces of the sample were placed in a 6 well plate containing 5ml of warm of R10-HS medium containing 100IU/ml IL-2 (Peperotech™) + IL-7 10ng/ml (Peprotech™). 2.5ml of medium was aspirated every 72 hours and replaced with the same volume containing IL-2 100IU/ml + IL-7 10ng/ml. After 14 days non-adherent cells were washed off the plate and filtered through a 70µm filter. Cells were counted, rested overnight in R10-HS and stimulated in the morning for intracellular cytokine staining.

2.2.8 Enzyme-linked Immunosorbent Assay (ELISA)

Human ELISA Ready-SET-Go! (eBioscience) kits were used in all experiments. Washing steps were performed in PBS + 0.05% Tween-20 (Sigma). 96 well flat bottom plates (Nunc) were coated with 100µl/well capture antibody and incubated overnight at 4°C. Plates were washed 5 times with 300µl/well wash buffer and blocked for an hour with assay diluent. Standards were prepared with the top standard at a concentration of 500pg/ml. The top standard was double diluted to give a 7-point curve, ranging from 500pg/ml to 7.81pg/ml. After 5 washes 50µl/well of samples, standards or matrix medium

was added and plates were incubated overnight at 4°C. Samples were either added neat or diluted in assay diluent, as determined by titration experiments. After 5 washes, 100µl/well of detection antibody was added and incubated for an hour at room temperature. After a further 5 washes 100µl/well of avidin-HRP was added and plates were incubated for 30 minutes at room temperature. After a further 7 washes 100µl/well substrate solution was added and incubated in the dark at room temperature for 15 minutes. 50µl/well 2M sulphuric acid (Fisher Scientific) was added to stop the assay. Plates were read at 450nm on the 6800 plate reader (Bio-Rad), using the microplate manager 5.2.1 software. Files were imported into Microsoft Excel for analysis.

2.2.9 RNA extraction

The AllPrep DNA/RNA/miRNA kit (Qiagen) was used for RNA extraction. All names of buffers are those assigned by the manufacturer and the details of the kit are available at www.Qiagen.com, catalogue number 80224). 10µl of β2-mercaptoethanol was added for every 1ml of RLTplus lysis buffer. FACS sorted cells were spun down and re-suspended in 350µl of RLTplus buffer and transferred to 2ml tubes. Samples were then stored at -80°C for batched RNA extraction.

On the day of the extraction cells were allowed to warm to room temperature over one hour. Homogenization of the sample was carried out using the QIAshredder (Qiagen). The defrosted cell lysate was placed into the QIAshredder spin column which was placed in a 2ml collection tube. The column was spun at maximum speed for 2 minutes in a microcentifuge. The

lysate was collected for RNA purification. Cells were placed in an AllPrep DNA mini spin column, placed in a 2ml tube, and centrifuged for 30 seconds at full speed. 50 µl of proteinase K was added to the flow through followed by 200µl of pure ethanol. The mixture was incubated at room temperature for 10 minutes. After 10 minutes 400µl of pure ethanol was added and mixed well. 700µl of this mixture was transferred to an RNeasy mini spin column placed in a 2ml collection tube. The column was spun at maximum speed for 15 seconds and flow-through discarded. This step was repeated with the remainder of the mixture. Then, 500µl of buffer RPE was added to the RNeasy spin column and column was centrifuged for 15 seconds at full speed with the mixture discarded.

10µl DNase I stock solution was diluted in 70µl of RDD buffer. 80µl of the diluted DNase I was added onto the RNeasy mini spin column and incubated for 15 minutes at room temperature. After 15 minutes, 500µl of FRN buffer was added to the RNeasy mini spin column and the mixture was centrifuged at maximum speed for 15 seconds. The flow-through was then added back to the RNeasy mini column and centrifuged at maximum speed for 15 seconds. The flow-through was discarded. 500µl of RPE buffer was then added to the column before centrifuging at full speed for 15 seconds. The flow-through was discarded. 500µl of pure ethanol was added to the column to wash the membrane. The flow through was discarded and the column was re-spun in a new collection tube to eliminate any carryover of ethanol.

Finally, 35µl of RNase-free water was added to the column which was placed in a new collection tube and spun for 5 minutes at maximum speed. The eluted RNA flow-through was put through the column a second time to improve yield. The RNA amount was quantified by nano-drop and the RNA samples stored at -80°C for storage until ready for sequencing.

2.2.10 RNA sequencing

RNA sequencing was carried out at the Wellcome Trust Centre for Human Genetics core facility. RNA underwent quality control testing using the Agilent Technologies 2200 TapeStation system at the core facility followed by cDNA library preparation. Paired end sequencing was performed at 100 base pairs on each side of the DNA fragment on the hiSeq 4000 platform. Only samples with greater than 10 million reads were used in analysis.

2.3 Analytical methods

Prism version 6 was used for statistical analysis. For data on CD4+ T cell phenotype, in chapter 3, box-and-whisker plots were used. These show the smallest observation (sample minimum), lower quartile, median, upper quartile and largest observation (sample maximum).

When comparing two unpaired groups with normal distribution of data a two-tailed unpaired t test was used for statistical analysis. When comparing two unpaired groups with data that was not normally distributed a Mann-Whitney

test was used. For paired data a paired t test and Wilcoxon matched-pairs signed rank tests were used for parametric and non-parametric data respectively.

When comparing more than two unpaired groups, a one-way analysis of variance (ANOVA) with Bonferroni's correction was used for parametric data. Where the data was not normally distributed a Kruskal-Wallis test with Dunn's multiple comparison analysis was used for statistical analysis. For matched data with more than two groups, a one-way ANOVA with Greenhouse-Geisser correction was performed in parametric data sets. In non-prarametric data a Friedman test with Dunn's multiple comparison analysis was performed. Significance was defined as $p \leq 0.05$.

Bioinformatic analysis of RNA sequencing samples was carried out by Dr Irina Pulyakhina at the Wellcome Trust Centre for Human Genetics using validated packages in R. 20-80 million fragments were sequenced per sample. Reads were mapped to human genome reference sequence GRCh37 with tophat2 (Kim et al. 2013), deduplicated using samtools v.1.2 (H. Li et al. 2009). Gene counts were retrieved using htseq-cout *(Anders, Pyl, and Huber 2015)* and the Ensembl gene annotation. DESeq2 *(Love, Huber, and Anders 2014)*, an R package, was used for differential gene expression analysis with the false discovery rate set at <0.05 after corrections for multiple comparisons.

Chapter 3: Identification and characterisation of GM-CSF producing cells in SpA and inflammatory arthritis

3.1 Introduction

AS has long been associated with inheritance of the Human Leukocyte Antigen (HLA) allele B27 but more recent Genome Wide Association Studies (GWAS) have highlighted several other important genes involved in the interleukin-17 (IL-17) and interleukin-23 (IL-23) inflammatory axis, (D. M. Evans et al. 2011). These GWAS observations have been confirmed by functional studies showing increased numbers of circulating T-helper 17 CD4 cells (Th17s) in the peripheral blood of patients with AS (Sarkar, Cooney, and Fox 2010; Shen, Goodall, and Hill Gaston 2009). This IL-17/IL-23 inflammatory axis is also implicated in auto-inflammatory disorders affecting the eyes, skin and gut (Miossec, Korn, and Kuchroo 2009). Manipulating this axis using monoclonal antibodies to block the main effector cytokine IL-17A has proved to be successful in human trials for AS (Baeten et al. 2013).

In addition to Th17s, CD161-expressing CD8 T cells (Walker et al. 2012), γδ T-cells (Kenna et al. 2011) and innate lymphoid cells (Cua and Tato 2010) have also been shown to be producers of the key inflammatory cytokine IL-17A. The latter innate lymphoid cells (ILCs) which share the same intracellular master transcription factors as T helper lymphocytes (Spits and Cupedo 2012) have been described in the gut (Geremia et al. 2011), lungs (Monticelli et al. 2011) and skin (Salimi et al. 2013) but there is only one report of their presence in the joint in psoriatic arthritis (Leijten et al. 2015).

Furthermore, within the CD4 T-cell compartment, Th17 cells show considerable plasticity (Y. Lee et al. 2012) and IL-17A cytokine production is often coupled with other inflammatory cytokines such as IL-22 (Bluestone et al. 2009), IFN-γ (Ghoreschi et al. 2010) and GM-CSF (El-Behi et al. 2011) within the same cell. Murine models of neuro-inflammation suggest that co-expression of IL-17A and GM-CSF by CD4 cells marks out a pathogenic subset of Th17 cells (Shiomi et al. 2014), (El-Behi et al. 2011). Therapeutic antibodies targeting GM-CSF are already in clinical trials in human diseases. The targeting of GM-CSF in RA is largely based on observations in mouse models of arthritis (Greven et al. 2014). The relevance of GM-CSF production in spondyloarthitis remains unknown but studies using the SKG-mouse model of SpA have shown GM-CSF to be a key driver of inflammation and its neutralisation using monoclonal antibodies to be effective (Shiomi et al. 2014).

3.2 Aims

The aim of this chapter was to characterise the lymphocyte population making GM-CSF in the peripheral blood and joints of patients with AS compared to RA and healthy donors.

The key questions were:

- Which lymphocyte populations are making GM-CSF upon ex-vivo stimulation of PBMCs and which is the predominant GM-CSF-producing cell type in the peripheral blood?

- What is the surface and functional phenotype of the GM-CSF producing lymphocyte population and which signals are controlling the expression of this cytokine?

- Which lymphocyte population is the predominant producer of GM-CSF in the inflammed joint and what it the role of innate lymphoid cells?

3.3 Results

3.3.1 CD4 T cells are the predominant producers of GM-CSF amongst PBMCs in SpA and healthy donors

Many cell types have been reported to be capable of producing GM-CSF in the literature (Cornish et al. 2009). In order to investigate the predominant cell type capable of producing this cytokine upon ex-vivo stimulation of PBMCs with PMA and ionomycin, I performed detailed phenotyping of AS PBMCs using CyTOF. Cell surface markers TCR-γδ, CD3, CD4, CD8, CD11b, CD11c, CD14, CD16, CD20, CD23, CD27, CD34, CD45, CD45RA, CD56, CD117, CD127, CD161, CD196 and CD336 were combined with intracellular staining for GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22 and TNF-α.

Figure 3.1 shows ViSNE, a principal component-derived high dimensional analysis, which plots and clusters all the cells according to the variance in the sample (Amir et al. 2013). All live GM-CSF positive cells were gated and includedin the analysis which demonstrates CD4 cells to be the predominant population producing GM-CSF. The other main populations producing GM-CSF are CD8 cells, and NK cells. There is minimal contribution to this GM-CSF pool from CD14 cells or CD20 lymphocytes.

Results were validated using flow cytometry with surface markers for TCR-γδ, CD3, CD4, CD8, CD56 and intracellular staining for GM-CSF, IFN-γ, IL-17A and IL-22. Figure 3.2A shows the gating strategy and figure 3.2B shows increased ex-vivo live cell GM-CSF production in AS. 3.2C shows CD4 cells

to be the largest GM-CSF producing population in healthy donors. Flow Cytometry validation also shows γδ-T cells to be producers of GM-CSF which was not observed in the CyTOF staining.

Figure 3.1

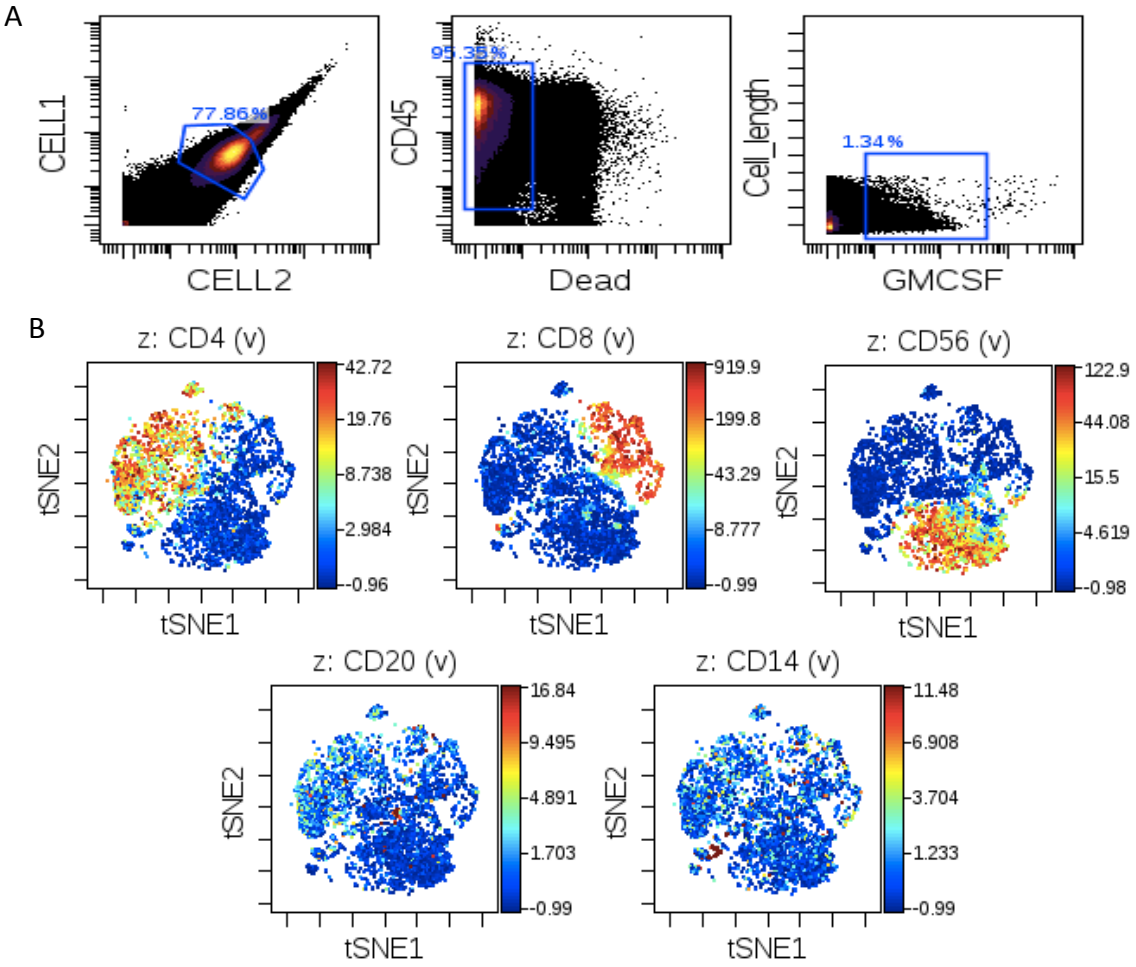


Figure 3.1. Principal component ViSNE analysis of CD45+GM-CSF+ AS PBMCs stimulated with PMA and ionomycin.
1 million AS PBMCs from frozen were stained for intracellular and extracellular markers. Cells were gated on single cell, live, CD45+ GM-CSF+ (A) before running the ViSNE analysis based on all other parameters in the CyTOF staining. B shows that in this individual CD4 cells are the main producers of GM-CSF in addition to a significant contribution from the CD8 and CD56 pool. There is only a very relatively minor contribution from CD20+ B cells and CD14+ monocytes. Cytobank software was used for the analysis.

Figure 3.2

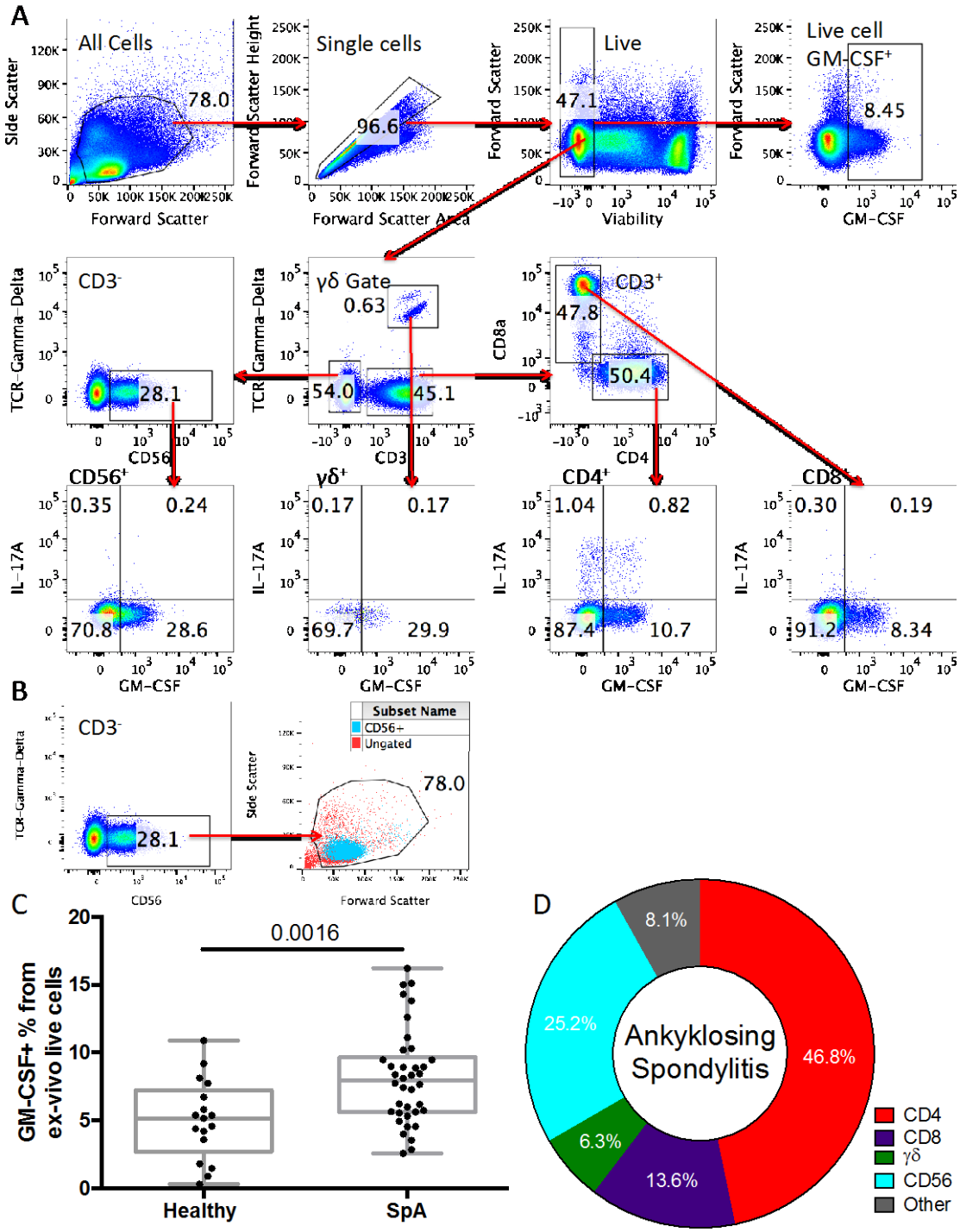

A
All Cells
78.0
Side Scatter
Forward Scatter
Single cells
96.6
Forward Scatter Height
Forward Scatter Area
Live
47.1
Viability
Live cell
GM-CSF+
8.45
GM-CSF
CD3-
28.1
TCR Gamma-Delta
CD56
γδ Gate
0.63
54.0
45.1
CD3
CD3+
47.8
50.4
CD8a
CD4
CD56+
0.35
0.24
70.8
28.6
IL-17A
GM-CSF
γδ+
0.17
0.17
69.7
29.9
CD4+
1.04
0.82
87.4
10.7
CD8+
0.30
0.19
91.2
8.34
B
CD3-
28.1
CD56
Subset Name
CD56+
Ungated
78.0
Side Scatter
Forward Scatter
C
GM-CSF+ % from ex-vivo live cells
0.0016
Healthy
SpA
D
Ankyklosing Spondylitis
46.8%
25.2%
8.1%
6.3%
13.6%
CD4
CD8
γδ
CD56
Other

Figure 3.2. Flow Cytometry data confirms CD4 cells are the main ex-vivo producers of GM-CSF upon stimulation of PBMCs.
A. Representative staining from AS patient showing the full panel gating strategy. B shows CD3-CD56+ population back-gated onto all cells to confirm by forward and side scatter that these are lymphoid cells and not myeloid cells. C expansion of live GM-CSF positive PBMCs in AS (n=38) compared to healthy donors (n=17) (unpaired T tests, all data points shown with box representing interquartile range and whiskers showing maximum and minimum). D shows mean percentage of CD4, CD8, γδ-T cells (GD) and CD56+ cells within live GM-CSF+ PBMCs in AS (n=9).

3.3.2 CD4 T cells producing GM-CSF and IL-17A are significantly expanded in AS compared to RA and healthy donors

Since CD4 cells are the main producers of GM-CSF in ex-vivo stimulated AS PBMCs I decided to compare ex-vivo stimulated PBMCs from healthy donors, AS patients and RA controls. 38 patients with AS attending the clinic in Oxford were recruited prospectively from November 2013 to June 2015. 14 Patients with RA were also recruited and 17 healthy volunteers (table 3.1). PBMCs were isolated directly ex-vivo and rested overnight. On the following day the cells were stimulated with PMA and Ionomycin for 4 hours in the presence of brefeldin A and monensin. See chapter 2 for detailed methods. Cells were stained with a basic panel for surface phenotype markers CD3, CD4, TCR-γδ, CD161 and a viability stain. Intracellular staining was performed for IL-17A, IL-22, IFN-γ and GM-CSF. A number of cells were also stained for CD56.

Figure 3.3 shows significantly increased percentages of CD4 cells positive for IL-17A, IL-22 and GM-CSF in AS compared to RA and healthy donors. There was no significant difference in the percentages of IFN-γ-producing CD4 T cells between the three groups. The mean percentage of CD4+GM-CSF+ cell in AS was 8.1 (SD=3.6) compared to 4.5 (SD=2.6) in healthy donors and 2.6 (SD=1.8) in RA. The mean percentage of CD4+IL-17A+ cells in AS was 1.2, this was significantly greater than 0.7 in both healthy donors and RA.

Table 3.1

	AS (n=38)	**Rheumatoid arthritis (n=14)**	**Healthy controls (n=17)**
Age, mean (range) years	49.6 (22-71)	64.6 (49-82)	37.3 (28-62)
Sex, male/female	27/10	6/8	11/6
HLA-B27+ no. (%)	27* (77%)	n/a	n/a
RF/CCP+ no. (%)	n/a	10 (71%)	n/a
BASDAI, mean (range)	3.44 (1-10)	n/a	n/a
DAS28-CRP, mean (range)	n/a	3.38 (1.7-5.33)	n/a
DMARD therapy, current (previous)	6 (0)	13 (0)	n/a
Anti TNF therapy, current (previous)	10 (2)	0	n/a
CRP, mean (range)	17.18 (0.2-98.4)	17.86 (0.7-97.4)	n/a

Table 3.1 Baseline characteristics of study participants

Figure 3.3

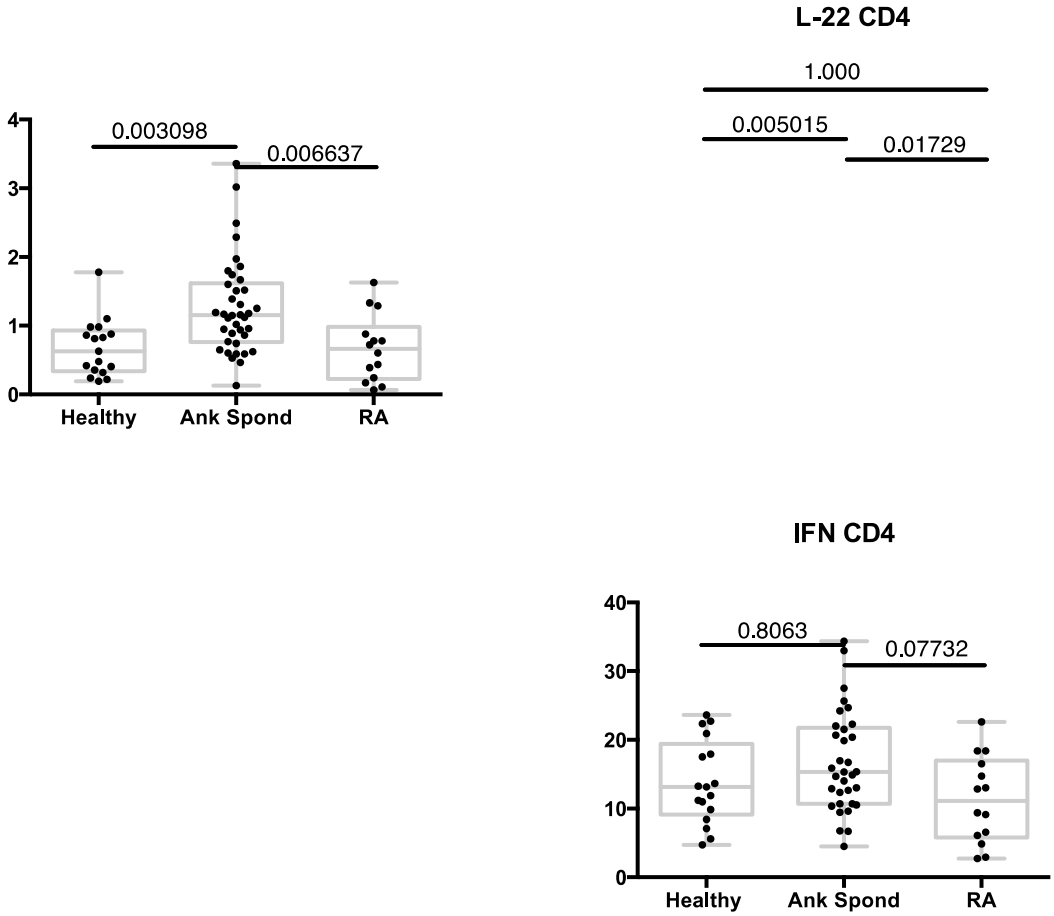


Figure 3.3. GM-CSF and IL-17A producing CD4 cells are expanded in AS compared to healthy controls and RA.
Ex-vivo stimulated PBMCs from patients with AS (n=38), RA (n=14) and healthy controls (n=17) were gated on $CD3^{+}CD4^{+}$ cells and the percentage of CD4 cell expressing IL-17A, GM-CSF, IL-22 and IFN-γ was determined. All data points presented with box plots. GM-CSF, IL-17A and IL-22 positive CD4 cells are significantly expanded in AS compared to RA and healthy controls. There was no significant difference in the percentages of IFN-γ positive cells (P>0.05). Statistical significance was determined using a Kruskal-Wallis test with p values calculated using Dunn's multiple comparisons test.

3.3.3 CD8 and γδ-T, but not NK cells show increased IL-17A and GM-CSF production in AS

Cytokine production from CD8 cells was also measured in the same cohort of patients and controls and for a subset of the same cohort, cytokine production was also measured in γδ-T cells and CD56-positive NK cells. The percentage of IL-17A, IL-22 and GM-CSF-positive CD8 cells from peripheral blood of patients with AS was significantly increased compared to both healthy donors and RA disease controls (figure 3.4). In addition, IFN-γ positive CD8 cells are also significantly increased in both AS and RA compared to healthy donors.

In the δγ T cell compartment, the percentage of IL-17A and IL-22-positive cells was significantly increased in AS compared to healthy donors and RA patients (figure 3.5). There is no difference in the percentages of IFN-γ positive cells.

There was no significant difference in the cytokine profile of $CD56^{+}$ NK cells between AS and healthy donors (figure 3.6). The only observed significant difference was an increase in IFN-γ production in AS NK cells compared to RA disease controls.

Figure 3.4

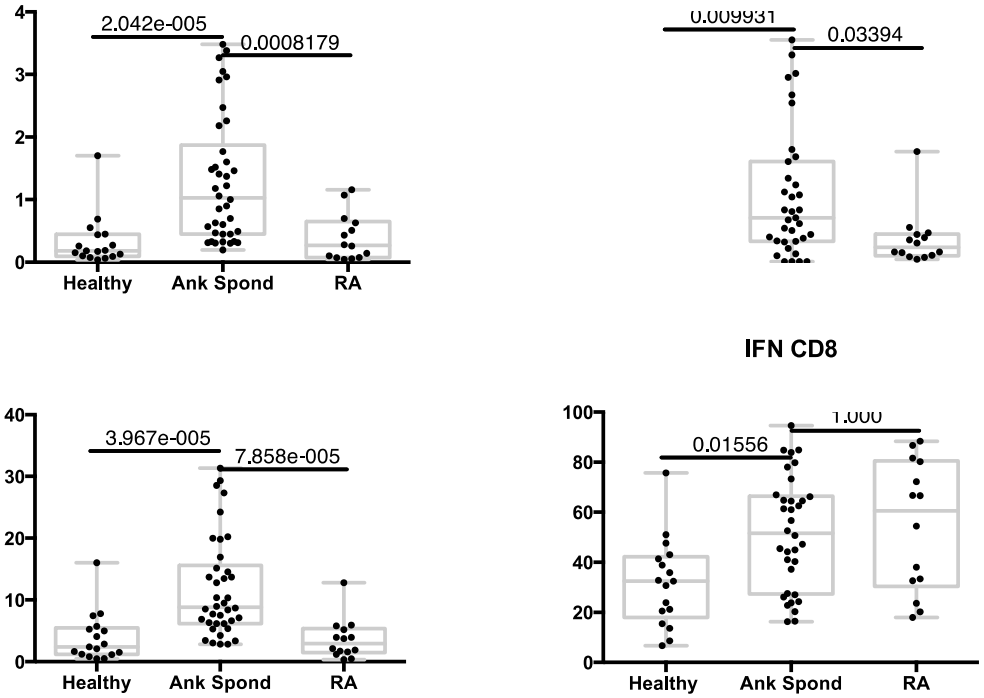


Figure 3.4. Percentage of GM-CSF and IL-17A producing CD8 cells are increased in AS compared to healthy controls and RA.
Ex-vivo stimulated PBMCs from patients with AS (n=38), RA (n=14) and healthy controls (n=17) were gated on $CD3^{+}CD8^{+}$ cells and the percentage of CD8 cell expressing IL-17A, GM-CSF, IL-22 and IFN-γ was determined. All data points are presented with box plots. GM-CSF, IL-17A and IL-22 positive CD8 cells are significantly expanded in AS compared to RA and healthy controls. IFN-γ positive CD8 cells are significantly increased in AS and RA compared to healthy controls cells. Statistical significance was determined using a Kruskal-Wallis test with p values calculated using Dunn's multiple comparisons test.

Figure 3.5

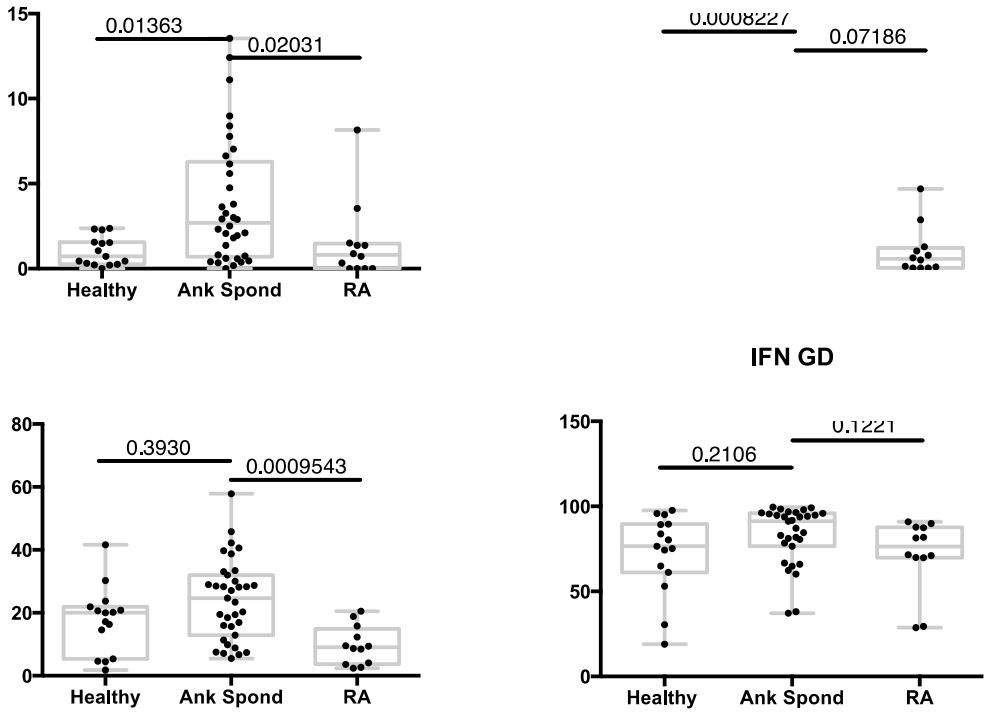


Figure 3.5. Percentage of IL-17A producing γδ-T cells (GD) are expanded in AS compared to healthy controls and RA.
Ex-vivo stimulated PBMCs from patients with AS (n=34), RA (n=12) and healthy controls (n=15) were gated on $CD3^{+}\gamma\delta^{+}$ cells and the percentage of GD cells expressing IL-17A, GM-CSF, IL-22 and IFN-γ was determined. All data points are presented with box plots. IL-17A and IL-22 positive GD cells are significantly expanded in AS compared healthy controls. There was no significant difference in the percentages IFN-γ positive and GM-CSF positive GD cells between AS and healthy controls but there a significant increase in GM-CSF production in AS compared to RA. Statistical significance was determined using a Kruskal-Wallis test with p values calculated using Dunn's multiple comparisons test.

Figure 3.6

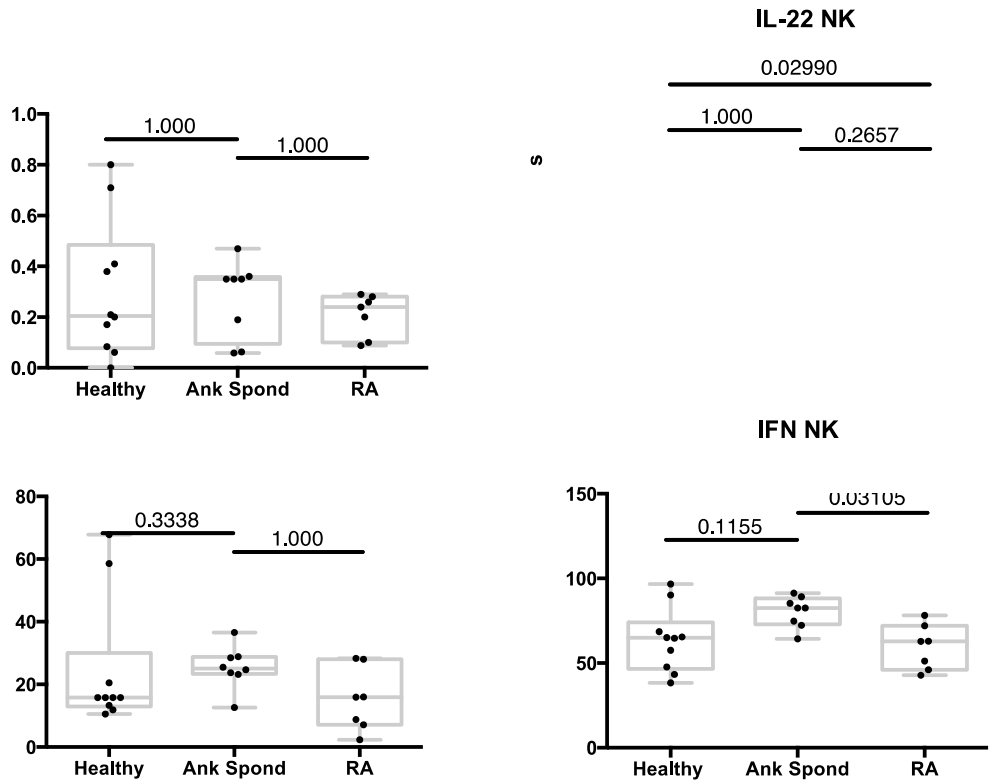


Figure 3.6. The cytokine phenotype of NK cells in AS does not differ significantly compared to healthy donors.
Ex-vivo stimulated PBMCs from patients with AS (n=8), RA (n=7) and healthy controls (n=10) were gated on $CD3^{-}CD56^{+}$ cells and the percentage of NK cells expressing IL-17A, GM-CSF, IL-22 and IFN-γ was determined. All data points are presented with box plots. There was no difference in the NK cytokine profile between AS and healthy donors. Statistical significance was determined using a Kruskal-Wallis test with p values calculated using Dunn's multiple comparisons test.

3.3.4 The IL-17A/GM-CSF double positive subset is expanded in AS CD4, CD8, γδ-T cell and NK cell compartments in AS

Polyfunctional lymphocytes, in particular those that co-express IL-17A and GM-CSF, have been shown to be particularly pathogenic in mouse models of autoimmunity (El-Behi et al. 2011; Shiomi et al. 2014). Given the overall increase in IL-17A and GM-CSF in the CD4 and CD8 cells, I next decided to look at the production of these two cytokines in combination across the four studied subsets of lymphocytes. Ex-vivo PBMCs from AS, RA and healthy donors were stimulated with PMA and ionomycin as above and the percentage of IL-17A and GM-CSF double positive cells was determined in each of the lymphocyte compartments.

I observed a statistically significant increase in the percentage of IL-17A/GM-CSF double positive CD4, CD8, δγ-T cell and NK cells in AS compared to healthy donors and RA disease controls (figure 3.7). Next I looked at whether GM-CSF was being produced by IL-17A-producing Th17 cells or IFN-γ-producing Th1 cells. I used a Boolean gating approach to look at the production of IL-17A, IFN-γ and GM-CSF in isolation or in combination with the other cytokines. Figure 3.8 shows the relative mean overlap of cytokine production between the various T helper subsets (n=28). Percentages are based on the total number of cytokine producing CD4 cells. I observe that GM-CSF is expressed in combination with both IFN-γ and IL-17A but also independently of these two subsets. The overlap of IL-17A and GM-CSF was greater than that of IL-17A and IFN-γ even though the percentage of IFN-γ producers was nearly twice that of GM-CSF producers (76.2% vs 37.6% respectively).

In order to address the question of whether GM-CSF-producing CD4 are more likely to be related to IFN-γproducing Th1 cells or IL-17A-producing Th17 cells I performed linear regression analysis of the total percentage of GM-CSF positive cells against the percentage of IL-17A and IFN-γ positive cells in the CD4 compartment (Figure 3.9). A positive correlation is observed between GM-CSF and both IL-17A and IFN-γ but the correlation between GM-CSF and IFN-γ is much stronger with an R^2 value of 0.564 versus 0.165.

Next I performed linear regression analysis of the IL-17A/GM-CSF double positive subset against total IL-17A and total GM-CSF. Once again a positive correlation was observed in both cases. However the correlation of IL-17A/GM-CSF double producing CD4 cells versus IL-17A had an R^2 value of 0.658 while the IL-17A/GM-CSF double producing population versus total GM-CSF had an R^2 value of 0.3. There was no correlation of GM-CSF percentage with age in any of the three patient/donor groups.

Figure 3.7

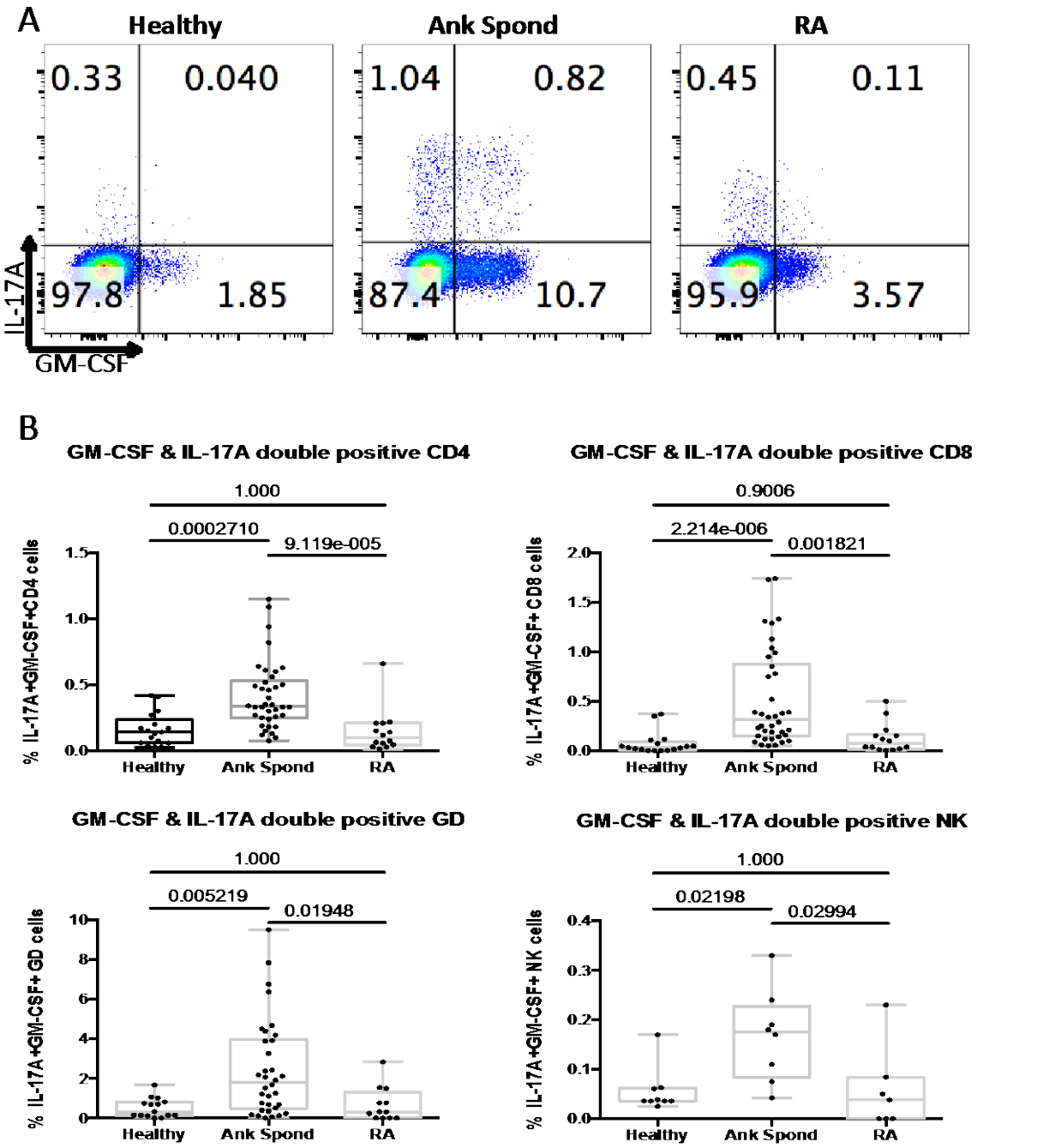


Figure 3.7. Increased percentage of IL-17A/GM-CSF double positive cells in AS CD4, CD8, γδ-T cell and NK cell compartments.

A representative flow cytometry plots showing IL-17A and GM-CSF staining in healthy, AS and RA PBMCs gated on CD4. Ex-vivo stimulated PBMCs from patients with AS, RA and healthy controls were gated on $CD3^+CD4^+$, $CD3^+CD8^+$ (AS n=38, RA n=14, HC n=17 for CD4 and CD8), $CD3^+\gamma\delta^+$ (AS n=34, RA n=12, HC n=15) and $CD3^-CD56^+$ (AS n=7, RA n=8, HC n=10) cells and the percentage IL-17A and GM-CSF co-producing cells was determined within each compartment. A significant increase in double positive cells was observed across all 4 lymphocyte compartments in AS compared to healthy donors and RA disease controls. All data points are presented with box plots. Statistical significance was determined using a Kruskal-Wallis test with p values calculated using Dunn's multiple comparisons test.

Figure 3.8

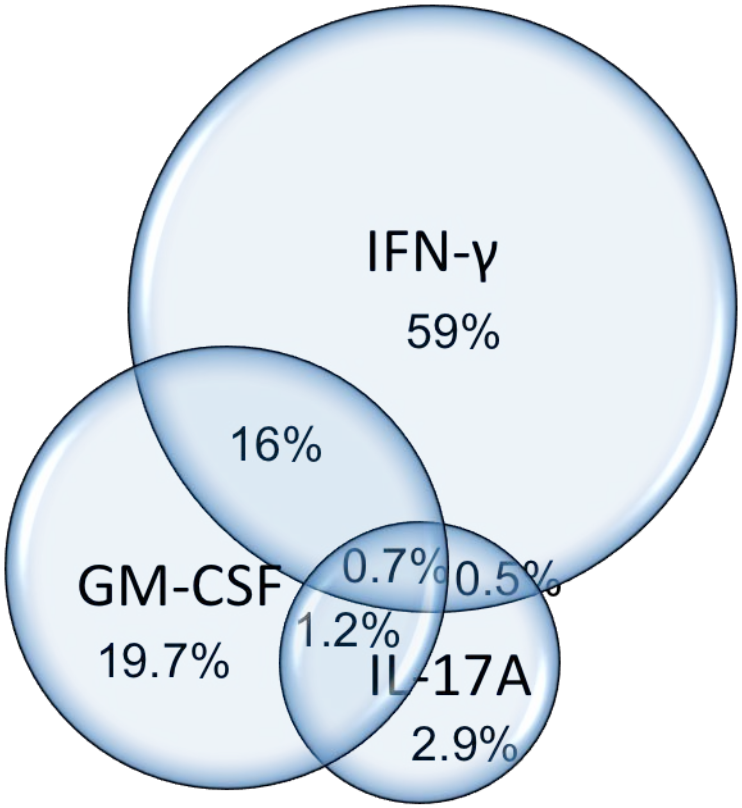


Figure 3.8. CD4 cytokine production in AS shows considerable functional overlap. Venn diagram representing mean expression of GMCSF, IL-17A and IFN-γ from CD4 cells in AS. Cytokine expression was determined by Boolean gating on flowjo® software and combinations of cytokines were derived. All CD4 cells expressing at least one of the three cytokines were included in the pooled analysis from 24 AS PBMCs gated on CD4. Cytokine expression is represented as % cells expressing each cytokine from all cytokine expressing CD4 cells. The area of the circles is adjusted according to relative expression of each cytokine compared to all cytokine-expressing CD4 population.

Figure 3.9

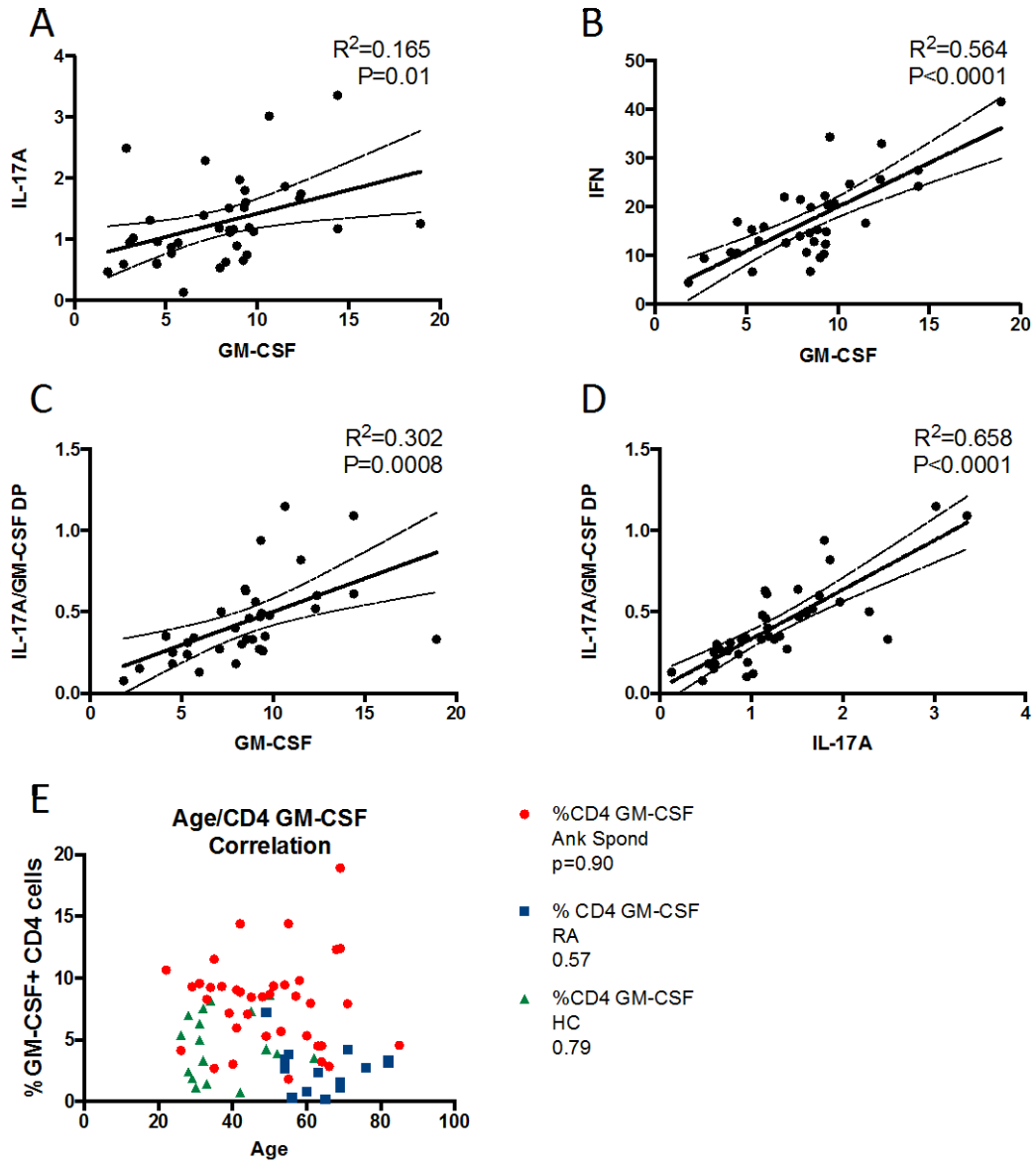


Figure 3.9. Total CD4 GM-CSF production correlates with total IFN-γ production while IL-17A/GM-CSF double producing cells correlate more closely with total IL-17A production. GM-CSF does not correlate with Age.
Linear regression analysis was performed on PMA/ionomycin activated PBMCs from AS patients gated on CD4 cells. The total percentage of CD4-positive GM-CSF-positive cells was plotted against IL-17A (A, n=38) and IFN-γ (B, n=34). In the bottom tow panels IL-17A/GM-CSF-double positive CD4 cells were plotted against overall CD4 cell GM-CSF (C, n=38) and IL-17A (D, n=38). All data points shown plus line of best curve and 95% confidence intervals. E The percentage of GM-CSF positive CD4 cells was plotted against the patient/donor age to test if age is a variable.

3.3.5 Surface phenotype of GM-CSF positive CD4 cells

I wanted to better understand the surface phenotype of the GM-CSF producing subset of cells. Firstly I observed all the GM-CSF expression ex-vivo was from the CD45RA-negative compartment indicating that these cells have a memory phenotype (figure 3.10). Next I looked at surface expression of the natural killer family receptor KIR3DL2. This receptor has been shown to recognise aberrantly folded HLA-B27 (Chan et al. 2005). I observed a threefold increase in GM-CSF expression in KIR3DL2 positive CD4 cells compared to total CD4 populations in the same patients (23% vs 7.3%, figure 3.11). Expression of KIR3DL2 also marked out a GM-CSF-enriched population within CD8 and γδ-T cells (figure 3.11A). I also observed a significant enrichment of GM-CSF expression in CD4 cells within the CD161 positive compartment. There was no significant difference between GM-CSF enrichment in the KIR3DL2 and CD161 compartments but cells positive for both KIR3DL2 and CD161 were significantly enriched for GM-CSF production compared to KIR3DL2 or CD161 alone (figure 3.11C).

Next I looked at the surface expression of the classical Th17 surface markers CD161 and CCR6. As expected ex-vivo IFN-γ producing Th1 cells had a low (<20%) expression of CD161 and CCR6 in combination compared to with 60% co-expression in Th17 cells expressing these markers in combination. GM-CSF single-producing cells expressed a similar percentage of cell-surface CCR6 and CD161 to IFN-γ-single producers but GM-CSF/IL-17A double

producers resembled classic Th17s (highly positive for CCR6 and CD161) with high expression of CCR6 and CD161 in combination (figure 3.12, n=8).

Figure 3.10

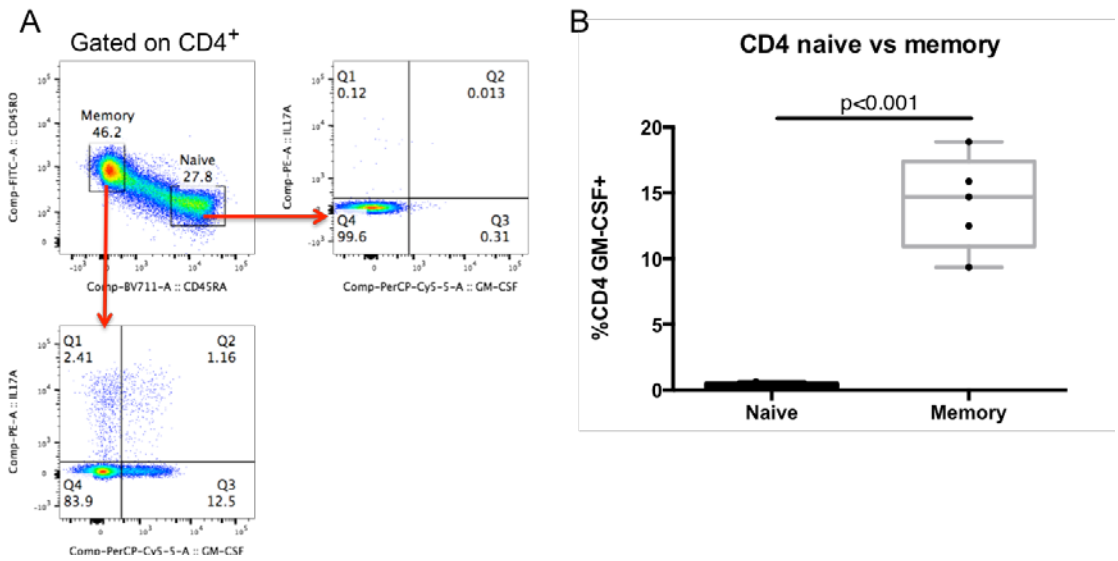


Figure 3.10. CD4 GM-CSF producing cells are all in the memory compartment. PBMCs were stimulated ex-vivo with PMA/ionomycin and $CD3^{+}CD4^{+}$ cells were gated according to $CD45RA^{+}CD45RO^{-}$ naïve cells and $CD45RA^{-}CD45RO^{+}$ memory cells. A shows representative flow cytometery plot and gating strategy. B shows data from 5 individuals. All data points presented with mean and standard deviation in box and maximum and minimum points in whiskers. Statistical significance was determined using a paired T test.

Figure 3.11

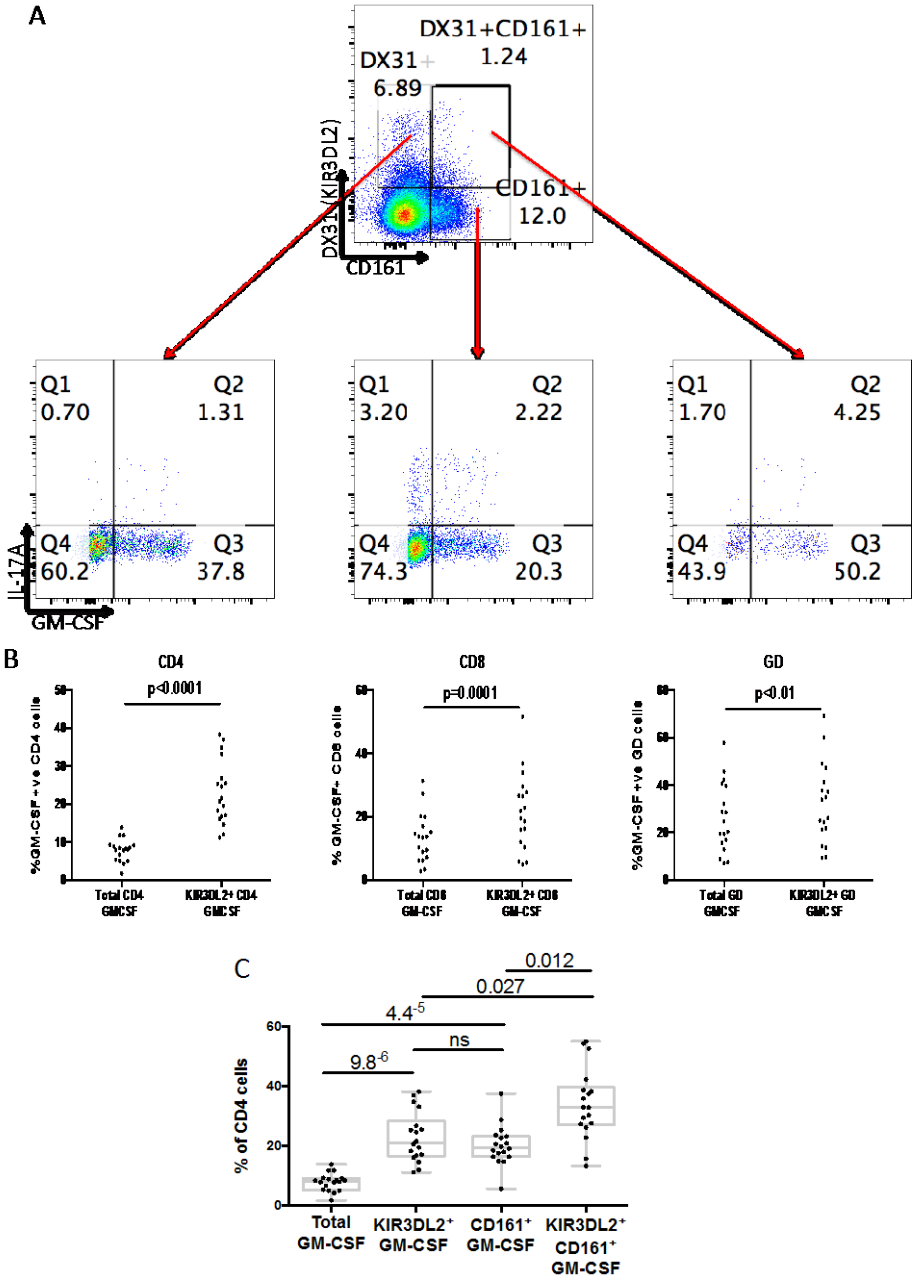


Figure 3.11. KIR3DL2 and CD161 positive cells are enriched for GM-CSF production. A. Representative flow cytometry plots of AS PBMCs gated on CD4 stained for DX31 (KIR3DL2) and CD161 showing IL-17A and GM-CSF staining in DX31^{+}, CD161^{+} and DX31^{+}CD161^{+} cells. B KIR3DL2 expressing CD4, CD8 and γδ-T cells in AS cells express higher levels of intracellular GM-CSF compared to overall CD4, CD8 γδ-T cells in the same individuals. B. CD161^{+} CD4 cells also express higher levels of GM-CSF compared to overall CD4 populations. The level of GM-CSF expression in CD161 positive CD4 cells is comparable to KIR3DL2 expressing cells but highest in the DX31^{+}CD161^{+} cells (n=18, RM one-way ANOVA).

Figure 3.12

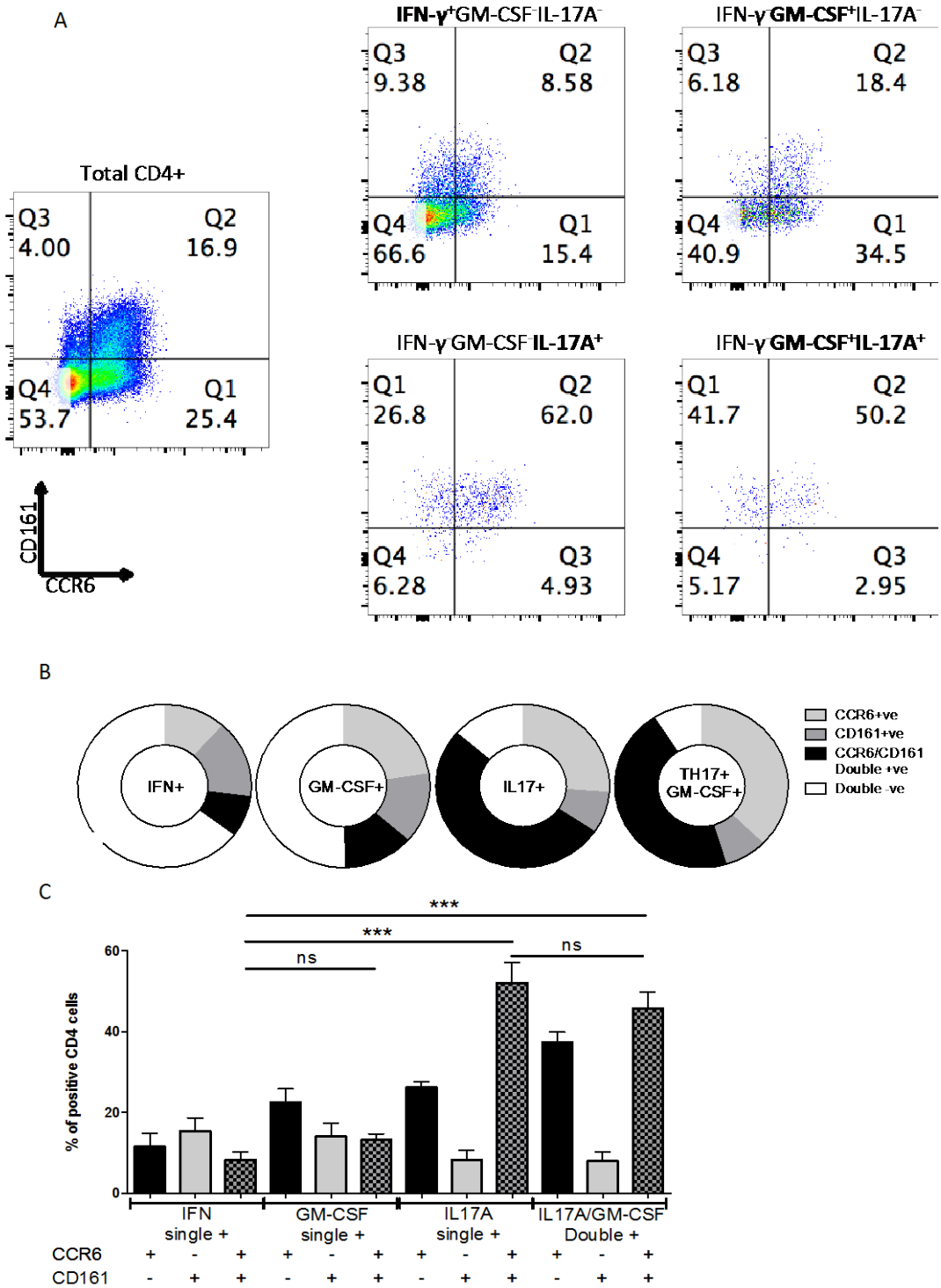


Figure 3.12. IL-17A/GM-CSF double-producing cells express Th17 surface markers CCR6 and CD161 in similar levels to IL-17A single producing cells.
A Representative flow cytometry plots of bulk AS PBMC CD4 cells stained for CCR6 and CD161 plus cells gated on cytokine production. B GM-CSF single positive cells have a similar level of CCR6 and CD161 expression to Th1 cells with a CD161/CCR6 double expression at around 15% while classical Th17 cells had significantly higher CD161/CCR6 double expression at around 50% (n=8, Friedman's non-parametric multiple comparison test). IL-17A/GM-CSF double producers had high levels of CD161/CCR6 double expression similar to Th17 cells. C CCR6 and CD161 expression of Th1 cells, GM-CSF single producing cells, Th17 cells and GM-CSF producing Th17 cells based on mean surface expression (n=8).

3.3.6 Phenotype of lymphocytes from spondyloarthritis joints

To study lymphocyte populations in the joint I looked at surgically derived synovial tissue of SpA patients undergoing joint replacement procedures (n=5). Inflammed synovium was explanted in the presence of IL-2 and IL-7 and the lymphocytes which migrated out of the tissue were harvested after 14 days and re-stimulated with PMA and ionomycin (figure 3.13A). For full methods details see chapter 2. GM-CSF production was observed to be around four fold higher in CD4 cells derived from synovium compared to AS PBMCs (figure 13.3B). I also observed significantly higher percentages of CD4 cells expressing GM-CSF in combination with IL-17A, compared to healthy PBMCs but not compared to AS PBMCs (figure 13.3C).

Next I studied ex-vivo CD4 cell responses from re-stimulated PBMCs with pared synovial fluid mononuclear cells (SFMCs) from the same SpA patient. Firstly I used CyTOF to compare and confirm that CD4 cells are the predominant GM-CSF producing lymphocyte in the inflamed synovium. Cell surface markers TCR-γδ, CD3, CD4, CD8, CD11b, CD11c, CD14, CD16, CD20, CD23, CD27, CD34, CD45, CD45RA, CD56, CD117, CD127, CD161, CD196 and CD336 were combined with intracellular staining for GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22 and TNF-α. ViSNE principal component anaylsis was done after gating on all live $CD45^+$ GM-CSF producers. Figure 3.14A shows overall more GM-CSF production in the ex-vivo synovial fluid cells, with CD4 cells as the dominant producing cell in both PBMCs and SFMCs. CD8 and CD56 account for the majority of the other GM-

CSF producing cells in the synovium. Figure 3.14B shows the overlap of GM-CSF production with the cytokines IL-17A, IL-4, IL-10, TNF-α and IFN-γ. I observed a small but very discreetly clustered population of IL-17A-producing GM-CSF positive cells however TNF and IFN-γ co-production with GM-CSF seemed to be more diffuse.

In order to quantify the GM-CSF and IL-17A production from the synovial fluid, I performed flow cytometry analysis on 5 matched PBMC/SFMC SpA samples ex-vivo. I observed increased expression of GM-CSF from the CD4 and CD8 cells in the SFMC compared to the PBMCs. In the CD4 compartment there was also a significant increase in the SFMC percentage of IL-17A/GM-CSF double positive cells compared to the PBMC CD4 cells as demonstrated by the representative flow cytometry plots gated on CD4 (figure 15A) and the combined data for CD4 and CD8 cells in figure 15B.

Figure 3.13

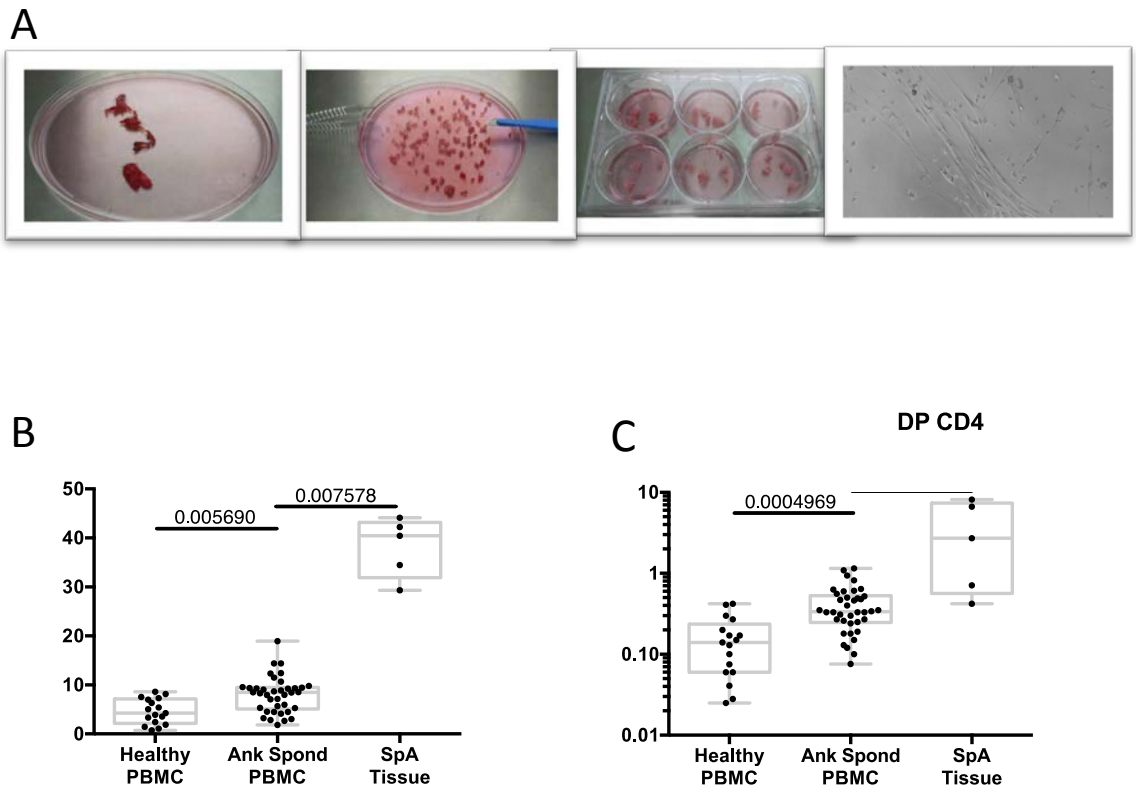


Figure 3.13. Elevated overall GM-CSF from inflamed synovium-derived CD4 lymphocytes.
A Shows stages of explant culture system used to obtain synovial tissue CD4 cells. Surgical samples are manually dissected to 3-4mm pieces and placed in a 6 well plate with RPMI medium containing 10% human AB serum and 100 IU/ml IL-2. Medium is replenished every 72 hours and non-adherent synovial tissue cells are harvested at day 14. B. Intracellular GM-CSF expression from synovial derived cultured CD4 cells (n=5) compared to ex-vivo CD4 cells from healthy controls (n=17) and AS (n=38) PBMCs (Kruskal-Wallis test with p values calculated using Dunn's multiple comparisons test). C. Intracellular co-expression of GM-CSF and IL-17A from ex-vivo CD4 cells of healthy controls, AS PBMCs and cultured SpA STMCs (Kruskal-Walis).

Figure 3.14

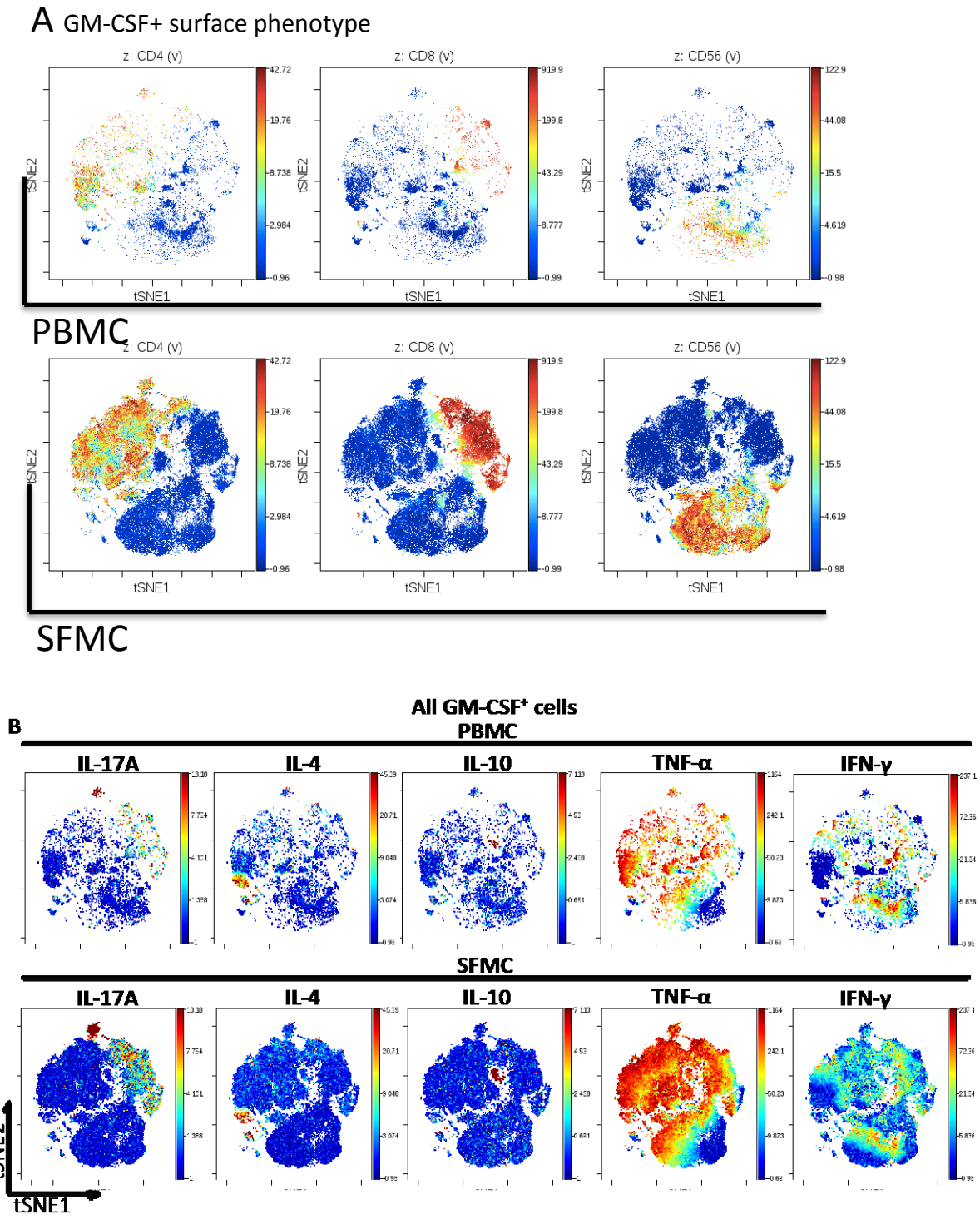


Figure 3.14. Principal component CyTOF ViSNE analysis of ex-vivo AS PBMCs (donor AS1453) with matched SFMC from the same patient stimulated with PMA and ionomycin.

Cells were gated on single cell, live, CD45+ GM-CSF+ before running the principal component analysis based on all other parameters in the CyTOF staining. 3.1A shows increased numbers of GM-CSF producing CD4, CD8 and CD56 cells in the SFMCs compared to the PBMCs but in both samples the CD4 cells are the main producers of GM-CSF. Panel B shows overlap of GM-CSF producing cells with the cytokines IL-17A, IL-4, IL-10, TNF-α and IFN-γ. Cytobank software was used for analysis.

Figure 3.15

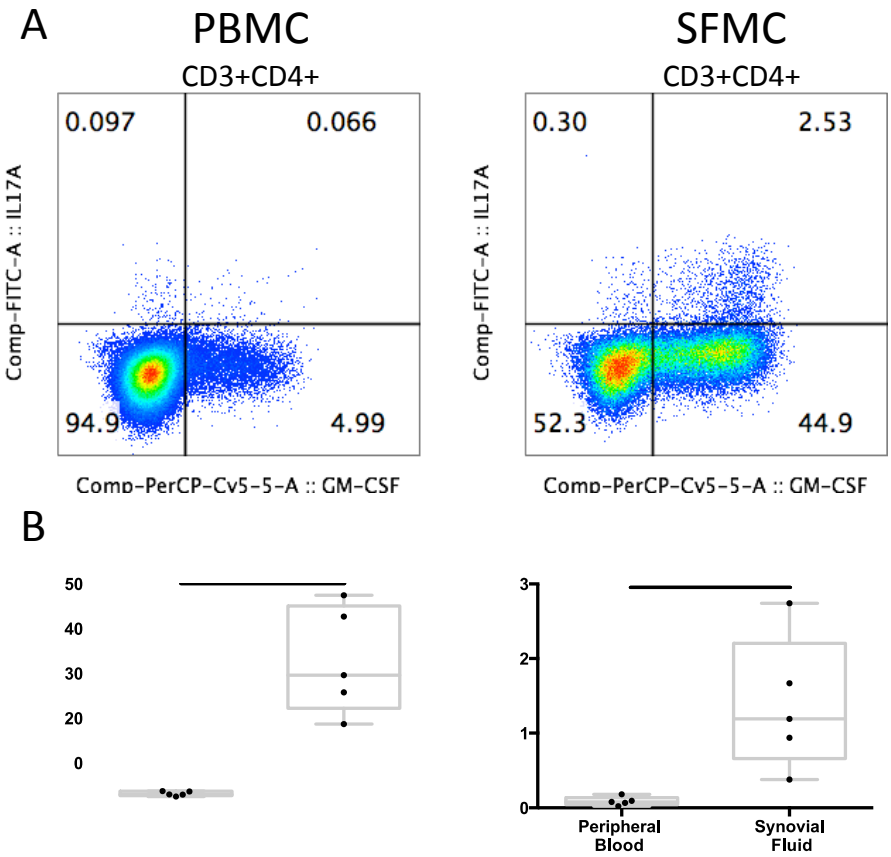


Figure 3.15. Increased ex-vivo GM-CSF from synovial fluid CD4 and CD8 cells compared to matched PBMCs.
A. Representative flow cytometry plots of ex-vivo activated PBMCs and SFMC from an AS patient gated on $CD3^{+}CD4^{+}$ cells. IL-17A is plotted against GM-CSF. B shows data from 5 matched PBMC/SFMC samples from SpA patients. The percentage of GM-CSF-positive CD4 and CD8 cells is significantly increased in the SFMCs compared to the PBMCs. The percentage of IL-17A/GM-CSF co-producing cells is also significantly increased in the synovial fluid CD4 cells but no the CD8 cells. N=5, paired t test.

3.3.7 Innate lymphoid cells are present in the inflamed joint and produce GM-CSF upon stimulation

Innate lymphoid cells are a newly described subset of cells thought to contribute to inflammation in a tissue-specific way. I observed by CyTOF the existence of a subset of GM-CSF producing cells in the synovial fluid that are negative for the lineage markers CD3, CD8, CD11b, CD11c, CD14, CD20, CD34 and γδ-TCR (figure 13.16). These cells are also positive for CD127 (IL-7R), which is the key defining marker of the innate lymphoid cells.

I further investigated the existence of this cell type in human joints by flow cytometry. Figure 13.17 shows the gating strategy employed to identify these cells. I gated on lineage-negative (CD3, CD5, CD8, CD11b, CD11c, CD14, CD19, CD20, CD34 TCR-γδ) CD45 positive live cells which also express the IL-7 receptor alpha chain. Even though this is a rare population, I identified ILCs in mononuclear cells derived from inflamed synovial tissue explants. These cells are generally divided into three main subtypes depending on surface expression of C-Kit and CRTH2. ILC type 3 cells express C-Kit and the majority also express NKp44 (figure 3.17). The KNp44 subset is known as NCR^{+}. ILC type 2 cells express CRTH2. I did not observe any CRTH2 expression on ILCs in the joint and therefore subsequently included CRTH2 into the lineage cocktail to exclude these cells from analysis. I observed ILC type 3 cells to be the predominant population in the ILC pool of patients with inflammatory arthritis (n=7), (figure 13.18B) while ILC1 cells were the predominant population in the peripheral blood in AS (In a different cohort of patients). Functional interrogation of these cells by PMA and ionomycin

stimulation revealed GM-CSF to be the most abundantly expressed cytokine both in ILC type 1 and type 3 cells (figure 13.18C-D).

Figure 3.16

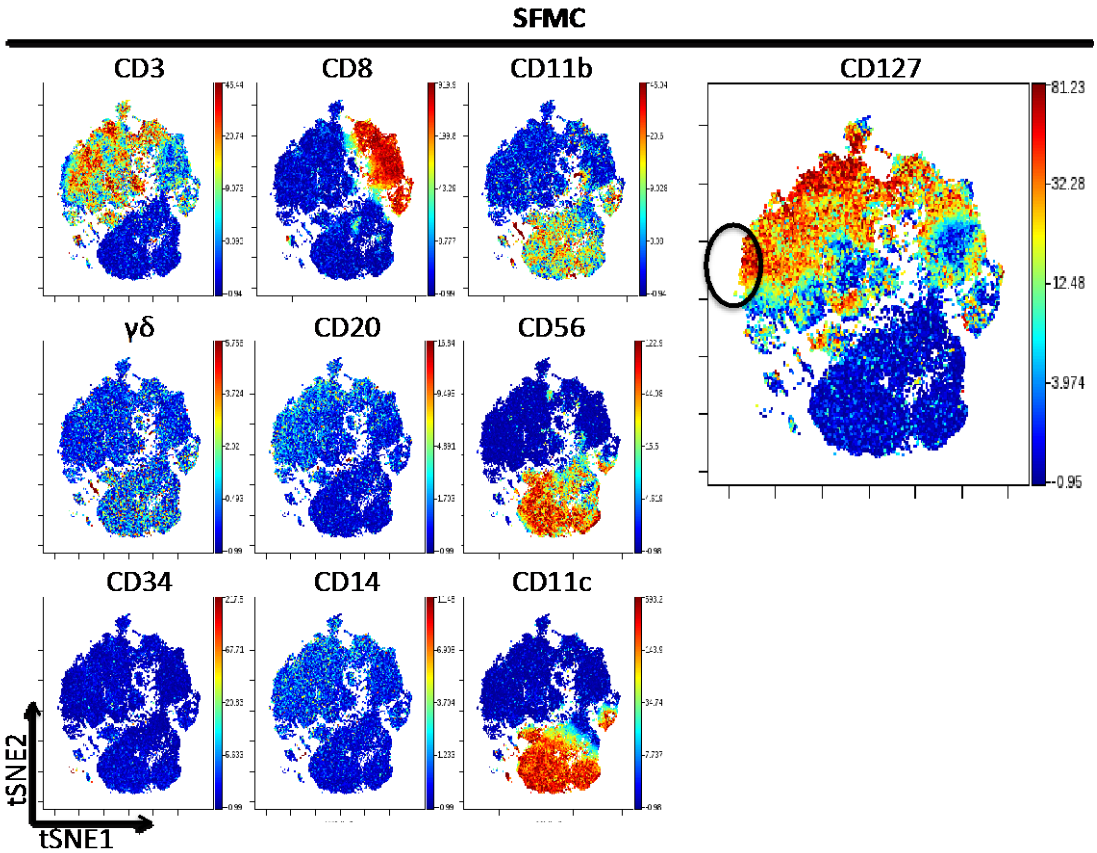


Figure 3.16. Principal component CyTOF ViSNE Analysis of ex-vivo AS SFMCs demonstrates a lineage-negative, IL7R positive GM-CSF producing subset.
Cells were gated on single cell, live, CD45+ GM-CSF+ before running the principal component analysis based on all other parameters in the CyTOF staining. Lineage markers CD3, CD8, CD11b, TCR-γδ, CD20, CD56, CD34, CD14 and CD11c were used to identify a lineage negative, IL7R positive subset of cells within the GM-CSF producing population. Cytobank software was used for analysis.

Figure 3.17

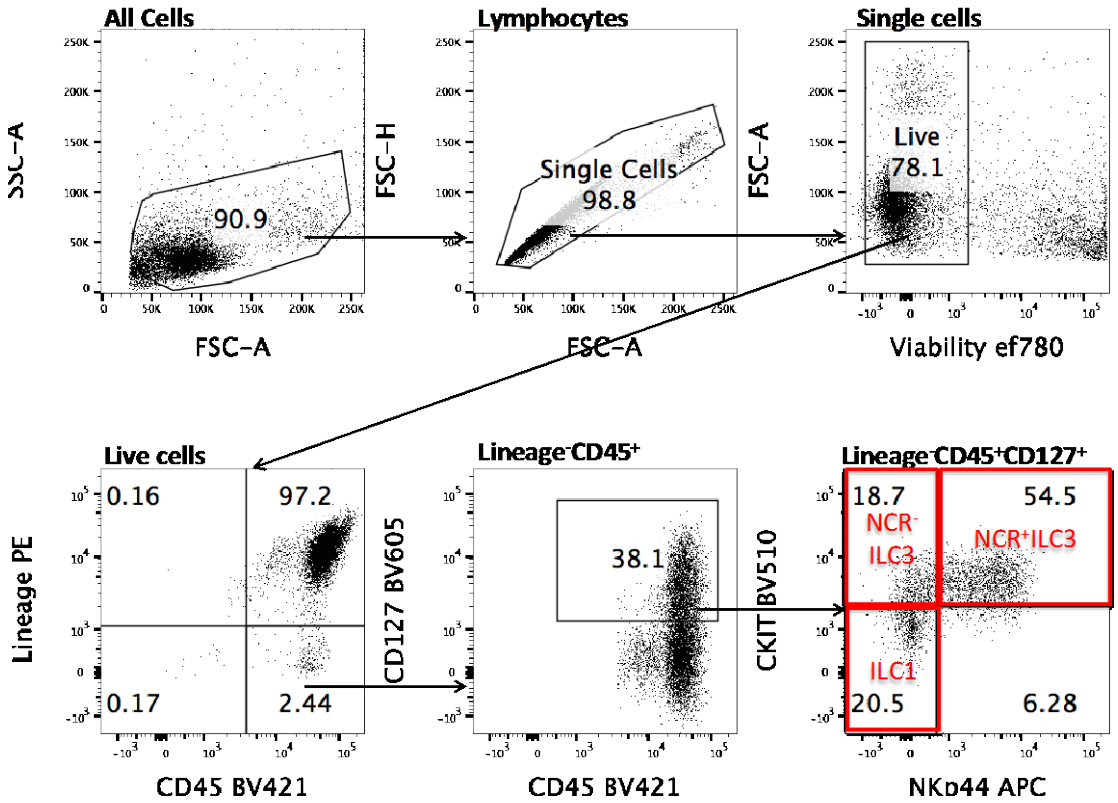


Figure 3.17. Gating strategy for identifying ILC populations
Gating strategy for identifying innate lymphoid cells from STMC cultures. Lineage negative (Lineage cocktail: CD3, CD5, CD8, CD11b, CD11c, CD19, CD20, CD34, TCR-γδ, CRTH2) CD45 positive cells were then gated on IL7R expression. ILC1 subset defined as $Lin^-CD45^+IL\text{-}7R^+CKIT^-$ and ILC3 subset defined as $Lin^-CD45^+IL\text{-}7R^+CKIT^+$. ILC3 population further subdivided into NCR^- and NCR^+ subpopulations based on NKp44 expression.

Fig 3.18

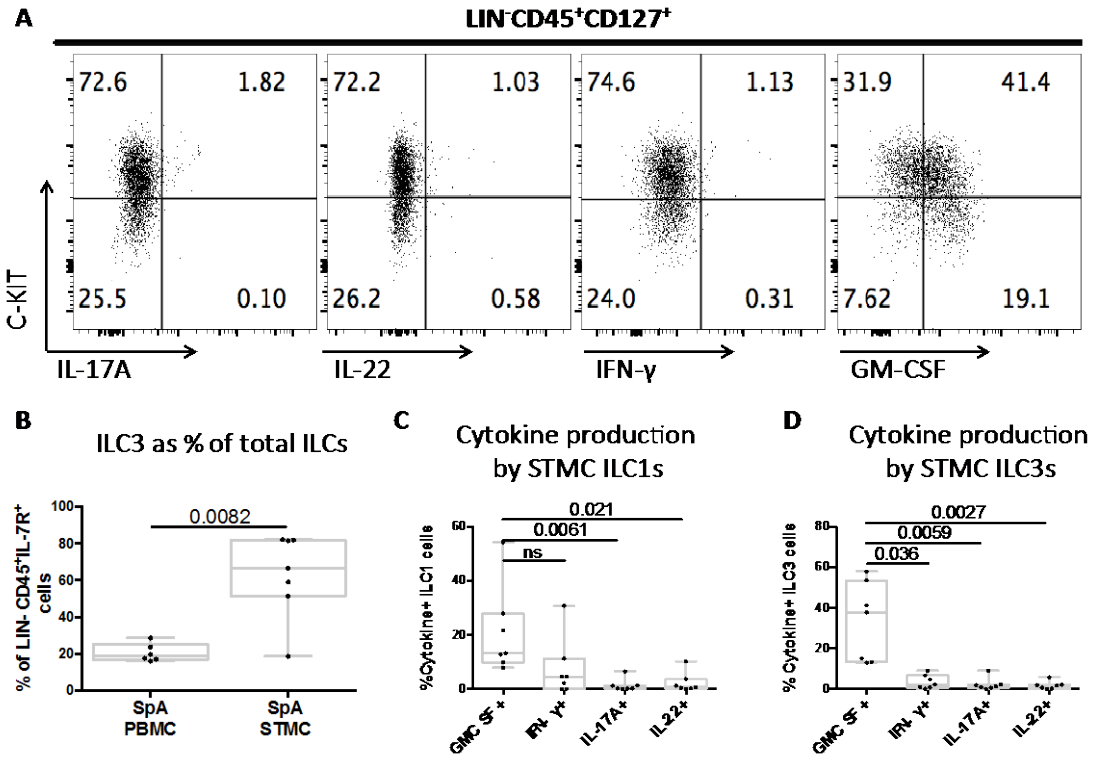


Figure 3.18. Type 3 innate lymphoid cells (ILC3) are enriched in the joint and produce GM-CSF abundantly.

A. Representative flow cytometry plots of ILC cytokine staining with C-KIT used to identify all (NCR^+ and NCR^- ILC3 populations). B. Relative enrichment of ILC3 populations as percentage of total ILCs in ex-vivo peripheral AS PBMCs (n=6) compared to inflammatory arthritis STMCs from a different cohort of patients (n=7) (unpaired t test). C-D STMC ILC1 and ILC3 cytokine producing frequencies from 7 patients with inflammatory arthritis (4 SpA, 3RA) (Friedman's non-parametric multiple comparison test).

3.3.8 IL-7 promotes GM-CSF production from CD4 cells in blood and synovial tissue blood

In order to investigate the factors driving GM-CSF production from CD4 cells in the joint I looked at the role of IL-7. IL-7 is a stromal-derived cytokine that has been shown to be expressed in the inflammed joint (Hartgring et al. 2009). I isolated CD4 cells from 7 healthy donors by negative magnetic bead selection. Cells were cultured with or without the presence of recombinant human IL-7 (10ng/ml) for one week (with anti-CD2/CD3/CD28 beads at a bead to cell ratio of 1:5 in the presence of 20 IU of IL-2). Figure 3.19 shows that IL-7 specifically enhances the GM-CSF production by CD4 cells, with a doubling of the number of cells expressing this cytokine. There was no statistically significant effect of IL-7 on IL-17A, IL-22, IFN-γ, TNF or IL-17A/GM-CSF double positive cells.

Next I investigated the effects of adding recombinant human IL-7 to explant STMC cultures from patients with inflammatory arthritis. 10ng/ml of IL-7 was added into the explant culture system from the beginning and added to subsequent medium used to top up the cultures every 72 hours. After two weeks cells were harvested. The addition IL-7 resulted in a significant increase in the percentage of GM-CSF positive cells. There was also a statistically significant increase in the percentage of IL-22-positive cells but no difference in IFN-γ, IL-17A and IL-17A/GM-CSF double positive cells. In addition, supernatants from explant cultures of patients with inflammatory arthritis were collected prior to PMA/ionomycin stimulation in order to quantify the baseline production of GM-CSF in the system. The addition of IL-7

significantly increased the amount of GM-CSF secreted into the explant culture when measure by ELISA (figure 3.20B)

Next I tested the effects of neutralising IL-7 made by stromal cells in the STMC cultures. A STMC culture was setup using tissue from an AS patient with the addition of a monoclonal anti-IL-7 antibody at 5µg/ml or its isotype control at the same concentration. The antibody and isotype were added at the same concentration to all subsequent media used to replenish the cultures. After two weeks non-adherant STMC were harvested and stained for flow cytometry. There was a marked absence of any STMC cells in the cultures containing anti-human IL-7 (figure 3.21).

Figure 3.19

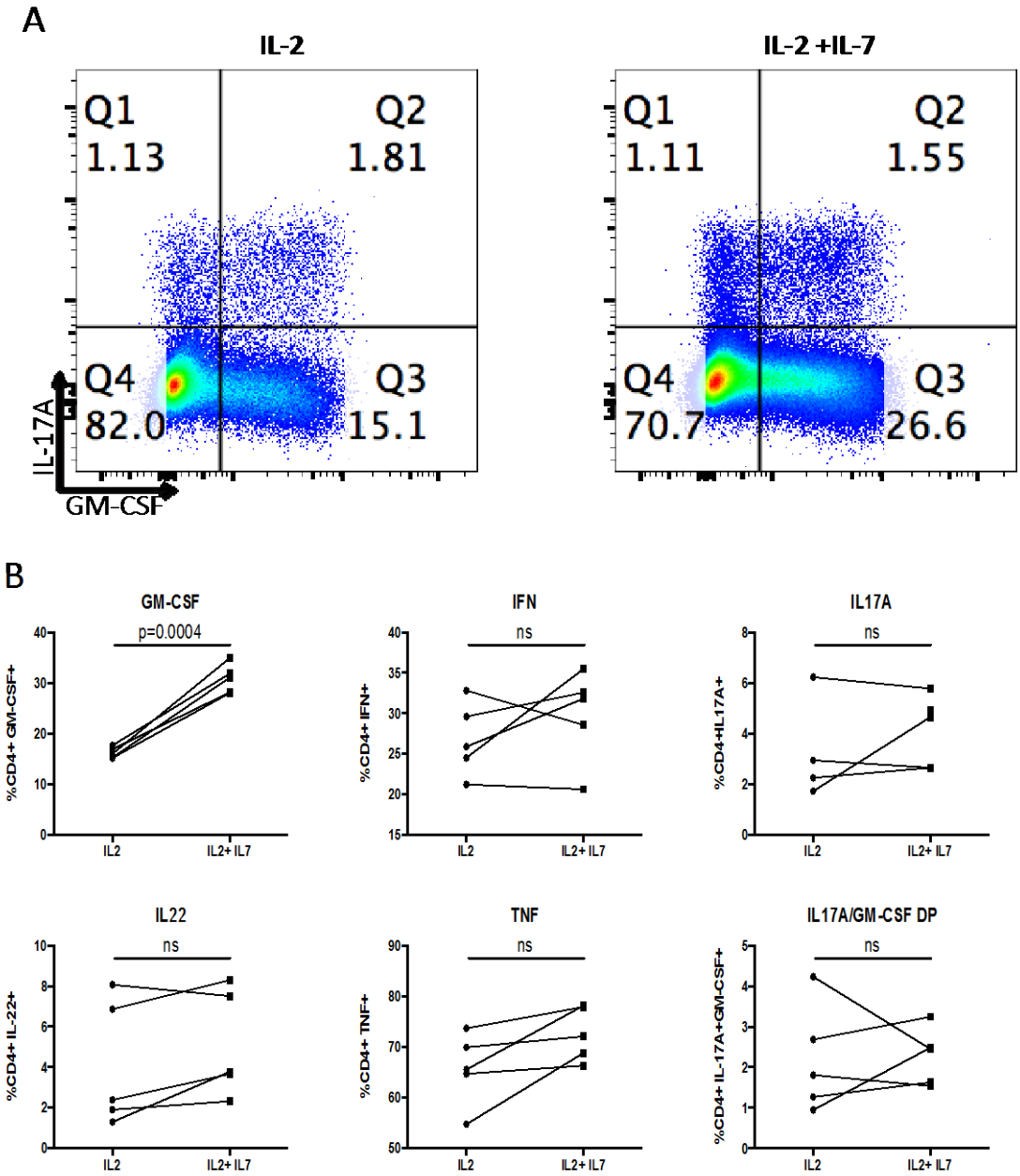


Figure 3.19. IL-7 promotes GM-CSF production from PBMC-derived CD4 cells.
A Representative flow cytometry plots, gated on live cells, of CD4 cells stimulated for one week in the presence of IL-2 alone or IL-2 with the addition of IL-7. B PBMCs from 7 healthy donors were isolated by negative selection and cultured for one week with or without 10ng of recombinant human IL-7. Cells were cultured and activated with anti CD2/3/28 beads at a ratio of 1 bead to 5 cells and were supplemented with 20 IU/ml of IL-2. At the end of the culture cells were re-stimulated with PMA/ionomycin and intracellular cytokine staining was performed. Statistical significance was determined using a paired t-

Figure 3.20

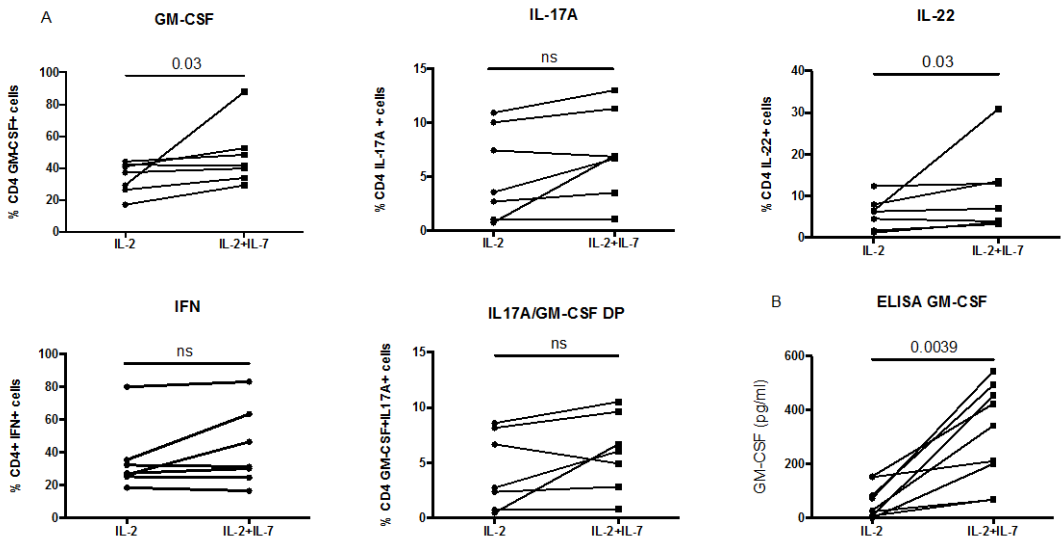


Figure 3.20. IL-7 promotes GM-CSF production in synovial tissue-derived CD4 cells. A- Surgical tissue from 7 patients with inflammatory arthritis undergoing joint replacement surgery was explanted with or without 10ng/ml of recombinant human IL-7. After two weeks of culture the STMCs were re-stimulated with PMA/ionomycin and intracellular cytokine staining was performed. Data derived after gating on $CD3^{+}CD4^{+}$ cells. B- supernatant from explant cultures was collected after two weeks and GM-CSF quantified by ELISA. Data from 9 independent experiments from 5 patients with inflammatory arthritis. Statistical significance was determined using a paired Wilcoxon test.

Figure 3.21

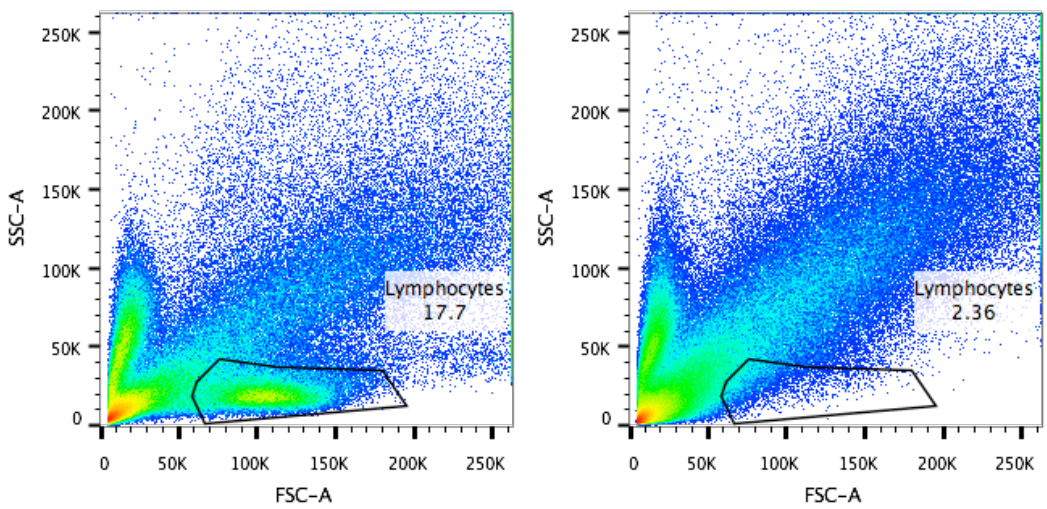


Figure 3.21. IL-7 is required for the survival of lymphocyte populations in STMC explant cultures.
Surgical tissue from a patient with spondyloarthritis undergoing joint replacement surgery was explanted with or without 5µg/ml of a monoclonal antibody against human IL-7 (Biolegend). After two weeks of culture the STMCs were stained for flow cytometry. A marked absence of cells in the lymphocyte gate was noticed in the presence of anti-IL7 coupled with increased proliferation of stromal cells.

3.3.9 Transcriptional profile of GM-CSF-producing CD4 cells

To understand the transcriptional profile of GM-CSF-producing T cells, RNA sequencing was carried out on cytokine-captured cells. CD4 cells were magnetically isolated by negative selection from lymphocyte cone PBMCs from 4 donors (purity >95%). CD4 cells were rested overnight and on the following day stimulated with PMA and ionomycin for two hours and triple cytokine capture assay was performed for IFN-γ, GM-CSF and IL-17A followed by fluorescent cell sorting into pure cytokine positive cells. (figure 3.22) For detailed methods see chapter 2, materials and methods. In summary 5 sorted populations from all 4 donors passed quality control and were run for sequencing. Post sequencing samples with less than 10 million (2 of 20) mapped reads were excluded from analysis.

Figure 3.23 focuses on some key known genes expected to be differentially expressed between the different sorted subsets in order to validate the sort. In particular I observed IFNG and TBX21, the genes encoding IFN-γ and T-bet, to be upregulated in IFN-γ single positive cells. Meanwhile IL17A and RORC, the genes encoding IL-17A and RORγt are elevated in IL-17A single positive cells. GM-CSF single positive cells show lower levels of IFNG expression compared to IFN-γ single positive cells but high levels of CSF2 (gene encoding GM-CSF) and high levels of TBX21. In addition, they show higher RORC compared to IFN-γ single positive cells. IL-17A/GM-CSF double positive cells show the highest expression of IL17A and CSF2, even higher than IL-17A single positive and GM-CSF single positive populations

respectively. In addition, the double positive cells also show a higher expression of TBX21 compared to IL-17A single positive cells.

Differential gene expression analysis was carried out for each single-producing subset of cells compared to CD45RA naïve cells. The false discovery rate (FDR) was set at <0.05. Figure 3.24A shows a unique gene expression for each of the single positive populations in addition to shared expression of some genes. The IL-17A single positive cells had more expression overlap with the GM-CSF single positive cells while the GM-CSF single positive cells had the greatest overlap with the IFN-γ single positive cells. The GM-CSF single producing cells also had a unique gene expression profile with 431 genes expressed uniquely by this subset. Figure 3.24B looks at the differential gene expression between GM-CSF and IL-17A single positive cells and IL-17A/GM-CSF double positive cells. This shows the double positive cells as having 340 uniquely expressed genes compared to the two single producing subsets.

Gene clustering analysis in figure 3.25 shows a unique cluster of gene expression for each of the 5 subsets of cells. In terms of clustering of the populations, the CD45RA naïve cells cluster on their own while the IFN-γ and GM-CSF single positive populations cluster together. IL-17A single positive and IL-17A/GM-CSF double producers also cluster together indicating that IL-17A/GM-CSF double producing cells have a transcription profile that is more aligned with IL-17A single producing cells rather than GM-CSF single producing cells.

Figure 3.22

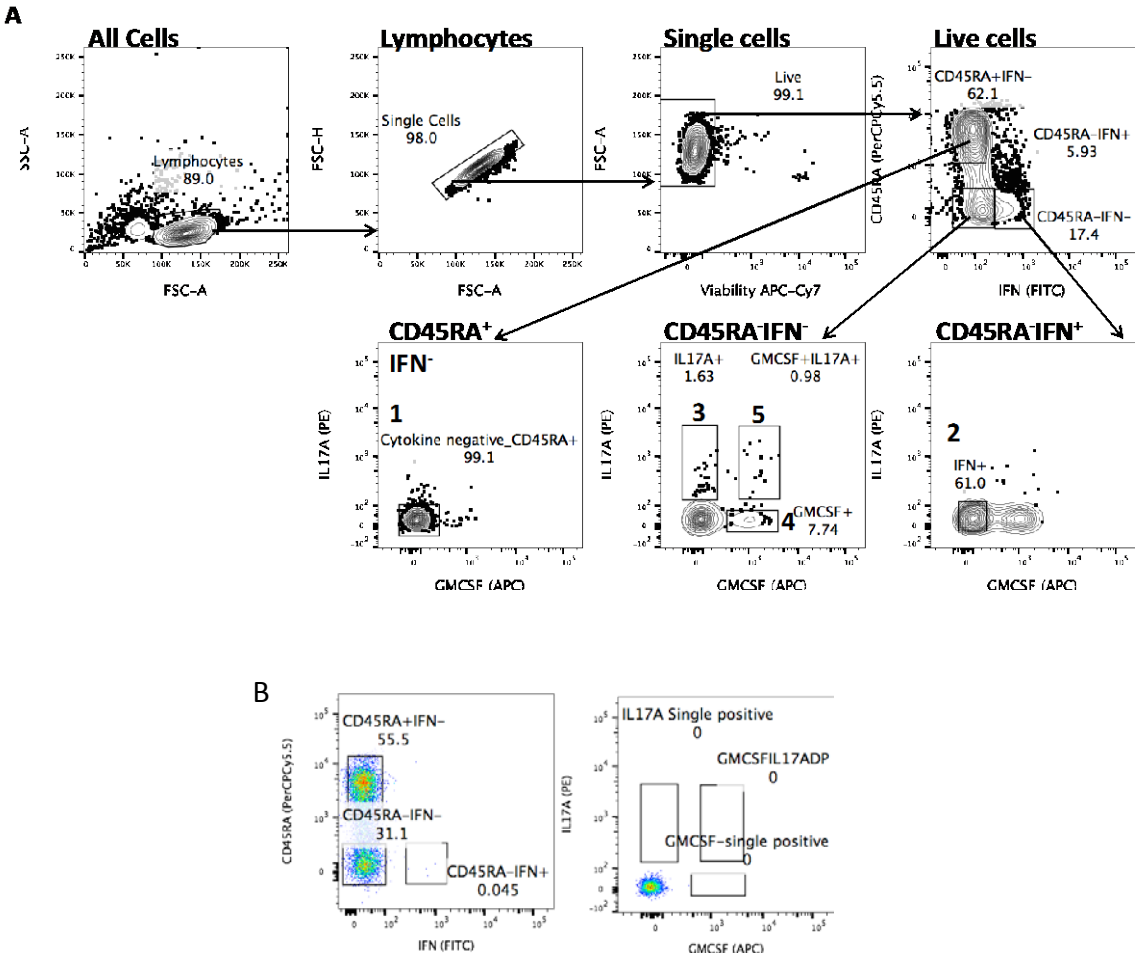
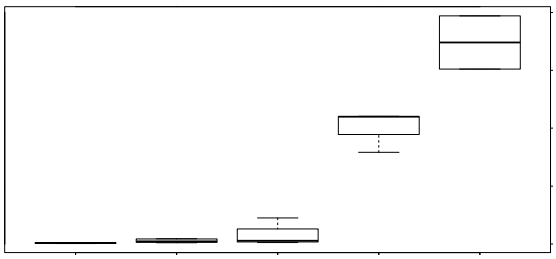


Figure 3.22. Sorting strategy for triple cytokine capture.
PBMCs were obtained from a lymphocyte cone by density centrifugation and CD4 cells were isolated by magnetic bead negative isolation on the same day. The following day cells were stimulated with PMA/Ionomycin and triple cytokine capture was performed for IL-17A, IFN-γ and GM-CSF. 5 populations (numbered 1-5) were sorted according to the gating strategy above (A). Live cells were first gated according to CD45RA and IFN-γ. $CD45RA^{+}IFN\text{-}\gamma^{-}$ cells were then sorted according to a IL-17A/GM-CSF negative gate (population 1: triple –ve CD45RA +ve). $CD45RA^{-}IFN\text{-}\gamma^{+}$ cells were sorted according to a IL-17A/GM-CSF double negative gate (population 2: CD45RA –ve IFN-γ single +ve). $CD45RA^{-}IFN\text{-}\gamma^{-}$ cells were sorted into 3 populations. (population 3: CD45RA –ve IL-17A single +ve; population 4: CD45RA –ve GM-CSF singe positive; Population 5: CD45RA –ve IL-17A/GM-CSF double +ve). B shows an unstimulated control from the same individual.

Figure 3.23



Cyt$^-$ IFN-γ$^+$ GM-CSF$^+$ IL-17A$^+$ IL-17A$^+$ GM-CSF$^+$

Figure 3.23. Sort validation by RNA seq.
RNA extracted from sorted populations was sequenced and the expression of IFNG, IL17A, and CSF2 genes was plotted for each group of sorted cell. IFNG gene expression was highest in the IFN-γ-positive cells, IL17A gene was highest in the IL-17A single-positive and IL-17A/GM-CSF double positives, and CSF2 was highest in the GM-CSF-positive and IL-17A/GM-CSF double positives. On the right sided panels mRNA expression of RORC, TXB21 and PTPRC (marker of naïve CD4 cells) was quantified in the 5 sorted populations. RORC was highest in the IL-17A/GM-CSF double positives followed by the IL-17A single positives while TBX21 is highest in GM-CSF single positives followed by IFN-γ single positives. PTPRC is highest CD45RA+ triple cytokine negative CD4 cells.

Figure 3.24

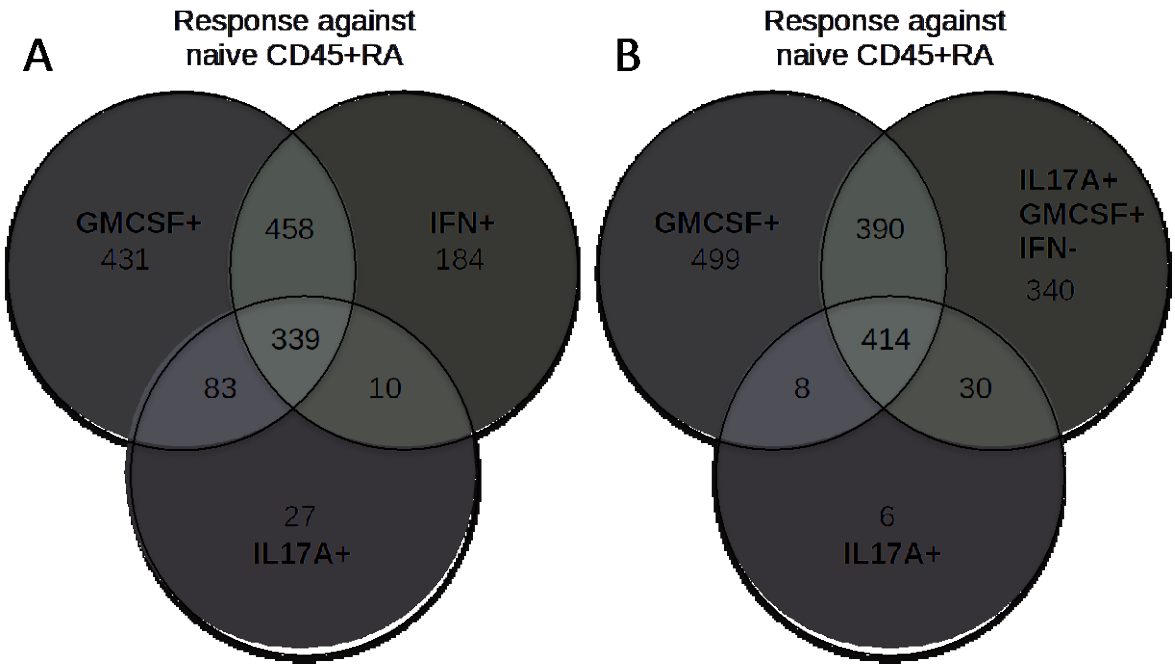


Figure 3.24. Differential gene expression by subsets of cytokine producing lymphocytes.
Differential gene expression was carried out on the subsets of cytokine producing cells compared to cytokine negative CD45RA naïve cells. A. The three single producing subsets of cells are plotted showing the number of commonly and differentially expressed genes. B. GM-CSF single positive and IL-17A single positive gene expression is plotted against IL-17A/GM-CSF double positive cells. False discovery rate for this analysis was set at <0.05%.

Figure 3.25

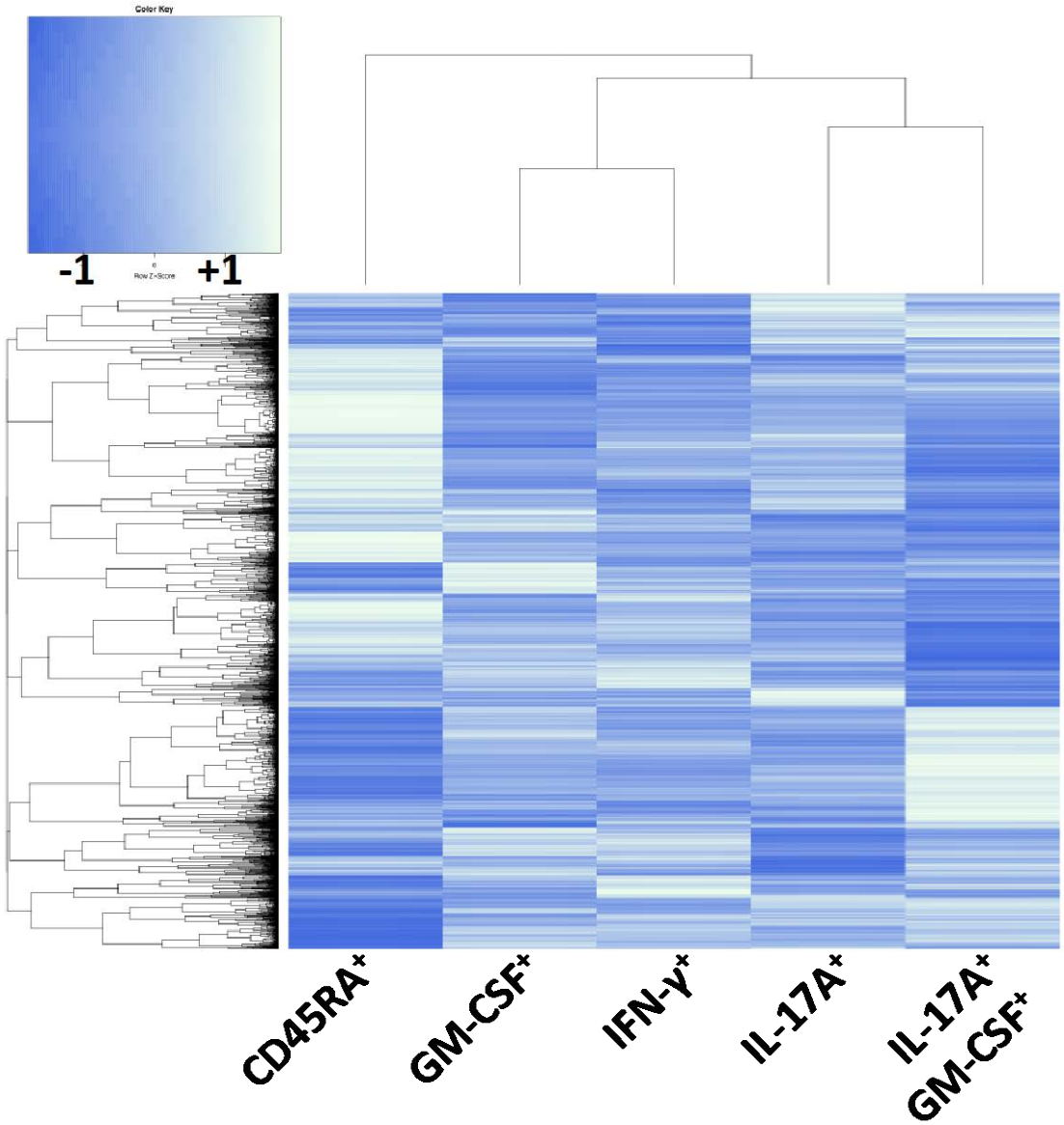


Figure 3.25. Gene clustering heatmap.
Unbiased gene clustering analysis of the 5 sorted subsets of cells shows GM-CSF single positive and IFN-γ single positive cells clustering together while IL-17A single positive and IL-17A/GM-CSF double positive cells cluster together. In addition, there is clear clustering of activated genes that define each subset.

3.3.10 The main cellular targets of GM-CSF in ex-vivo PBMC and SFMCs are $CD14^+$ monocytes

GM-CSF is a cytokine that is known to have its effects predominantly on myeloid cells (Gearing et al. 1989) and signals via STAT5 phosphorylation (Okuda, Foster, and Griffin 1999). In order to investigate the ex-vivo effects of GM-CSF in PBMC and SFMC populations in AS, I carried out staining for pSTAT-5 after exposure to recombinant human GM-CSF. PBMC and SFMC were starved by incubation in R0 medium for 4 hours then stimulated with 50ng/ml of GM-CSF (or 10ng/ml of IL-7 as a control) for 15 minutes. Results were analysed according to cell type (figure 3.26) and demonstrated a noticeable shift in pSTAT5 induction in $CD14^+$ cells in the peripheral blood and the joint after GM-CSF. No induction of CD4, CD8, CD56 or CD20 cells was seen with GM-CSF but CD4 and CD8 cells were shown to be able to induce pSTAT5 after IL-7 incubation (figure 3.27).

Figure 3.26

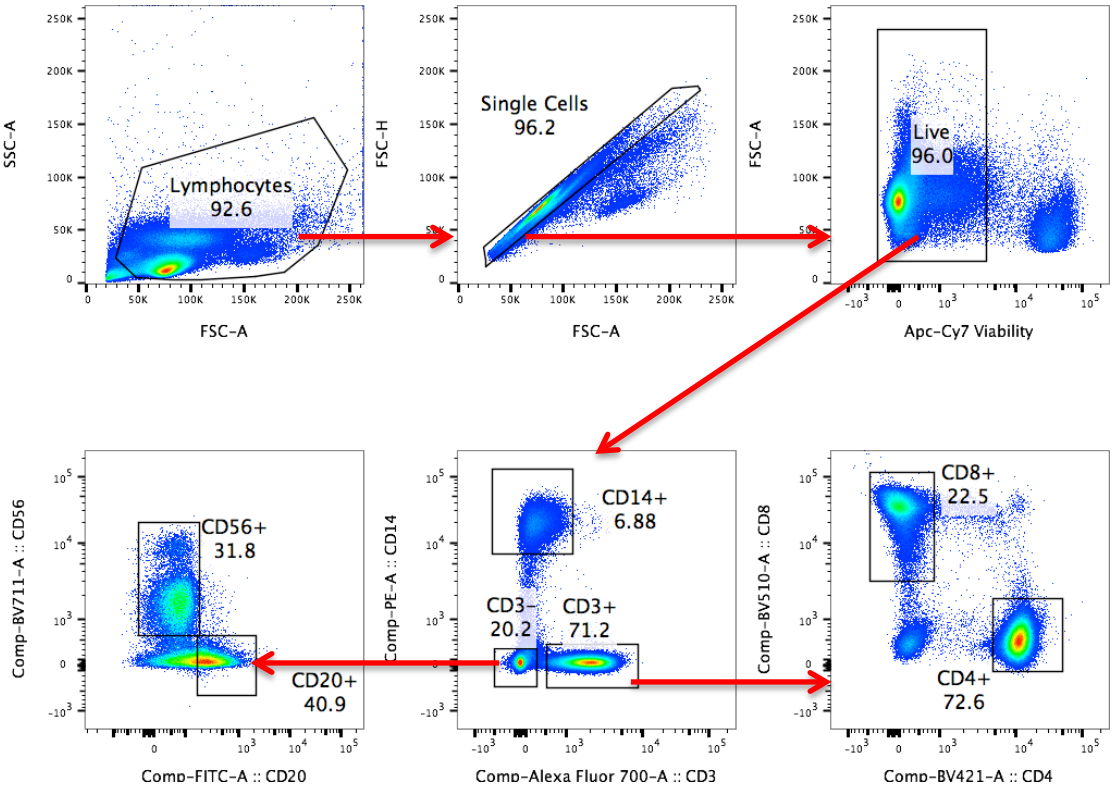


Figure 3.26. Gating strategy for phosho-STAT5 staining.
Matched PBMCs and SFMC were obtained from an AS patient by density centrufigation. Cells were starved for 4 hours in R0 medium then re-suspend in medium containing 50ng/ml of recombinant human GM-CSF for 15 minutes before being placed on ice. Cells were then stained for surface markers followed by an intracellular phospho-STAT5 staining. Above is a representative FACS plot form PBMCs showing the gating for the different

Figure 3.27

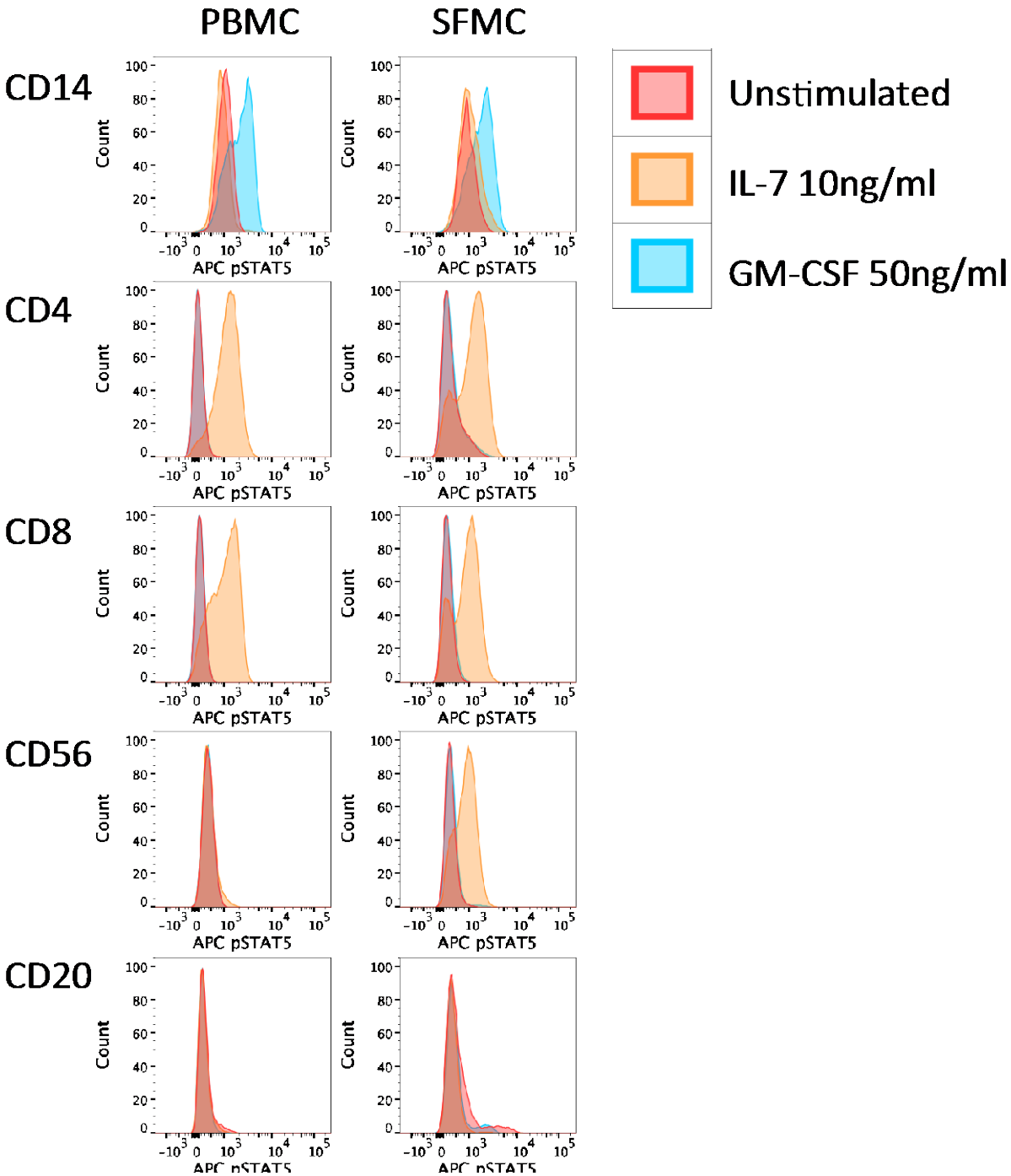


Fig 3.27. Phospho-STAT5 staining of PBMCs and SFMCs shows CD14 monocytes to be the main population responding to GM-CSF.
Matched PBMCs and SFMC were obtained from an AS patient by density centrufigation. Cells were starved for 4 hours in R0 medium then re-suspend in medium containing 50ng/ml of recombinant human GM-CSF or 10ng/ml of recombinant human IL-7 for 15 minutes before being placed on ice. Cells were then stained for surface markers followed by an intracellular phospho-STAT5 staining. Phospho-STAT5 signal was plotted in unstimulated (red) and after GM-CSF (blue) or IL-7 (Yellow) exposure as overlaid histograms according to the various cell types. Phospho-STAT5 (pSTAT5) induction was clearly seen in response to GM-CSF only in the $CD14^{+}$ cells in the blood and synovial fluid while pSTAT 5 is observed CD4 and CD8 cells after IL-7 stimulation in the PBMC and CD4, CD8 and CD56 induction in the synovial fluid. Representative plots shown from 3

3.4 Discussion

The data presented in this chapter confirm the expanded CD4 Th17 compartment observed by other groups (Shen, Goodall, and Hill Gaston 2009; Sarkar, Cooney, and Fox 2010), and further show for the first time an increase of GM-CSF producing CD4 cells in AS compared to healthy donors and RA disease controls. The observation of expanded CD4 type 17 responses in the peripheral blood of patients with AS highlights the importance of this inflammatory axis for the development of therapeutics (Baeten et al. 2015). The expansion of the GM-CSF compartment was mediated by memory CD4 cells, which predominantly produce GM-CSF independently of IL-17A. One possible explanation for these findings would be that patients with AS simply have expanded numbers of circulating memory CD4 cells, however there is no published data on this in the current literature. Moreover my data suggest that the overall ability of CD4 results to produce other cytokines such as IFN-γ does not differ in AS compared to healthy donors or RA disease controls. This suggests that an altered T cell cytokine phenotype likely accounts for the differences in the group rather than absolute numbers of cytokine-producing memory CD4 cells.

A surprising finding is that the numbers of circulating CD4 cells expressing GM-CSF in the RA disease controls was significantly lower compared to AS. This is especially surprising given that anti-GM-CSF monoclonal antibodies are currently in clinical trials in RA (Behrens et al. 2014; Burmester et al. 2013). However, a role for circulating GM-CSF-producing CD4 cells in RA has not to my knowledge been published. The efficacy of the antibody may

therefore be predominantly due to the local effects of GM-CSF in synovial tissue where increased numbers of cells expressing the GM-CSF receptor have been described in both RA and PsA (Greven et al. 2014). The efficacy of anti-GM-CSF therapy has been described in the collagen-induced arthritis models (Cook et al. 2001). Moreover the role of circulating Th17 cells has been previously investigated in RA with conflicting results. Jandus et al published in 2008 that the number of circulating Th17 (determined by intracellular cytokine staining) was elevated in AS but not RA (Jandus et al. 2008), while Shen et al published the following year expanded percentage of CD4+ T cells both in AS and RA compared to controls. My results seem to support the findings of Jandus et al and suggest an increased percentage of Th17 cells is a feature of AS but not RA.

One possible confounding factor accounting for the differences between the groups may be the sex and age differences between the different groups. The AS cohort has an average age of 50 while the RA cohort is significantly older with a mean age of 65. The healthy donors have a mean age of 37, therefore if younger age predisposed to increased circulating GM-CSF, one would expect the healthy donors to be the group with the greatest percentage of $CD4^+$ GM-CSF-producing cells. Moreover there was no correlation between age and the percentage of GM-CSF-producing CD4 cells in any of the groups (figure 3.9E).

The flow cytometry and CyTOF data presented in this chapter was all generated by activating cells with PMA and ionomycin. It can be argued that

this is a supra-physiological stimulation for T cells and does not reflect the in-vivo stimulation of these cells. However, the use of PMA and ionomycin allows me to take a snapshot of the ex-vivo potential of the T cells and since both patient and control PBMCs are treated in exactly the same way, then any differences observed between the groups would be valid. Moreover, the data in figure 3.20B shows there is spontaneous secretion of GM-CSF from patient derived synovial cultures, therefore suggesting GM-CSF is likely to be a relevant cytokine in the disease setting.

My data also show GM-CSF is abundantly expressed by CD4 cells at the site of inflammation, both in paired synovial fluid mononuclear cells and in explant-derived synovial tissue cells. A clear limitation of the work is that I am unable to compare the inflammed joint responses with those from non-inflammatory disease controls. While patients with non-inflammatory joint diseases often do get small joint effusions, these are acellular in nature and therefore cannot be used for comparison.

I confim in human joints the existence of lineage-negative, IL-7R-positive innate lymphoid cells. In particular, the type 3 ILCs seem to be more abundant in the joints of patients with SpA compared with peripheral blood. Classically, C-Kit-expressing ILC type 3 cells are though to contribute to the cytokine environment through the production of IL-17A and IL-22 in response to tissue stress (Geremia et al. 2011; Spits and Cupedo 2012). Previously they have been shown to be important mediators of barrier integrity in the gut (Geremia et al. 2011). ILC3 cells derived from the joint show only very low levels of IL-

17A and IL-22 expression but abundant expression of GM-CSF when stimulated with PMA and ionomycin. ILC type 1 cells are also present in the joint and are capable of producing IFN-γ but the cytokine they express most frequently is GM-CSF. Thus GM-CSF seems to be an important mediator in both innate and adaptive immunity during inflammation.

The pathogenic role of T cell-derived GM-CSF has gained prominence with GM-CSF shown to be driving type-17 neuroinflammation in a mouse model of neuroinflammation (El-Behi et al. 2011). Neutralisation of GM-CSF has been shown to be sufficient to halt mouse inflammatory disease (El-Behi et al. 2011; Shiomi et al. 2014). T cell specific GM-CSF knockout mice are protected from disease (Codarri et al. 2011) suggesting T cell derived GM-CSF is important in driving pathogenesis in these mouse models. The pathogenic role of GM-CSF is thought to primarily occur through its effects on pro-inflammatory monocyte differentiation (Gordon and Martinez 2010). I confirm in my results that the principle population responding to the GM-CSF both in the blood and the joint are myeloid derived CD14 cells. A recent report has suggested that GM-CSF may additionaly play a role in mouse gut pathogenesis through its effects on other myeloid populations such as Eosinophils and myeloid precursors (Griseri et al. 2015). The data I present in this thesis do not exclude a role for Eosinophils since they were largely excluded through the density centrifugation technique used for PBMC and SFMC isolation. Eosinophills tend to have density greater than the histopaque and therefore are excluded along with neutrophils and red blood cells.

In terms of GM-CSF relevance to human disease, elevated GM-CSF-producing T cells have been shown in the joints of patients with Juvenile Inflammatory Arthritis (JIA) (Piper et al. 2014). Moreover a greatly increased percentage GM-CSF-producing CD4 cells have been found in the CSF of patients with MS compared to non-MS CSF (Noster et al. 2014). In addition, CD4 and CD8 cells expressing GM-CSF have been shown to be elevated in the peripheral blood of patients with MS compared to healthy donors and MS patients treated with IFN-β therapy (Rasouli et al. 2015).

It remains unclear whether GM-CSF production independent of IL-17A is contributing to the disease process or if GM-CSF pathogenicity occurs in the context of Th17s. More evidence seems to be pointing towards GM-CSF-producing CD4 cells representing a distinct effector phenotype with a recent report suggesting that GM-CSF production is more aligned with a STAT5 rather than a STAT3 programme (Noster et al. 2014). IL-7 is implicated as a driver of STAT-5 mediated inflammation in the mouse EAE model (Sheng et al. 2014). Indeed my data showing IL-7 to be enhancing the production of GM-CSF from CD4 cells, both in isolation and in an inflamed tissue explant system, would support this hypothesis. Other studies have shown GM-CSF production by CD4 cells to be under the influence of IL-1β (Lukens et al. 2012; Duhen et al. 2009) , with GM-CSF in turn boosting IL-1β production by myeloid derived cells (Khameneh et al. 2011). What is clear from my data is that the human CD4 compartment shows considerable capacity for multiple cytokine production and ex-vivo PBMC responses show GM-CSF production

occurring in isolation but also in combination with classic IFN-γ producing Th1 cells and classic IL-17A producing Th17s.

In peripheral blood CD4 cells the correlation of overall GM-CSF to overall IFN-γ is greater than the correlation to overall IL-17A, with the strongest correlation observed between total IL-17A and IL-17A/GM-CSF double producing cells. The percentage of these polyfunctional cells is greatly increased in the peripheral blood of patients with AS and especially in the inflamed synovial fluid and tissue. Phenotypic analysis of these GM-CSF/IL-17A double producers shows these cells to be very similar to classic Th17s, with high CCR6 and CD161 co-expression. This contrasts with GM-CSF single producers which have CD161/CCR6 expression comparable to Th1 cells. The surface phenotype and correlation data would suggest that these double producers originated from classic Th17 cells rather than arising from Th1-like GM-CSF single producers. The surface expression data are independently supported by the RNA sequencing gene clustering data which also show clustering of IL-17A single producers with IL-17A/GM-CSF double producers and the clustering of GM-CSF single producers with IFN-γ single producers.

Therefore in the context of human SpA I propose several roles for lymphocyte-derived GM-CSF. Firstly, there is an expansion of GM-CSF single producing cells in the CD4, CD8 and γδ compartments. This is largely independent of IL-17A and IL-7 may be a key driver of this subset. The abundance of IL-7 in the inflamed joint may account for the increase in GM-

CSF producing lymphocytes seen at this site (Rihl et al. 2008; Pickens et al. 2011). Moreover polymorphisms in the IL-7 receptor have been shown to be associated with AS (Cortes et al. 2013) and MS (Gregory et al. 2007). In addition there is an increase in IL-17A/GM-CSF double producing cells and their presence correlates with classic Th17. These may represent "pathogenic" Th17s and the drivers for their existence are not directly under the influence of IL-7 but perhaps under the influence of GM-CSF-driven pro-inflammatory monocytes. Finally, early production of GM-CSF from ILCs present in the joint may be crucial for starting a chronic inflammatory response that culminates in a STAT-5 mediated pro-inflammatory loop. Targeting GM-CSF has so far proved to be safe in human disease and clinical trials in AS may be warranted.

So far no master transcription factor regulating GM-CSF production by CD4 cells has been identified, therefore it is currently more helpful to study GM-CSF production in the context of CD4 plasticity rather than as a distinct effector phenotype. However, the RNA sequencing data I have generated suggests GM-CSF producing cells have a transcriptional profile that is distinct from IFN-γ positive cells and IL-17A producing cells suggesting they may be a distinct effector subset of CD4 cells. Gene clustering analysis has provided several candidate key genes that may be necessary to activate this subset of cells but these will need to be validated at a functional level. Additionally, there may be several genes that are important in activating a "pathogenic" programme within Th17 immunity. If the role of these are genes is validated in future studies, they may provide an attractive target for therapeutic

intervention with the goal of targeting “pathogenic” cells while maintaining normal immunity.

Figure 3.28

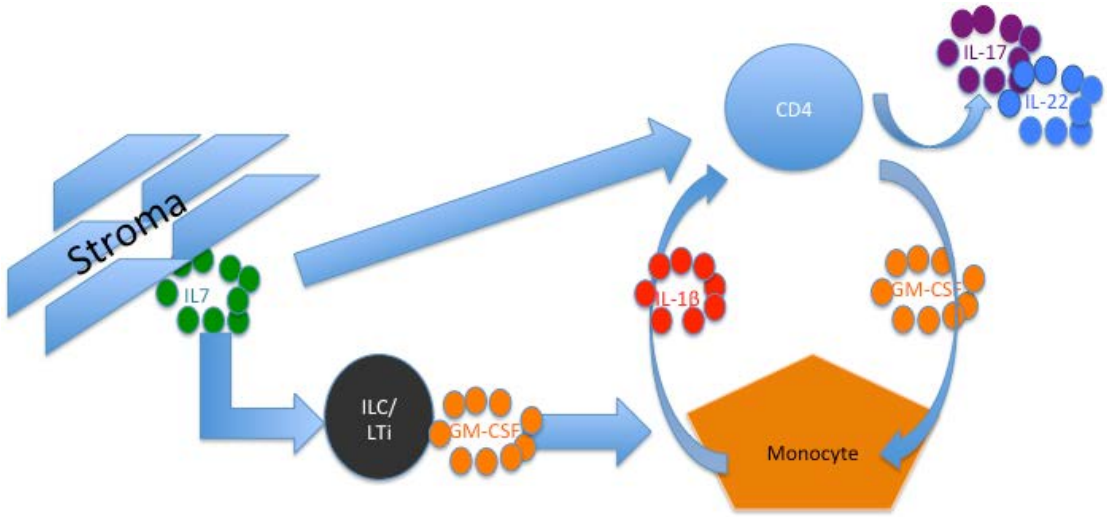


Figure 3.28. Proposed model for the role of GM-CSF in SpA joint disease pathogenesis

Joint-resident ILCs are early responders to tissue stress and can be activated through the action of stromal cytokines such as IL-7. One of their main effector functions is the production of GM-CSF which will influence the differentiation of monocytes and other myeloid populations trafficking into the joint. IL-7 will also promote GM-CSF production from trafficking CD4 cells. The increase of GM-CSF in the inflamed joint will drive pro-inflammatory phenotype of monocytes and set up a pro-inflammatory loop.

Chapter 4: The role of IL7R polymorphisms in inflammatory disease association

4.1 Introduction

Single nucleotide polymorphisms (SNPs) in the IL7Rα chain (CD127) have shown disease association in AS (Cortes et al. 2013) and other inflammatory diseases including MS (Gregory et al. 2007) and primary biliary cirrhosis (Mells et al. 2011). The MS-associated SNP rs6897932 on chromosome 5 is the best-studied. The high-risk common allele (C) encodes the amino acid threonine residue at position 244 in the 6th exon, while the rare protective allele T encodes Isoleucine (R. I. Mazzucchelli, Riva, and Durum 2012). The frequency of the minor protective allele (T) is 0.17 (http://www.ncbi.nlm.nih.gov/SNP). This SNP is located in a block of high linkage disequilibrium (LD), which includes the lead AS-associated SNP rs11742270 (located 6868 base pairs from the lead MS associated SNP). This region is in complete LD with an R^2 of 1.0 and D-Prime of 1.0 according to the 1000 genomes database from the broad institute (available via the SNAP website https://www.broadinstitute.org/mpg/snap).

The functional relevance of these polymorphisms and indeed their cellular specificity remains unclear. Studies looking at expression quantitative trait loci (eQTL) in a cell-specific and context-specific manner have been hugely informative in deciphering the relevance of common disease associated polymorphisms. One such recent study (Fairfax et al. 2014) looked at B cells, monocytes and NK cells and found a statistically significant ($P=5.9 \times 10^{-19}$) genotypic eQTL effect for IL7R RNA expression in CD14 monocytes after 24

hours of LPS stimulation but not at baseline. Meanwhile a paper examining eQTL in CD4 cells by Raj et al., (Raj et al. 2014) did not find an IL7R-associated eQTL. The findings of these two eQTL studies are very intriguing especially as the IL7R is thought to be integral to T cell biology but not commonly known to be expressed on CD14 monocytes (reviewed in Mackall, Fry, and Gress 2011; Bendall et al. 2011). Based on an effect size of 0.2, and power of 0.8, we estimated that we would need to recruit 42 individuals in order to see an effect based on linear regression model.

4.2 Aims

The aim of this chapter was to study cell surface expression of the IL7R using flow cytometry in a cohort of healthy individuals recruited from the Oxford Biobank on multiple cell types within baseline and stimulated PBMCs.

The Key questions were:

- What is the baseline IL7R expression in human PBMC subsets and does this change with stimulation?

- What are the effects of 24 hours of LPS stimulation on IL7R expression of CD14 monocytes and can we reproduce the RNA eQTL at protein level?

- What is the functional relevance of IL7R expression on monocytes?

4.3 Results

4.3.1 IL7R expression at baseline is predominantly on CD4 and CD8 cells but is induced on CD14 monocytes after LPS stimulation

In order to understand baseline IL7R expression in ex-vivo human PBMCs I used a multicolour flow cytometry panel staining for CD3, CD4, CD8, CD14, CD20 and CD56 (figure 4.1). At baseline I noted high levels of IL7R expression on CD4 and CD8 cells only. After 24 hours stimulation with 20ng/ml of LPS there was a marked induction of this receptor on CD14 monocytes. By contrast there was a down-regulation of IL7R observed in CD4, CD8 and CD56 cells. There was no observable effect of LPS on IL7R expression on CD19 B cells compared to unstimulated controls (figure 4.2). For this reason CD19 was not included in subsequent staining. I also tested the isotype control of the IL7R antibody on LPS-treated CD14 monocytes, to ensure specificity of the staining (figure 4.2B). Isotype controls were included in all subsequent stains and used to determine the gating of the IL7R expression.

Figure 4.1

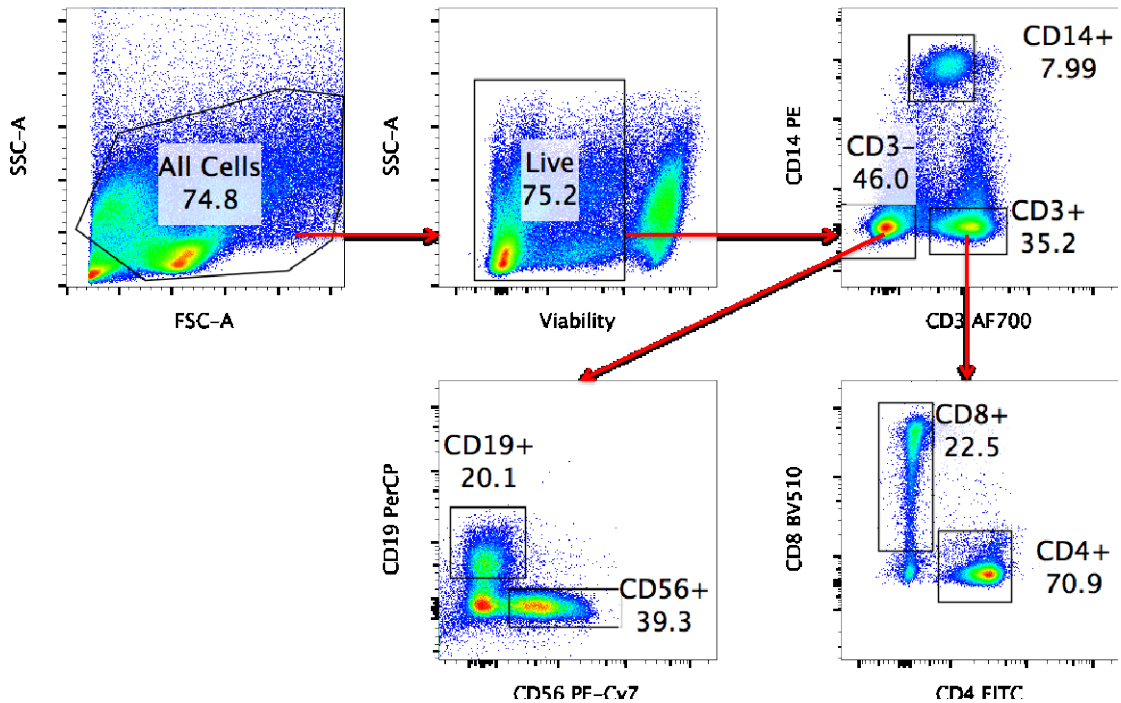


Figure 4.1. Gating strategy for donor PBMC surface staining.
Representative flow cytometery plot from biobank donor 534 showing gating strategy for identifying $CD14^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD19^+$ and $CD3^-CD56^+$ cell populations.

Figure 4.2

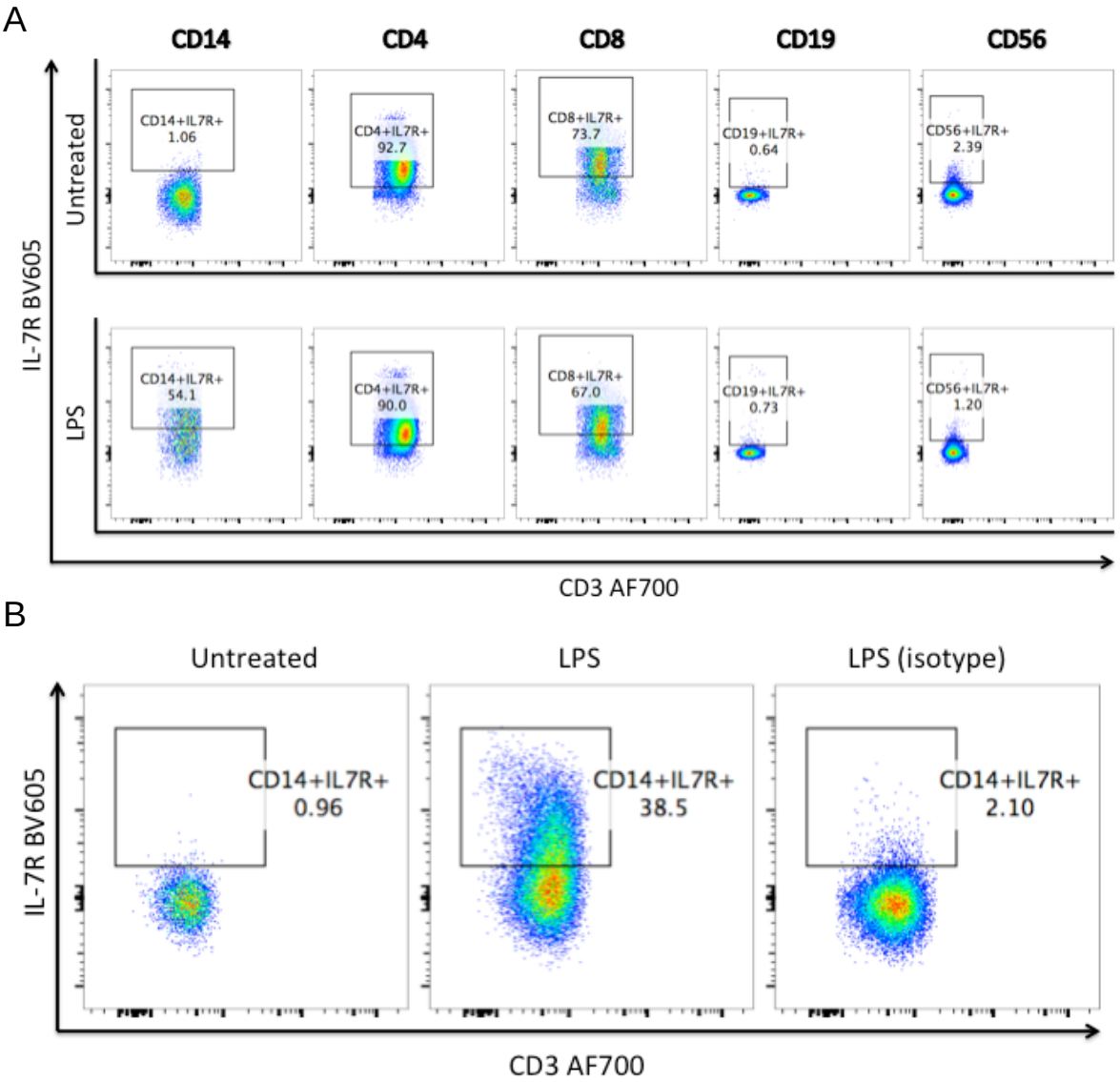


Figure 4.2. IL7R expression on different PBMC cell types at control and after 24-hour LPS treatment.
Cells were incubated in medium alone or in the presence of 20ng of LPS for 24 hours and then stained for surface markers as per figure 4.1 in addition to IL-7R (CD127) expression. **A** Representative flow cytometery plot from biobank donor 534 in panel A shows expression according to cell type with and without LPS in PBMCs. B CD14 monocytes were isolated from the same donor by positive selection and incubated overnight with LPS or medium. Representative plots show untreated, LPS 20ng/ml treated and LPS 20ng/ml treated stained with isotype control antibody for IL-7R.

4.3.2 IL7R expression on monocytes peaks at 6-24 hours post stimulation with LPS and is also induced by other TLR agonists

In order to understand the kinetics of IL7R expression on CD14 monocytes I conducted a time course experiment in two individuals (figure 4.3). IL7R baseline expression on CD14 monocytes was determined in PBMCs as per the gating strategy in figure 4.1. PBMCs were then stimulated with 20ng/ml of LPS and aliquots of cells were taken and stained at 2, 6, 24 and 48 hours after LPS stimulation. I observed a clear IL7R induction on the monocytes within 6 hours with a peak around 12-24 hours. Based on these results I decided to look at the 24 hour time-point for IL7R expression on the monocyte surface. This was the same time point as the one used to generate the gene array data in the study of Fairfax et al. 2014.

I next asked the question of whether the up-regulation of IL7R was specific to LPS monocyte activation via TLR4 (Schwartz 2001) or if other inflammatory stimuli could also have the same effect (figure 4.4). I therefore tested a TLR2 stimulus (Pam3cysk4) or a TLR7 agonist (imiquimod) in a cohort of 7 donors. IL7R expression was observed in response to all three TLR stimuli but only reached statistical significance for LPS and Pam3cysk4.

Figure 4.3

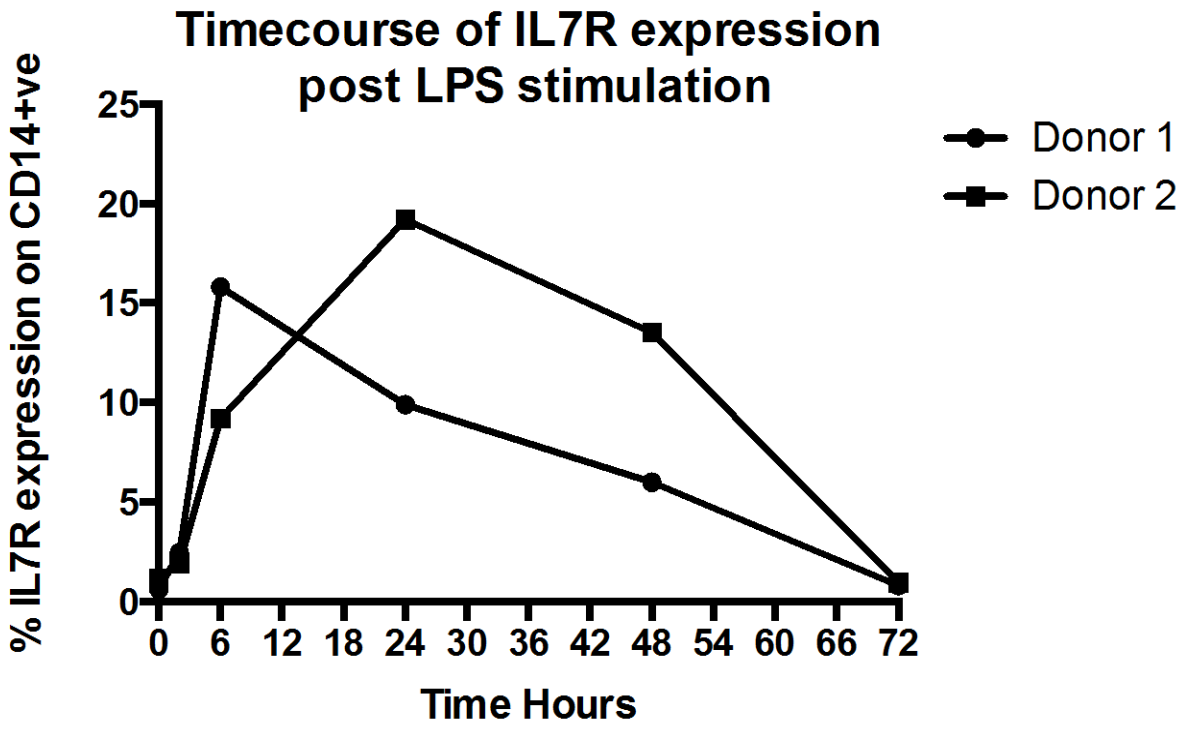


Figure 4.3. Time-course of IL-7R induction on CD14^{+} monocytes.
PBMCs from two donors were isolated by density centrifugation and rested overnight in R10 medium. The following day cells were stimulated with 20ng/ml of LPS and aliquots of cells were taken at 2,6,24,48 and 72 hours for IL7R staining. An unstimulated control was run at every time point. In the two individuals studied the peak IL7R induction is between 6 and 24 hours.

Figure 4.4

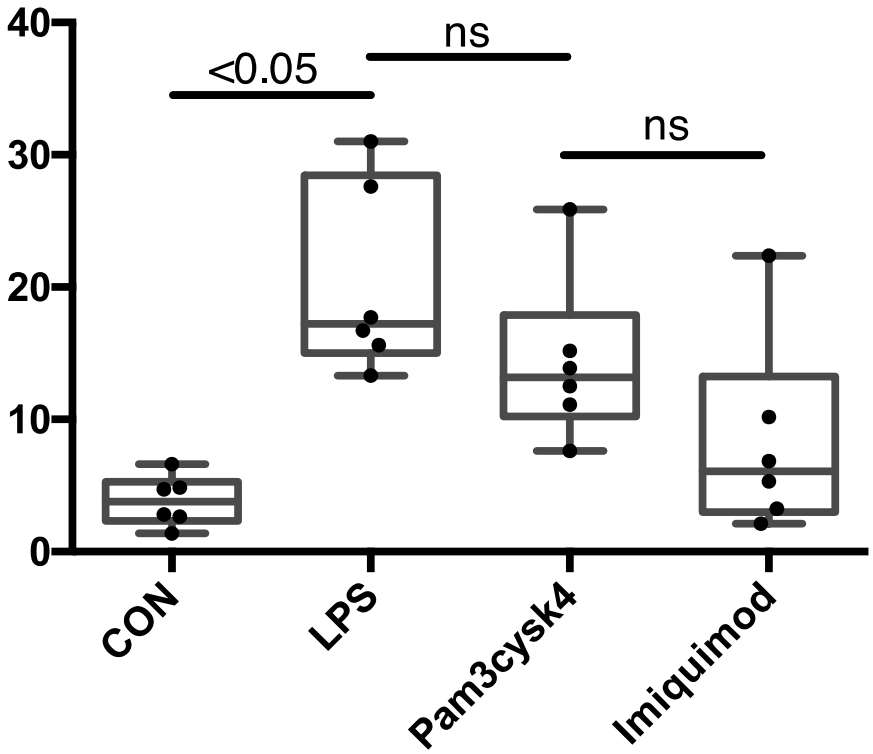


Figure 4.4. TLR2 agonists also induce IL7R expression on monocytes.
PBMCs from 6 biobank donors were isolated by density centrifugation and rested overnight in R20 medium. The following day cells were stimulated with 20ng/ml of LPS, 100ng/ml of Pam3cysk4 (TLR2 agonist) and 100ng/ml of imiquimod (TLR7 agonist) for 24 hours. Cells were stained and the percentage of IL7R+ cells in the CD14 gate was determined by flow cytometry. There was a statistically significant induction of IL7R after LPS and Pam3cysk4 stimulation. Statistical significance was determined using a Friedman's non parametric test.

4.3.3 IL7R surface expression shows response to LPS stimulus in myeloid and lymphoid subsets

IL7R expression was tested in a cohort of 103 PBMC biobank healthy donors after 24 hours of LPS stimulation versus medium alone incubator controls (figure 4.5). These experiments were carried out in 11 different batches. A very strong up regulation of IL7R on monocytes for all individuals was observed. In contrast IL7R expression on CD4 cells, CD8 cells and CD56 cells undergoes a relatively mild but clear down-regulation in response to 24 hours of LPS stimulation compared to incubator controls.

In parallel to the PBMCs LPS stimulation experiments, CD14 positive monocytes were also isolated from 85 individuals using positive selection magnetic bead separation (figure 4.6). A very robust induction of IL7R on isolated monocytes was again observed (figure 4.6A). There was a clear positive correlation between induction of IL7R in whole PBMCs and isolated monocytes in the same individual with an R^2 value of 0.43 (figure 4.6B).

Figure 4.5

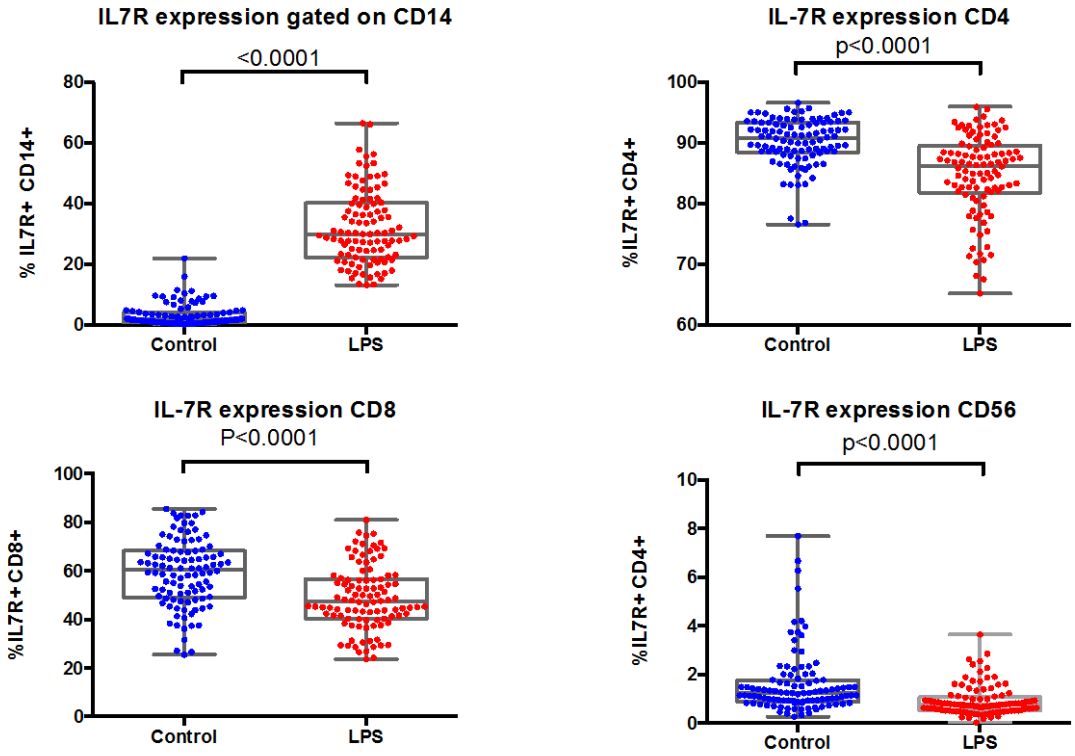


Figure 4.5. Differing effects of LPS stimulation on IL7R expression across PBMC subsets.
PBMCs from 103 biobank donors were stimulated with 20ng/ml of LPS for 24 hours and the percentage of IL7R expression was determined in CD14, CD4, CD8 and CD56 cells compared to untreated paired controls. Statistical significance was determined using a Wilcoxon non-parametric paired test.

Figure 4.6

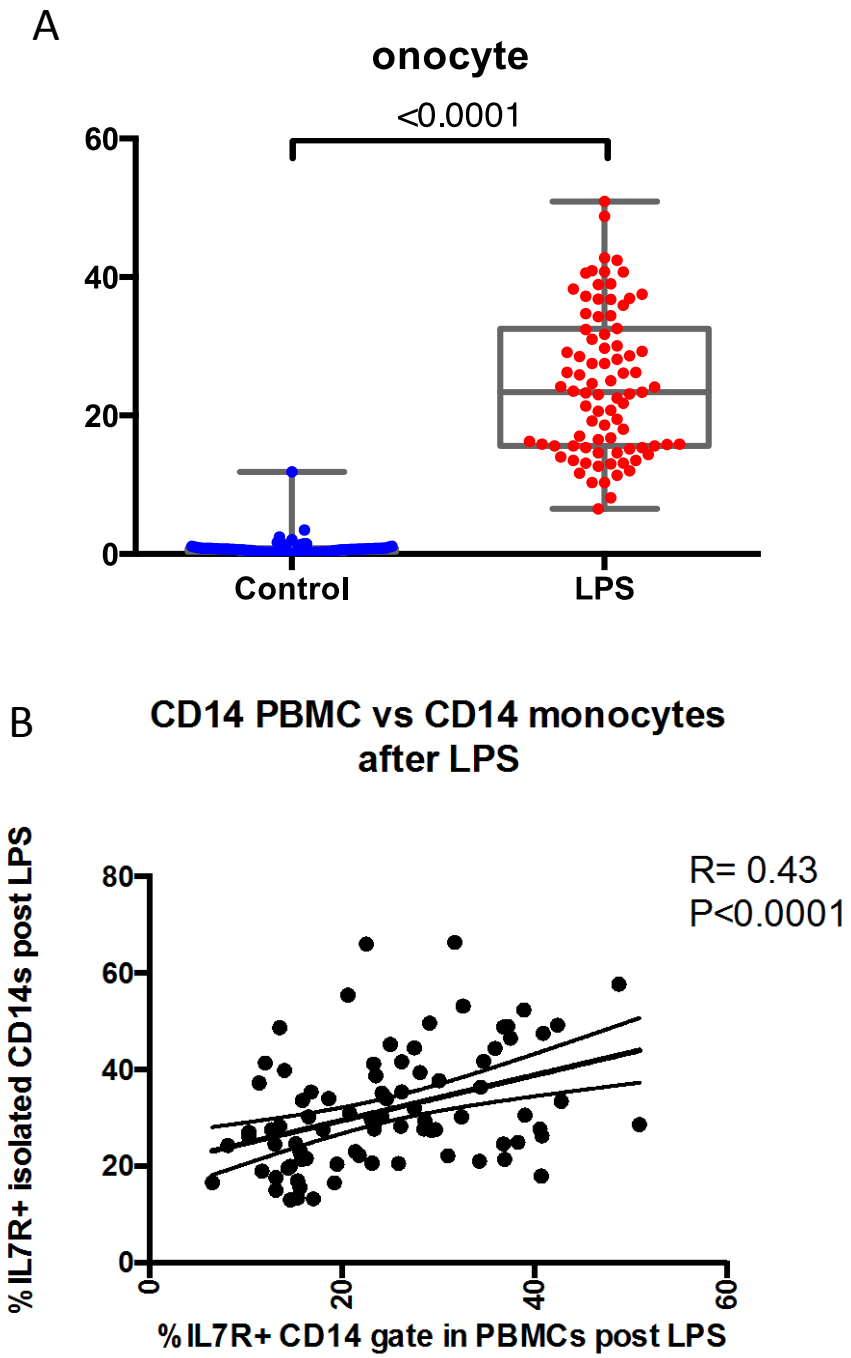


Figure 4.6. IL7R is upregulated on isolated monocytes after LPS stimulation and correlates with monocyte PBMC response in the same individuals.
A- Within the cohort of 103 individuals 85 individuals also had monocytes isolated by CD14 magnetic bead positive selection. Isolated monocytes were incubated for 24 hours with LPS versus incubator controls. Induction of IL7R was observed clearly with a P value of less than 0.0001 using a paired T test. B- In individuals who had both a PBMC and isolated CD14 LPS stimulated sample the correlation was measured between the two samples following LPS stimulation. A statistically significant positive correlation was observed using a Spearman r test with a R value of 0.43. Line of best fit and 95% confidence intervals were calculated using linear regression modelling.

4.3.4 Expression of LPS induced IL7R on monocytes is influenced by genotype

The cohort of 103 individuals recruited for the LPS stimulation experiments was carried out over 11 batches under identical experimental conditions and was blinded to genotype. After completion of the flow cytometry analysis the genotype of the biobank individuals was unblinded in order to assess the effects of genotype at the rs689732 SNP on IL7R expression. 52 individuals had the common CC genotype, 42 were heterozygous, and 9 had the rare TT variant. Figure 4.7 panels A and C show that there was no significant effect of genotype on IL7R baseline expression on monocytes within total PBMCs or isolated using positive selection.

After 24 hours of stimulation with LPS the genotype of the individuals has a significant effect on the level of IL7R expression on the surface. Figure 4.7B shows that homozygous carriers of the rare (AS-protective) allele TT have significantly higher expression of IL7R on the surface of CD14 monocytes compared to CC major allele homozygous carriers exposed to the same stimulation. This effect is also seen for LPS-stimulated isolated CD14 monocytes.

Within the other PBMC cell populations, no effect of genotype was observed on IL7R expression on unstimulated CD4, CD8 and CD56 cells (figure 4.8). Following LPS stimulation there was no effect of genotype on CD4 and CD56 cells but a significant effect was observed in CD8 lymphocytes, where the minor TT allele carriers showed lower expression of IL7R on CD8 cells

compared to the major CC allele carriers. This effect was in the opposite direction to the one observed in CD14 monocytes.

Figure 4.7

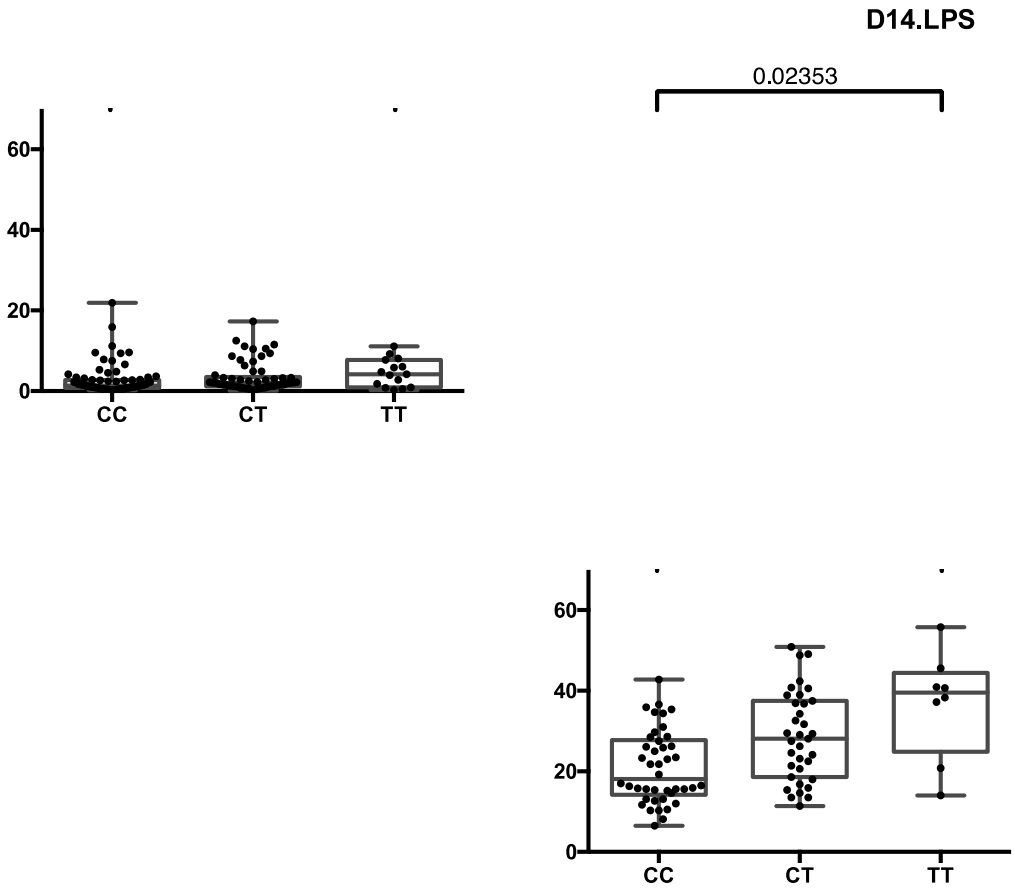
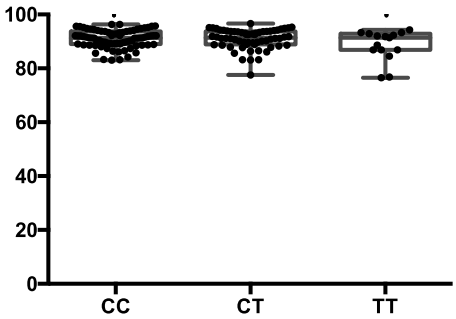


Figure 4.7. rs6897932 SNP genotype influences IL7R expression on monocytes following LPS stimulation.
Whole PBMCs (n=103, A & B) or positively selected, magnetically isolated CD14 cells (n=85, C & D) were incubated with or without 20ng/ml of LPS for 24 hours. There was no observed effect of genotype in untreated (UT) CD14 monocytes either within a PBMC culture or cultured independently of other PBMC populations (A & C respectively). After 24 hours of LPS stimulation a statistically significant genotypic effect is observed where homozygous carriers of the rare TT allele express higher levels of IL7R on the surface of the CD14 monocytes as measured by flow cytometry. This effect is significant both in the isolated monocytes (D) and monocytes cultured within PBMC populations (B). Statistical significance was calculated using a Kruskal-Wallis multiple comparisons test.

Figure 4.8



2

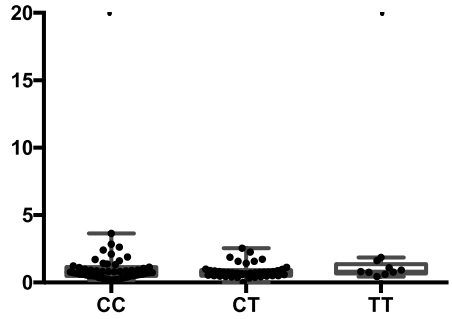


Figure 4.8. Genotypic effects of the rs6897932 SNP genotype influences IL7R expression on CD8 T cells after LPS stimulation.
IL7R surface expression on CD4, CD8 and CD56 subsets was measured by flow cytometry in whole PBMCs (n=103) incubated with or without 20ng/ml of LPS for 24 hours. There was no observed effect of genotype in untreated (UT) CD4, CD8 or CD56 cells. After 24 hours of LPS stimulation a statistically significant genotypic effect is observed in CD8 cells but not CD4 or CD56 subsets. In CD8 cells the homozygous carriers of the rare TT allele show lower levels of IL7R expression on the surface. Statistical significance was calculated using a Kruskal-Wallis multiple comparisons test.

4.3.5 Recombinant IL-7 in cultures down-regulates IL7R surface expression across all measured PBMC subsets

One approach to test whether the IL7R expression on the PBMC subsets is likely to be functional is to observe a physiological down-regulation of this receptor in response to recombinant human IL-7. This down-regulation has donors within the cohort of 103 donors, PBMCs were cultured with recombinant human IL-7 at a concentration of 10ng/ml in addition to LPS 20ng/ml for 24 hours. Figure 4.9 shows a significant down-regulation of surface IL7R in response to recombinant IL-7 in CD14, CD4, CD8 and CD56 cells compared to LPS alone.

Analysis of the results based on genotype (figure 4.10) shows the effects of polymorphisms in the RS689732 on the expression of the IL7R is maintained after down-regulation of the IL7R in CD14 monocytes. In CD4 and CD8 cells the down-regulation of the IL7R after culture with recombinant human IL-7 occurs across genotypes. In particular the effects of genotype on IL7R expression in CD8 cells observed after LPS stimulation is no longer seen after the addition of recombinant human IL-7.

Figure 4.9

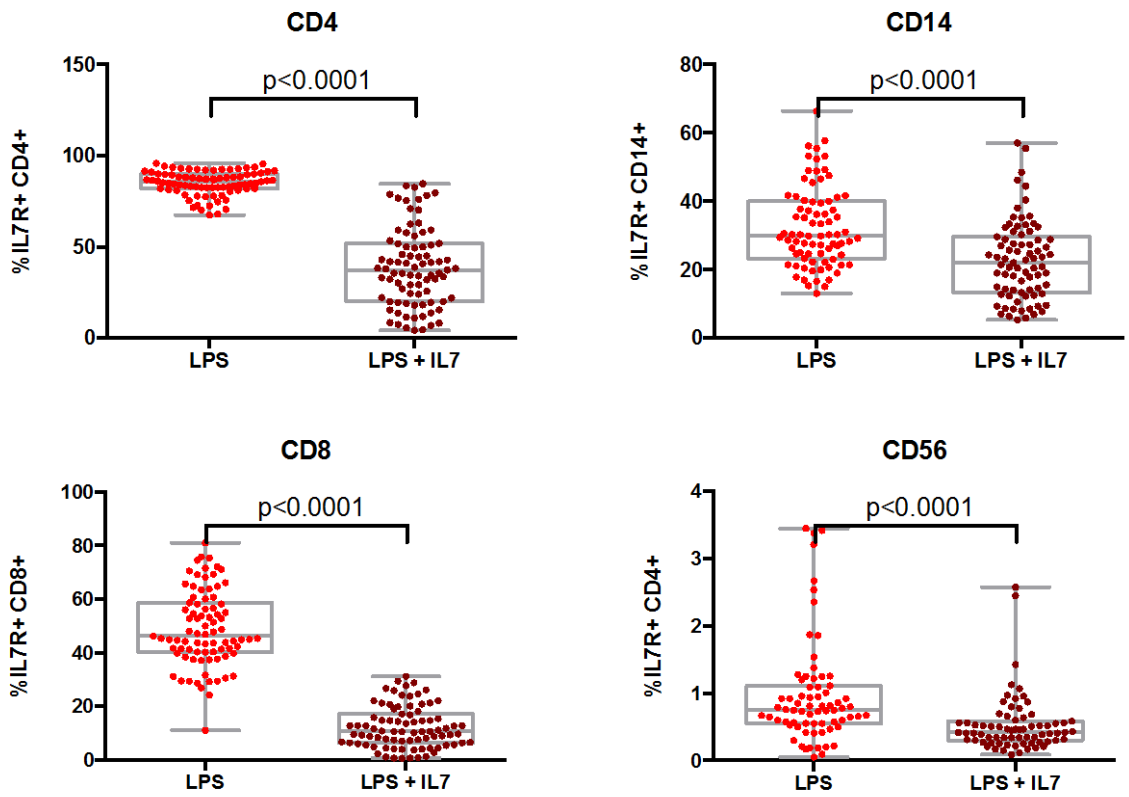
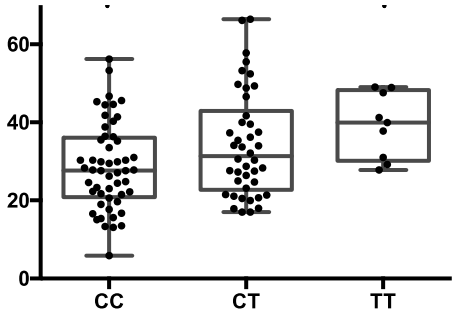


Figure 4.9. Recombinant IL-7 leads to downregulation of IL7R in CD14, CD4, CD8 and CD56 cells within LPS-stimulated PBMCs.
Recombinant human IL-7 was added to LPS-stimulated PBMC cultures in a subset of 83 donors within the cohort. The addition of recombinant IL-7 leads to a significant downregulation of the IL7R across all LPS-stimulated cells. Statistical significance was calculated using a paired T test.

Figure 4.10



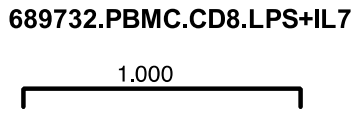


Figure 4.10. The genotypic influence on LPS-induced IL7R expression on monocytes is maintained after the addition of recombinant human IL-7.
Recombinant human IL-7 was added to LPS-stimulated PBMC cultures in a subset of 83 donors within the cohort and the results analysed according to RS689732 polymorphisms. There is overall down-regulation of the IL7R after the addition of IL-7 but the genotypic effect of LPS induced induction of IL7R was maintained despite the addition of recombinant IL-7. Higher surface levels of IL7R on monocytes are seen with the TT genotype compared to the CC genotype. The suppression of IL7R in CD4 and CD8 cells is seen across all genotypes after the addition of recombinant IL-7. Statistical significance was calculated using a Kruskal-Wallis multiple comparisons test.

4.3.6 TCR stimulation leads to down-regulation of IL7R expression on CD4 and CD8 cells but is not influenced by genotype

As no baseline effect of the rs689732 polymorphisms on the level of IL7R expression on CD4 and CD8 lymphocytes was observed, I decided to investigate whether there would be an effect after T-cell receptor (TCR) stimulation. Therefore PBMCs were stimulated for 24 hours with beads coated with anti-CD2/3/28 antibodies in a new cohort of individuals (23 CC, 16 CT, 5 TT) recruited from the biobank (figure 4.11). As previously noted, there was no genotypic effect on the level of IL7R expression in unstimulated CD4 and CD8 lymphocytes. After 24 hours of TCR stimulation a down-regulation of surface IL7R was observed in the lymphocytes, but once again this effect was not influenced by genotype.

Figure 4.11

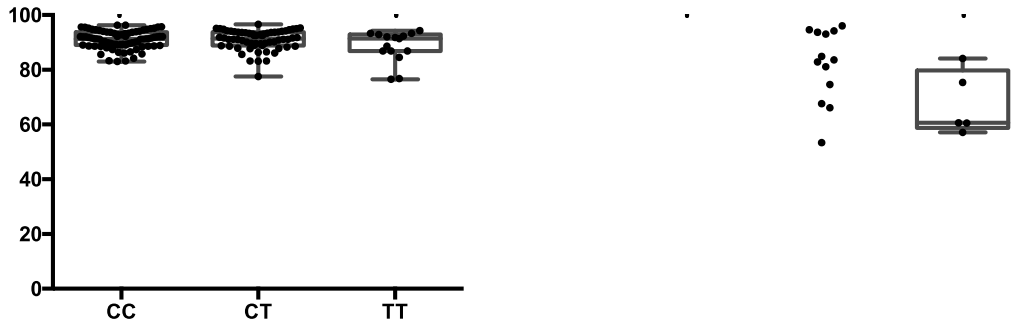


Figure 4.11. No observed genotypic effect after TCR stimulation on IL7R expression on CD4 and CD8 lymphocytes.
A new cohort of 44 individuals was recruited from the biobank in 4 batches. PBMCs were stimulated for 24 hours by beads coated anti CD2/3/28 antibodies at a ratio of 1 bead to 2 cells. At 24 hours IL7R surface expression was determined by flow cytometry. Results were unblinded to genotype at the end of the study. There was a down-regulation of IL7R on CD4 and CD8 lymphocytes after TCR stimulation. This down-regulation occurs across all gentoypes and no statistically significant difference was observed between differing genotypes at RS689732 polymorphism. Statistical significance was calculated using a Kruskal-Wallis multiple comparisons test.

4.3.7 The effects of LPS on monocyte IL7R expression are partially reversed by the neutralisation of TNF-α

In order to understand the regulation of LPS-induced IL7R expression on monocytes I reviewed the eQTL data set from the study by Fairfax et al (Fairfax et al. 2014). At mRNA level, expression of TNF-α at 2 hours was the best predictor of IL7R expression at 24 hours. The hypothesis therefore is that LPS-induced expression of IL7R on monocytes is driven by autocrine production of TNF-α. In a subset of 69 individuals from the original cohort LPS-stimulated PBMCs were also incubated with 5μg of the chimeric anti-TNF monoclonal antibody Infliximab.

The addition of Infliximab led to partial reversal of LPS-induced IL7R expression on monocytes (figure 4.12A). Moreover anti-TNF antibody addition also led to a slight but significant down-regulation of IL7R on CD4 and CD8 lymphocytes. The partial reversal of the LPS-induced IL7R expression in this subset of 69 donors led to a loss of the statistically significant genotypic effect observed with LPS stimulation alone (figure 2.12B).

Figure 4.12

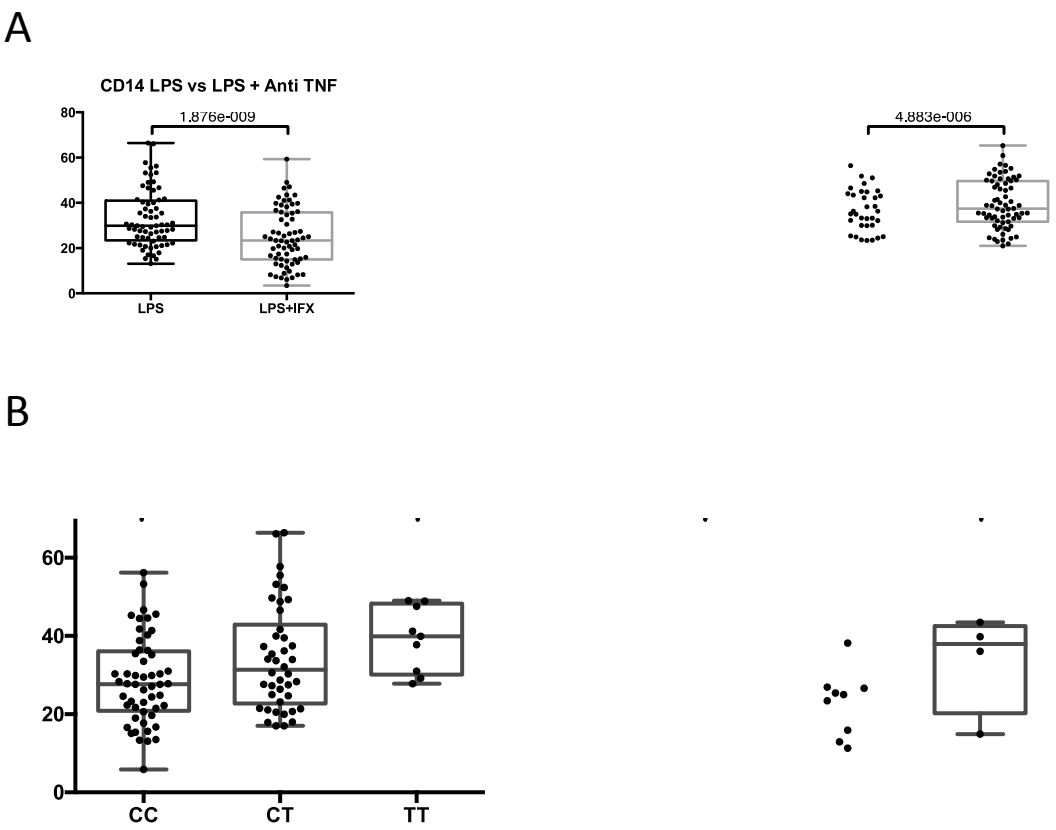
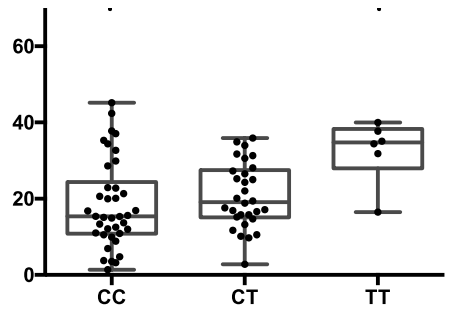


Figure 4.12. Anti-TNF antibody addition reverses LPS-induced IL7R expression induction on CD14 monocytes.
In a subset of 69 donors within the original cohort of 103 the neutralisation of TNF-α using 5μg/ml of Infliximab added to PBMC cultures showed a partial reversal of LPS-induced IL7R induction on monocytes. The addition of Infliximab also resulted in a minor but significant down-regulation of IL7R on CD4 and CD8 cells (Panel A, paired T-test). Analysis of the results based on genotype at the RS689732 polymorphisms showed a trend towards higher CD14 IL7R expression with the TT genotype but this was no longer statistically significant in this smaller cohort. Statistical significance was calculated using a Kruskal-Wallis multiple comparisons test.

4.3.8 Recombinant TNF promotes genotype-specific IL7R expression on monocytes independently of LPS

Since neutralisation of TNF-α can partially reverse IL7R expression on CD14 monocytes, I sought to investigate whether recombinant human TNF-α led to the up-regulation of IL7R on CD14 monocytes. In a new independent cohort of 78 individuals (recruited over 7 batches) PBMCs were cultured for 24 hours with 10ng/ml of recombinant TNF-α. Figure 4.13 shows that TNF addition led to up-regulation of IL7R on CD14 monocytes in a genotype-specific way. The effect of genotype on TNF-α-induced IL7R expression on monocytes at the rs689732 SNP polymorphism was in the same direction as the effect observed by LPS (figure 4.7). In both sets of experiments carriers of the TT allele showed significantly higher levels of IL7R on the surface of CD14 monocytes compared to CC allele. Once again there was no significant effect observed in the CD4, CD8 or CD56 cell subsets.

Figure 4.13



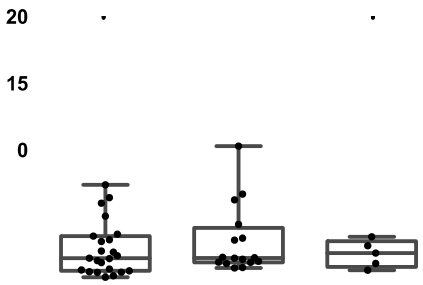


Figure 4.13. Recombinant TNF-α induces IL7R expression on CD14 monocytes in a genotype specific way.
PBMCs from a cohort of 78 donors recruited over 7 batches were stimulated with recombinant human TNF-α at a dose of 10ng/ml over 24 hours. Recombinant human TNF-α lead to the induction of IL7R on CD14 monocytes with carriers of the TT allele showing significantly greater TNF-α-induced IL7R induction compared with carriers of the CC allele. There were no statistically significant genotypic differences observed in the other studied PBMC cell subsets. Statistical significance was calculated using a Kruskal-Wallis multiple comparisons test.

4.3.9 Monocytes expressing IL7R are found in the inflamed joints of patients with SpA

Since polymorphisms of the IL7R are associated with many autoimmune inflammatory diseases and in particular ankylosing spondylitis, I sought to investigate whether this phenomenon of IL7R expression on CD14 monocytes might be relevant at the site of tissue inflammation in AS. Ex-vivo IL7R staining was carried out on matched PBMC and synovial fluid samples from 2 patients with AS recruited through the immune function in infllmmatory arthritis (IFIA) study. Figure 4.14 shows low levels of IL7R expression on monocytes from the peripheral blood of patients with AS. However, in both patients the matched synovial fluid samples showed marked ex-vivo IL7R expression on monocytes compared to the isotype control.

Figure 4.14

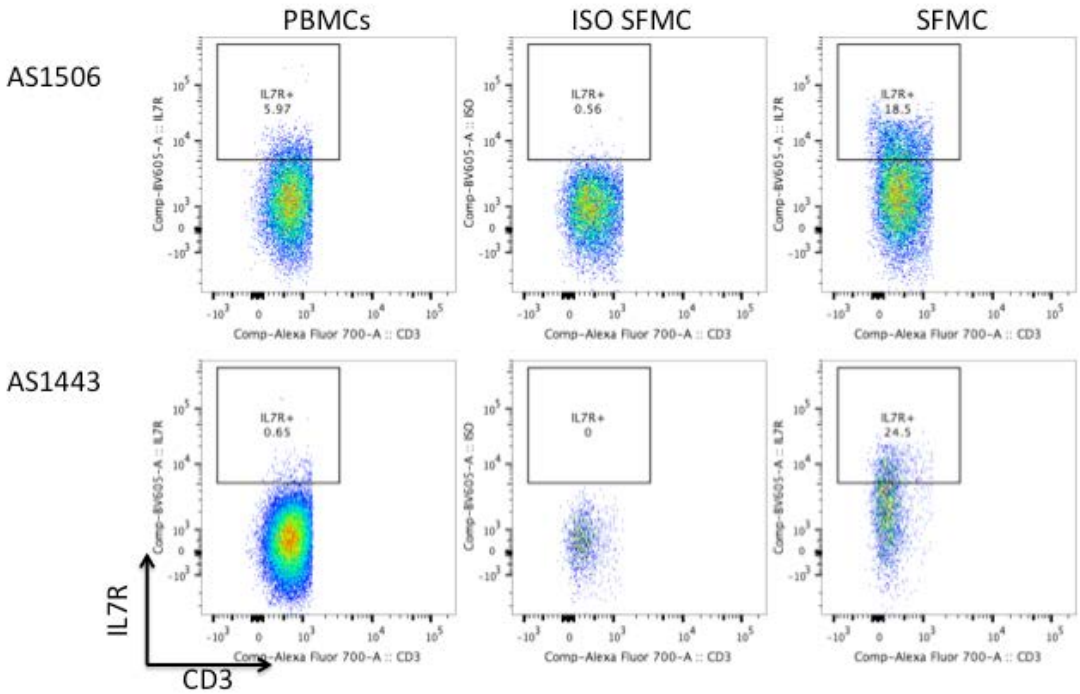


Figure 4.14. Ex-vivo AS CD14 synovial monocytes express IL7R.
Matched cryopreserved PBMC and synovial fluid mononuclear cell (SFMC) samples were stained for surface IL7R. IL7R staining was observed in CD14s cells from the synovial fluid. Middle panel shows isotype control staining of SFMC samples.

4.4 Discussion

My results show for the first time a genotypic effect of polymorphisms at the rs689732 SNP on monocyte IL7R expression after activation with LPS and TNF. Although IL7R signalling is not commonly associated with myeloid cell populations, It has been previously reported by several independent groups that myeloid populations do express the IL7R. Martinez et al profiled human monocytes after differing activation stimuli and showed IL7R to be one of the upregulated genes in pro-inflmmatory M1 signature (Martinez et al. 2006). Alderson and colleagues showed peripheral blood derived monocytes are able to respond to high doses of recombinant IL-7 (100ng/ml) in an in-vitro culture system (Alderson et al. 1991). In the context of inflammatory arthritis, it has been shown that macrophages derived from RA synovial fluid have high levels of IL7R (Pickens et al. 2011).

IL7R polymorphisms have been associated with multiple inflammatory diseases but the mechanisms through which these associations play out and indeed the relevant cell types have remained poorly understood. The assumption has always been that polymorphisms in the IL7R would have their primary effects through CD4 and CD8 lymphocytes since this cytokine is crucial for the development and expansion of these cells (Mackall, Fry, and Gress 2011). Indeed non-functioning mutations of the IL7R lead to severe combined immunodeficiency, highlighting the important role of IL-7 in T cell development and survival (Roifman et al. 2000).

When stimulating whole PBMCs with LPS my results show IL7R expression to be differentially regulated in the various leucocyte sub-populations. Whilst there is a robust induction on CD14 monocytes, there is a clear down-regulation from high baseline levels in CD4 and CD8 lymphocytes. The down-regulation of IL7R in lymphocytes after activation has been previously described previously, not just after IL-7 stimulation, but with other pro-inflammatory cytokines such as IL-6 (J.-H. Park et al. 2004). This cytokine is known to be produced by monocytes in response to TLR activation (Hartmann and Krieg 1999).

My results show that the LPS-induced induction of IL7R occurs both within a whole PBMC stimulation system and in isolated monocytes. This indicates that induction is predominantly intrinsic to monocytes and that other PBMC cell interactions are not required. Moreover the induction of IL7R in LPS-treated isolated CD14 monocytes and LPS-treated monocytes within PBMCs shows a strong positive correlation, suggesting that even within PBMCs, the induction of IL7R after LPS may be driven through direct action on the monocytes. In the array eQTL data-set by Fairfax et al., the stimulation of monocytes with LPS was carried out using isolated CD14 populations and the gene expression signature observed was thus due to a direct effect (Fairfax et al. 2014).

There was a clear and statistically significant effect of rs689732 genotype on LPS-induced IL7R induction both in the PBMC setting and in isolated monocytes. Homozygous carriers of the rare and protective TT allele showed

significantly increased LPS-induced IL7R expression, but this effect was not seen at baseline. The direction of this protein expression eQTL is the same as the gene array mRNA eQTL (Fairfax et al. 2014). Therefore I have confirmed the mRNA results at the cell surface expression level in an entirely new cohort of individuals and showed down-regulation of this receptor in response to IL-7 suggesting a functional relevance. Moreover, as with the gene array cohort, no significant genotypic effects were observed in untreated CD14 monocytes either in isolation or in the context of whole PBMC stimulation.

This paradoxical observation of a protective allele leading to increased expression of the potentially pro-inflammatory IL7R on the surface of monocytes is puzzling. The high AS-risk common major allele encodes a threonine at position 244 in the 6th exon of the extracellular domain (bordering the transmembrane region). This allele has been shown to be associated with a two-fold increase in the skipping of exon 6, resulting in an alternatively spliced variant and leading to a soluble form of the protein (Gregory et al. 2007). This may be an explanation for the observed lower IL7R surface expression seen with the CC risk allele since the flow cytometry assay does not take account of soluble IL7R. However if this was the case, then one might expect to see lower IL7R surface expression across the other PBMC populations. In reality, I observe a trend in the opposite direction in untreated CD8 cells, and statistically significant opposite effect with LPS-stimulated CD8 cells. It is not clear why an alternatively spliced, common TT variant supposedly resulting in more soluble IL7R, causes expression of more membrane-bound IL7R on CD8 but less membrane-bound IL7R on

monocytes. One possible explanation is cell-specific expression of transcription factors or splicing.

One alternative and as yet unproven hypothesis suggests that the disease-associated threonine residue causes gain of function at the IL7R and leads to increased signalling. Genetic sequencing data from childhood T-cell acute lymphoblastic leukemia has shown a somatic gain-of-function mutation at exon 6. Here an unpaired cysteine residue is introduced in the extracellular juxtamembrane/transmembrane region, leading to constitutive activation of the receptor (Zenatti et al. 2011). Since threonine is a polar amino acid whereas isoleucine is hydrophobic, one can postulate that the disease-associated threonine may alter the signalling strength. If this were indeed the case, it would still not explain the increased transcription of the IL7R specifically in monocytes after LPS stimulation. To formally test this hypothesis, one would have to clone the different receptor genotypes into a reporter system and directly measure increase in signalling.

An attractive hypothesis would be that IL7R expression on CD14 cells serves an immune-regulatory function, hence the observed higher surface expression with the protective allele. In a study by Guimond et al., it was shown that IL7R signalling in mouse dendritic cells (DCs) resulted in the down-regulation of MHC-II resulting in the diminished proliferation of CD4 cells in response to IL-7 (Guimond et al. 2009). This study provides a clear immunomodulatory feedback role for myeloid cells such as CD14 monocytes and DCs in response to IL-7 and would fit in with the observation of increased IL7R

expression on activated monocytes from homozygous carriers of the protective allele.

In order to test whether the IL7R on monocytes is indeed functional, I added recombinant human IL-7 to PBMC cultures (figure 4.10). Under normal circumstances IL-7 binding to IL7R on lymphocytes leads to down-regulation of that receptor (J.-H. Park et al. 2004). This is mediated through suppression of IL7R transcription and interestingly the mechanism of suppression differs between CD4 and CD8 cells (J.-H. Park et al. 2004). In lymphocytes this negative feedback loop is thought to be important for preventing unregulated expansion of lymphocytes. The addition of recombinant human IL-7 to the PBMC cultures resulted in a significant down-regulation of the IL7R on CD4 and CD8 cells as expected. In addition I also observed a down-regulation on the CD14 monocytes suggesting ligand engagement of IL7R on monocytes leads to an intracellular signalling process with an associated negative feedback loop. Interestingly this IL-7 mediated down-regulation of IL7R is not as marked as the down-regulation seen in lymphocytes. Moreover the genotypic differential expression of the IL7R on monocytes is maintained after IL-7 signalling.

In this study the eQTL seen for monocytes only occurs after appropriate stimulation by a TLR agonist, in this case LPS. While LPS is known to be a potent stimulus for CD14 cells, it is not the main stimulatory pathway for lymphocytes, even though human T cells have been reported to express TLRs (Funderburg et al. 2008). Therefore LPS stimulation may not be the

correct physiological way to reveal an activation-induced eQTL in lymphocytes. For this reason a second cohort of patients was recruited and PBMCs were stimulated for 24 hours with beads coated with CD2/3/28, a method previously used in several publications (E. V. Acosta-Rodriguez 2007). A significant down-regulation of IL7R expression was observed in both CD4 and CD8, cells but there was no statistically significant effect of genotype at the rs689732 SNP. However in both CD4 and CD8 cells there is a trend for lower IL7R expression following TCR stimulation in the TT protective allele. Unfortunately this second cohort may have been underpowered to detect a change and this will need to be followed up in future studies.

The signals driving IL7R expression on CD14 monocytes are not clear. One approach to deciphering this process was to go back to the gene array eQTL data-set by Fairfax et al (Fairfax et al. 2014). Pathway analysis suggested that expression of TNF-α at 2 hours was the strongest predictor of IL7R expression at 24 hours in isolated monocytes. This would suggest TNF-α production may be driving IL7R expression in an autocrine way. To test this hypothesis the anti-TNF-α antibody Infliximab was added to PBMC cultures. This antibody is used clinically to treat a host of inflammatory disorders (Feldmann 2002). Addition of this antibody to the PBMC cultures resulted in a partial reversal of the LPS-induced IL7R up-regulation. This suggests that although TNF has an influence, it is not the only pathway driving IL7R up-regulation in response to LPS.

The second approach taken was to test whether the addition of recombinant human TNF could induce IL7R expression on monocytes independently of LPS. For this purpose a second cohort of patients was recruited and PBMCs were stimulated with recombinant human TNF-α. In this cohort of 78 donors a clear genotypic effect of TNF-α-induced IL7R expression was seen. The effect was in the same direction as the first LPS-induced cohort, with the highest IL7R expression seen with homozygous TT rare protective genotype. This suggests that genotypically controlled IL7R expression on CD14 monocytes can be driven by TNF-α alone and that the loss of the genotypic effect with anti-TNF would imply that TNF is one of the key drivers of this expression.

Here I have demonstrated that the genotype-specific IL7R expression in CD14 monocytes in an in-vitro stimulation model. If myeloid IL7R expression is indeed relevant in SpA disease pathogenesis then CD14 cells expressing IL7R should be present in the inflammed joint. My ex-vivo staining of paired PBMCs and SFMC samples demonstrates clearly that CD14 cells in the joint do indeed express levels of IL7R comparable to in-vitro LPS and TNF-α stimulated CD14 cells. This confirms the observations by other groups who have, in rheumatoid arthritis, shown IL7R expression on myeloid cells in the infammed joints (Pickens et al. 2011; Hartgring et al. 2009). Immune cells trafficking into the joint will be exposed to a range of cytokines, in particular TNF-α (Punzi, Calò, and Plebani 2002). Therefore it is likely that CD14 cells are induced to express IL7R in response to factors such as TNF-α after arriving into the joint where there is also abundant IL-7 production (Hartgring et al. 2009; Pickens et al. 2011; van Roon et al. 2005).

In the context of SpA I now propose an updated model in figure 4.15. Tissue injury will activate ILCs through IL-7 production by stroma and this will lead to a STAT-5-mediated pro-inflmmatory loop described in my conclusions of chapter 3. However the monocytes trafficking into the inflammed joint will also be directly influenced by IL-7, after this receptor is induced on the surface in response to TNF-α. I hypothesise that IL-7 has an anti-inflammatory effect on monocytes. Therefore homozygous carriers of the TT protective allele will be able to resolve this inflammatory cycle more effectively while carriers of the CC disease-associated allele will be more likely to have a failure of resolution resulting in chronic inflammation.

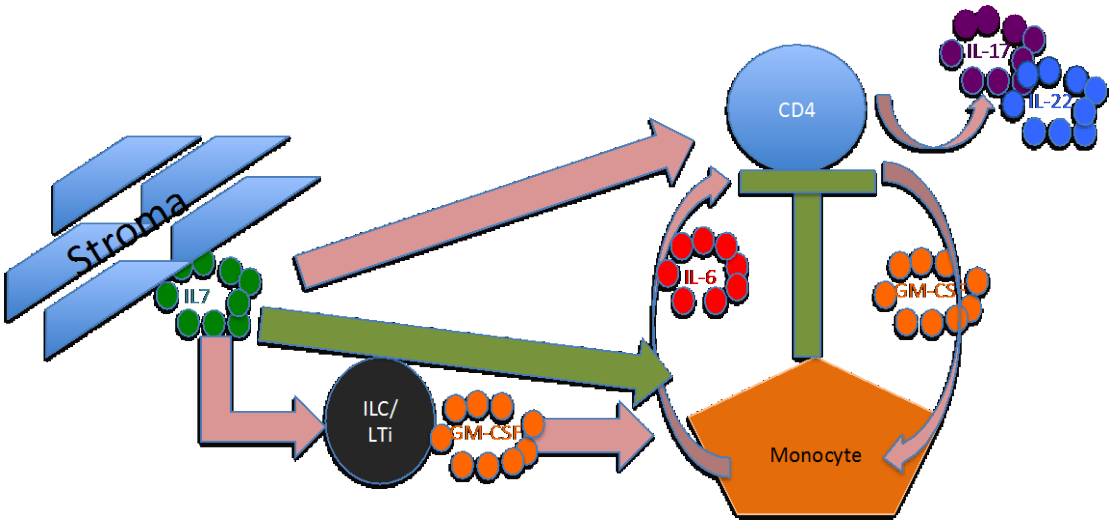


Figure 4.15. Proposed model for the role of IL-7 in SpA joint disease pathogenesis. IL-7 can act directly on ILCs populations and CD4 cells to induce GM-CSF which drives a proinflammatory monocyte/T cell loop. In addition IL-7 will also act on activated monocytes and may have an anti-inflammatory effect to limit excess immune activation. Carriers of the protective IL7R allele have higher monocyte IL7R expression which will allow for swift resolution, while carriers of the disease-associated allele have lower monocyte IL7R expression and therefore inflammation persists for longer.

Chapter 5: Using small molecule RORγt inhibitors to suppress in-vitro type 17 responses in spondyloarthritis

5.1 Introduction

IL-17A is the hallmark of type-17 immunity and is thought to play a pathogenic role in many immune-mediate inflammatory diseases including spondyloarhtritis (SpA), psoriasis and inflammatory bowel disease (IBD). Genome Wide Association Studies have linked multiple type-17 associated genes with SpA, psoriasis and IBD, including *IL23R* and *STAT3* (Danoy et al. 2010b, 3; Cortes et al. 2013; Bowes et al. 2015). Increased frequencies of T-helper type 17 (Th17) cells and enhanced levels of IL-17A in serum and synovial fluid have been found in SpA by several groups and were confirmed by my own findings in chapter 3 (Shen, Goodall, and Gaston 2009; Sarkar, Cooney, and Fox 2010; Baeten et al. 2013).

Monoclonal antibody therapy against IL-17A has been shown to be effective in treating patients with psoriatic arthritis (Mease et al. 2014) and SpA (Baeten et al. 2013). However clinical trials of agents targeting this pathway have failed to show significant benefit in RA patients (Genovese et al. 2013). In addition, treatment of IBD with anti-IL-17A not only proved to be ineffective, but also harmful for some patients (Hueber et al. 2012). These paradoxical effects of IL-17A neutralization can in some part be explained by the physiological role of IL-17A. IL-17A is highly conserved in evolution (Rast et al. 2006) and its principle function is to protect the host against extra-cellular bacterial and fungal infections (Miossec, Korn, and Kuchroo 2009). In the gut

it is thought to be important for maintaining epithelial barriers against the gut microbiome (Cua and Tato 2010).

Differentiation and function of type 17 cells is controlled by the key transcription factor retinoid-related orphan receptor-γt (RORγt) (Ivanov et al. 2006). RORγt orchestrates the differentiation of naïve T helper cells towards Th17 effector cells and is essential for the transcription of IL-17A (Ivanov et al. 2006). In addition to Th17s, CD161-expressing CD8 T cells (Walker et al. 2012), γδ T-cells (Kenna et al. 2011), mast cells and gut resident innate lymphoid cells (Cua and Tato 2010) have also been shown to make IL-17A and also express RORγt. RORγt therefore represents an excellent target to manipulate this inflammatory module for therapeutic benefit. Indeed, various small-molecule compounds targeting RORγt been shown to suppress IL-17A responses in mice including digoxin (Huh et al. 2011), SR1001 (Solt et al. 2011), TMP778 (Skepner et al. 2014), SR2211 (M. R. Chang et al. 2014) and TMP920 (Xiao et al. 2014). RORγt inhibitors have not been used in patients but TMP778 (Skepner et al. 2014) and SR2211 (Melton et al. 2013) have both been used to suppress human Th17 responses in vitro.

5.2 Aims & Objectives

To test the effects of two novel small molecule RORγt inhibitors (MRL367 and MRL248) on the peripheral blood and synovial fluid of patients with ankylosing spondylitis (AS).

1- To develop an assay for the expansion of patient-derived type 17 cells in-vitro.
2- To test the effect of the compounds on the production of IL-17A and other cytokines by blood lymphocytes of SpA patients.
3- Test the effect of the compounds on SpA synovial fluid mononuclear cell Th17 responses.

5.3 Results

5.3.1 Low dose T cell receptor stimulation promotes differentiation of naïve CD4 cells into Th17 cells

In addition to the three classical signals of naïve T cell differentiation (TCR engagement, co-stimulation & cytokines) (Murphy, Kenneth 2011), it has been suggested that the strength of TCR signalling also plays a role in the differentiation of Th17 cells (Purvis et al. 2010). I tested the effects of a standard human T cell expansion protocol (Miltenyi) on naïve T cells using anti-CD2/3/28 antibody coated beads at a ratio of 1 bead for every 2 cells versus a lower dose of beads to naïve T cells (1 bead for every 8 cells, n=1). I observed a clear increase in the percentage of naïve T cells staining for intracellular IL-17A in a one week culture system using the lower dose stimulation in the presence of Th17 skewing cytokines (IL-6, IL-1β, IL-23 and TGF-β) (figure 5.1).

Figure 5.1

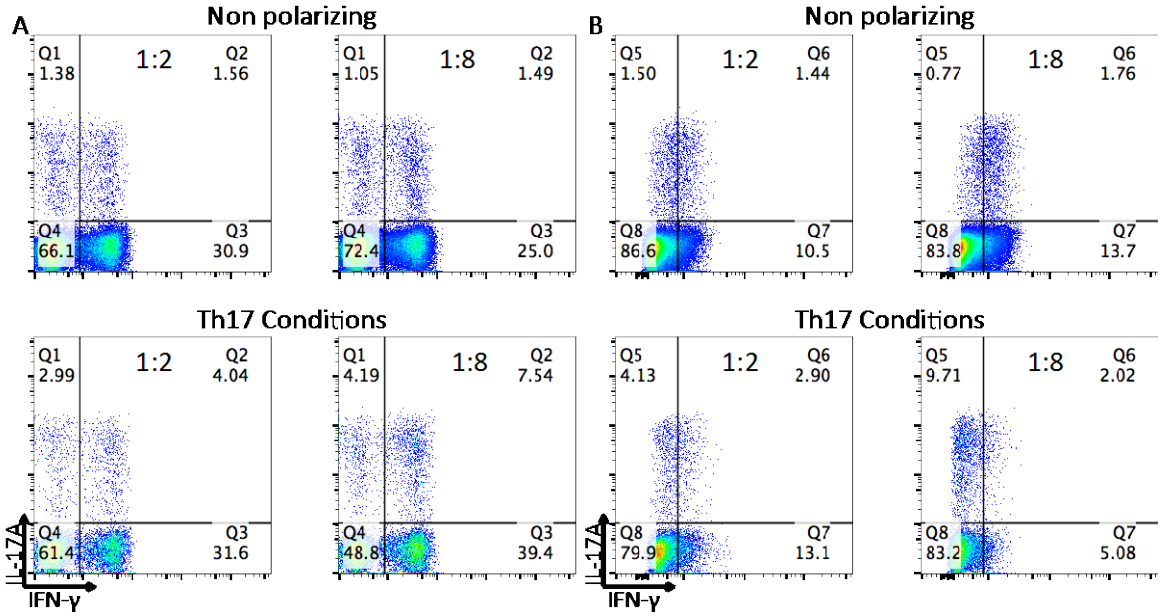


Figure 5.1. Polarisation of naïve CD4 cells towards Th17 is enhanced by reducing the TCR activating bead: cell ratio.

Naïve CD4 cells were isolated from whole healthy donor PBMCs by magnetic bead negative selection. 500,000 naïve CD4 cells were cultured for one week with TCR-activating miltenyi beads coated with anti CD2, CD3 and CD28 in a 48 well plate. Two different concentrations of beads were used; 1 bead to 2 cells (1:2) and 1 bead to 8 cells (1:8), as shown within each plot. Non-polarising conditions contained only IL-2 at a concentration of 100IU/ml while Th17 condition contained IL-1β, IL-6 and TGF-β all at 10ng/ml plus IL-23 at 20ng/ml. Cells were split on day 3 and on day 6 re-stimulated with PMA and Ionomycin for intracellular cytokine staining. A shows IL-17A plotted against IFN-γ and B shows IL-17A plotted against GM-CSF.

5.3.2 Th17 polarising cytokines show no effects in whole PBMC Th17 promoting cultures

Since the use of any therapy as a systemic oral agent would affect all PBMC populations (rather than just naïve CD4 T cells) I set out to develop a Th17 (CD4+IL-17A+) promoting culture system using whole PBMCs. Figure 5.2 shows representative flow cytometry plots using low dose versus standard TCR bead stimulation with Th17 promoting cytokines or non-polarising conditions (IL-2 alone). In the whole PBMC culture system I observed a similar percentage of Th17 cells after one week using both low and standard dose TCR stimulation (figure 5.2-A). In addition, there was no significant effect on Th17 percentages with the addition of pro-Th17 cytokines IL-6, IL-1β, IL-23 and TGF-β compared to IL-2 alone (figure 5.2-B). Finally I tested the duration of incubation on the percentage of Th17 cells and found no significant difference for a one-week incubation compared to a two-week incubation (figure 5.2-C).

Next I sought to find the optimal concentration of IL-2 and TCR beads in the one week culture system (using T25 flasks). PBMCs from a healthy donor were incubated for one week in different doses of IL-2 and at different TCR bead to cell ratios. TCR bead:cell doses tested were 1:10 (0.1), 1:20 (0.05) and 1:40 (0.025). In this set of experiments the optimum dose TCR ratio was 1:20 (figure 5.3B) and the optimum IL-2 concentration was 100 international units (figure 5.3A).

Finally, to test the reliability of the culture conditions in expanding Th17 populations from whole PBMCs, the Th17 percentage of CD4 cells was

determined ex-vivo in 6 patients with AS and compared to the percentage observed after one week in the culture system. I observe a statistically significant increase in the percentage of Th17 cells in all 6 donors (figure 5.4). Therefore these culture conditions were used in future experiments to test the effects of RORγt suppression.

Figure 5.2

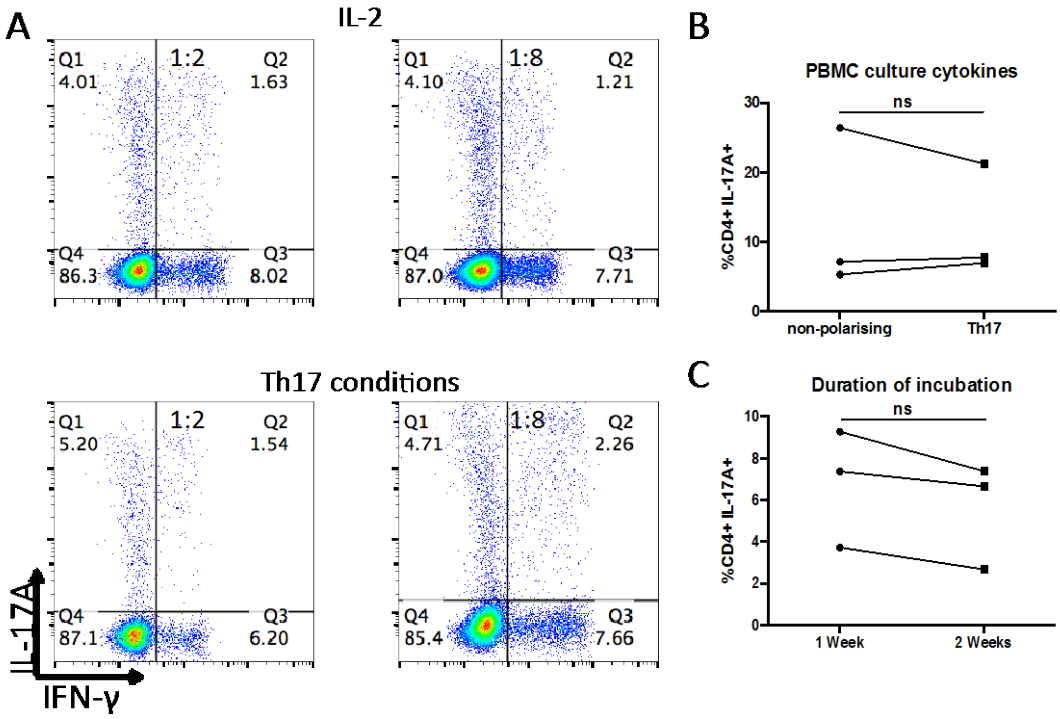


Figure 5.2. Polarisation of whole PBMCs towards Th17.
500,000 healthy donor PBMCs were cultured for one week with miltenyi beads coated with anti CD2, CD3 and CD28 in a 48 well plate. Two different concentrations of beads were used; 1 bead to 2 cells (1:2) and 1 bead to 8 cells (1:8). Non-polarising conditions contained only IL-2 at a concentration of 100IU/ml while Th17 condition contained IL-1β, IL-6 and TGF-β all at 10ng/ml plus IL-23 at 20ng/ml. Cells were split on day 3 and on day 6 re-stimulated with PMA and Ionomycin for intracellular cytokine staining. Panel A shows the representative flow cytometry plots of IL-17A plotted against IFN-γ. Panel B shows the percentage of IL-17A positive CD4 cells obtained data from 3 independent experiments using a 1:8 TCR stimulus. Panel C shows the percentage of IL-17A positive CD4 cells obtained after one week or two weeks of culture using non-polarising conditions and a 1:8 TCR stimulus. Statistical analysis was carried out using a paired T test.

Figure 5.3

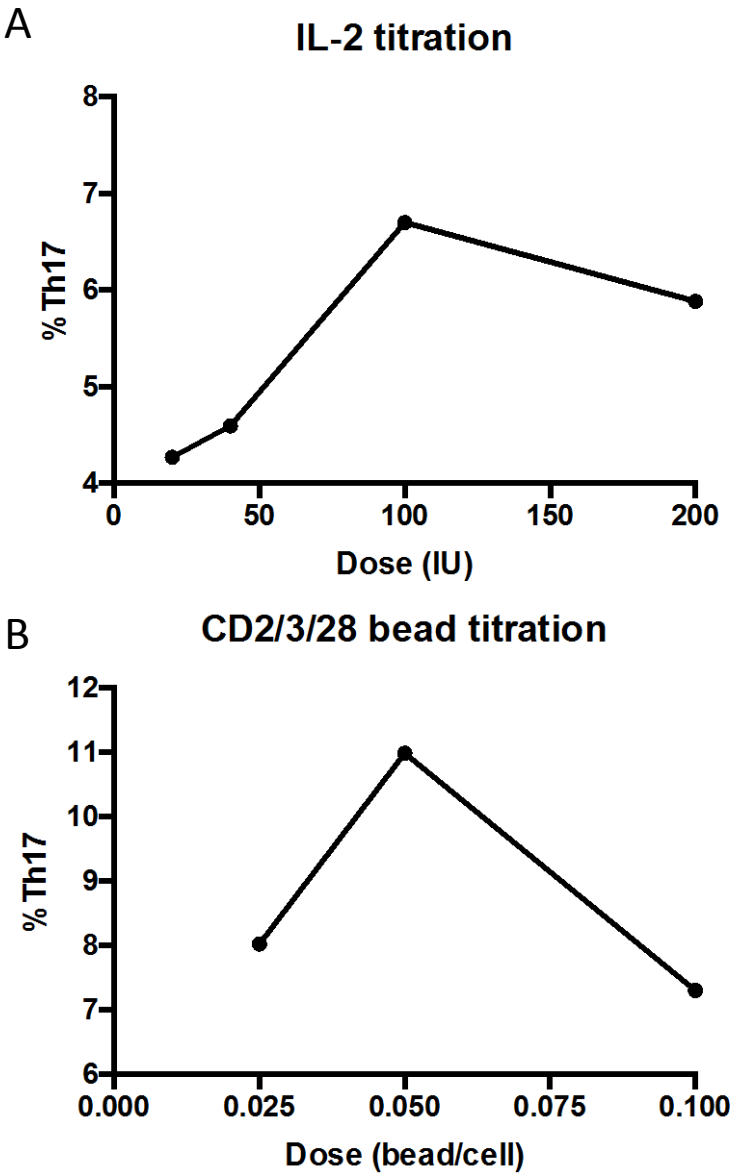


Figure 5.3. Optimisation of PBMC Th17 cultures in a T25 flask.
5,000,000 healthy donor PBMCs were isolated ex-vivo and cultured for one week with TCR-activating miltenyi beads coated with anti CD2, CD3 and CD28 in a T25 flask. Panel A shows the effects of differing doses of IL-2 on PBMCs from the same donor cultured with TCR-activating beads at a 1:10 concentration. Panel B shows the effects of three different concentrations of beads; 1:10 (0.1), 1:20 (0.05) and 1:40 (0.025) cultured in an IL-2 concentration of 100IU/ml.

Figure 5.4

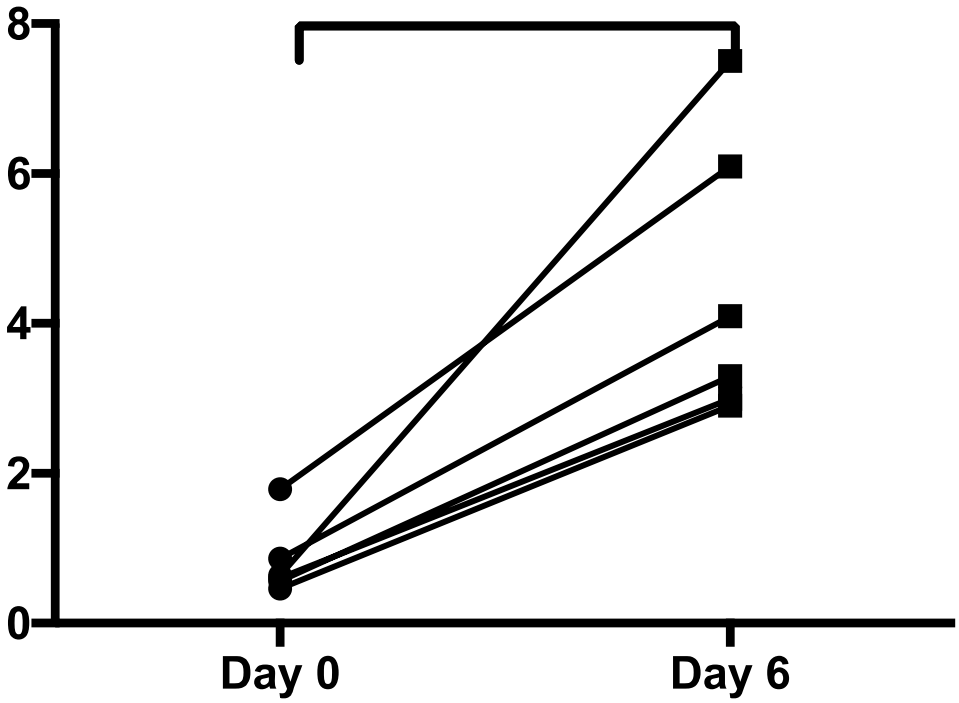


Figure 5.4. Culture of AS patients PBMCs in T25 flask with a 1:20 TCR bead dose and 100IU/ml of IL-2 leads to expansion of Th17 percentage compared to baseline.
5,000,000 PBMCs from AS patients PBMCs were cultured for one week with TCR-activating miltenyi beads coated with anti CD2, CD3 and CD28 in a T25 flask at bead concentration of 1:20 in the presence of 100IU/ml of IL-2. Cells were cultured in 5mls of R10 medium and the flask kept upright in the incubator. After 3 days 5mls of R10 medium containing 200IU/ml of IL-2 was added to the flask. At day 6 cells were re-stimulated with PMA and ionomycin in the presence of berfeldnin A and monensin for 4 hours and the percentage of IL-17A positive cells in the CD4 gate was determined and compared to the baseline ex-vivo percentage for each individual. The figure shows the results of 6 independent experiments and statistical significance was determined using a paired T test.

5.3.3 RORγt inhibitor compounds suppress IL-17A expansion in CD4 cells

Two RORγt inhibitor compounds (MRL-367 and MRL-248), available via a material transfer agreement between the University of Oxford and Merck pharmaceuticals, were tested on PBMCs derived from AS patients. The two compounds are structurally very similar and the only difference is an additional OH group on MRL-367 (figure 5.5A). The assay used is summarised in figure 5.5B.

Figure 5.5C shows the representative flow cytometry plots from one AS patient after a week-long culture with DMSO control or each of the inhibitors at three different doses. Both inhibitors show a dose-dependent reduction in the percentage of CD4 cells expressing intracellular IL-17A compared to control. The dose response relationship of the two compounds is similar and summarised in figure 5.5-D. In addition to the reduction in the percentage of Th17 cells within the CD4 pool, I also observed a reduction in the mean fluorescence intensity (MFI) of the intracellular IL-17A staining in the CD4 cells (figure 5.5E).

Figure 5.5

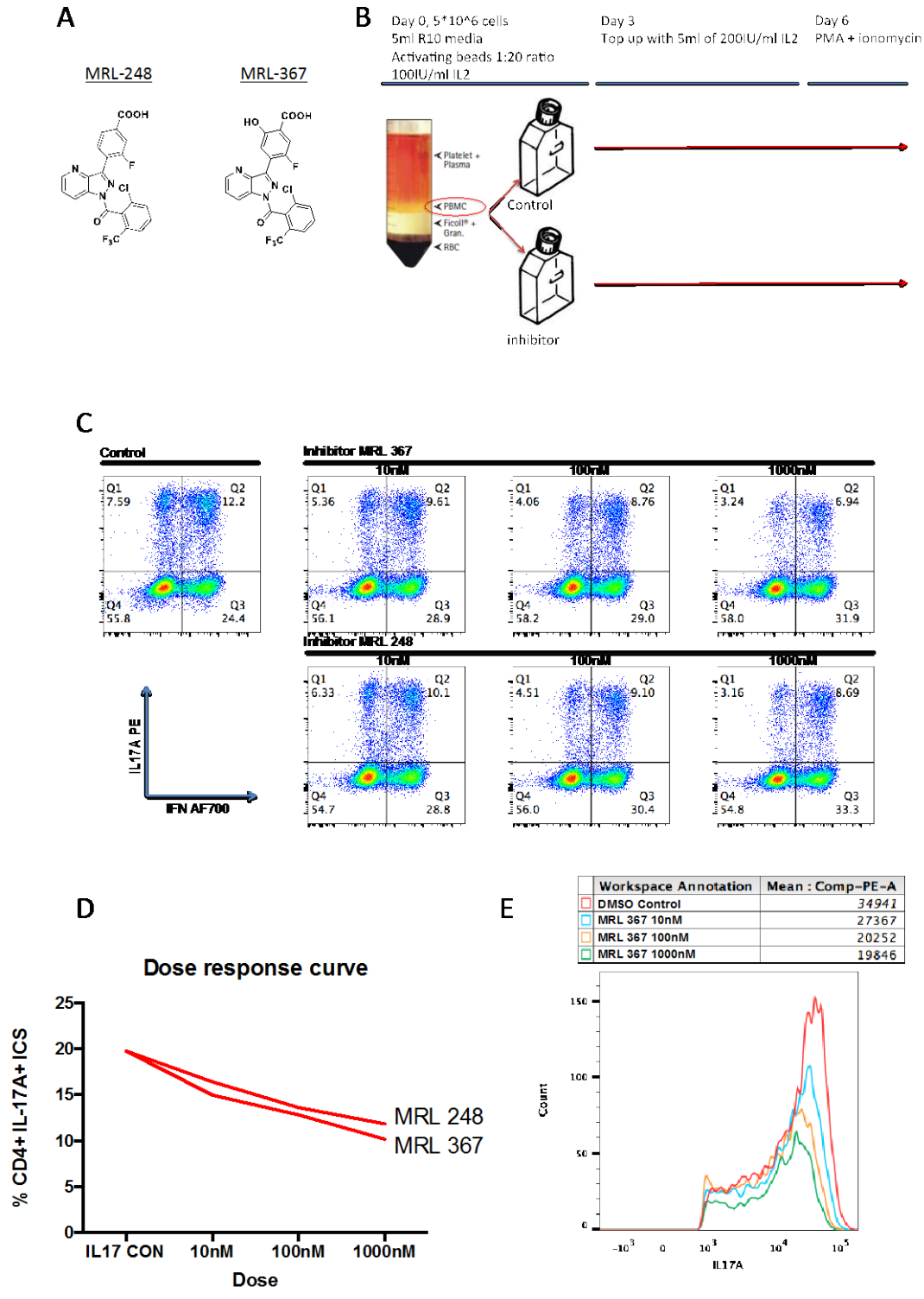


Figure 5.5. RORγt inhibitors MRL-367 and MRL-248 suppress IL-17A production from Th17 cells.

The structure of the two RORγt inhibitors MRL-248 and MRL-367 manufactured by Merck laboratories is shown in A and the summary of the assay is shown in B. C shows the representative flow cytometry data at day 7 using PBMC from an AS patient and each of the inhibitor used at 3 different doses (10nM/ml, 10nM/ml and 1000nM/ml). D summarises the percentage of Th17s on day 6 in culture with varying doses of the two inhibitors. E shows the mean fluorescence intensity of IL-17A positive CD4 cells cultured with three different doses of MRL-367 or control.

5.3.4 RORγt inhibition reduces both the percentage of Th17 cells in in-vitro Spondyloarthritis PBMC cultures and the amount of IL-17A secreted

The two RORγt compounds were tested using the in-vitro assay described above using PBMCs from 10 SpA patients. Figure 5.6-A shows the data for the 10 individuals at baseline and with each of the two inhibitors. Overall both inhibitors reduced the percentage of Th17 cells in all individuals but I did observe significant variation between individual patient donors. Figure 5.6-B summarises the data for all 10 patients, and shows that a statistically significant inhibition of Th17 cell percentage is achieved with both inhibitors compared to the DMSO control. There was no statistically significant difference in the inhibition seen with the two different compounds. 5.6-C shows the same data but standardised to the control level percentage of Th17 for each individual. Overall a mean suppression to approximately 50% of baseline was observed in the number of Th17. Both inhibitors suppressed the amount of secreted IL-17A measured by ELISA compared to control (figure 5.6-D). Finally I did not observe any difference in the cell viability of all cells after treatment with each of the inhibitors (figure 5.6-E).

Figure 5.6

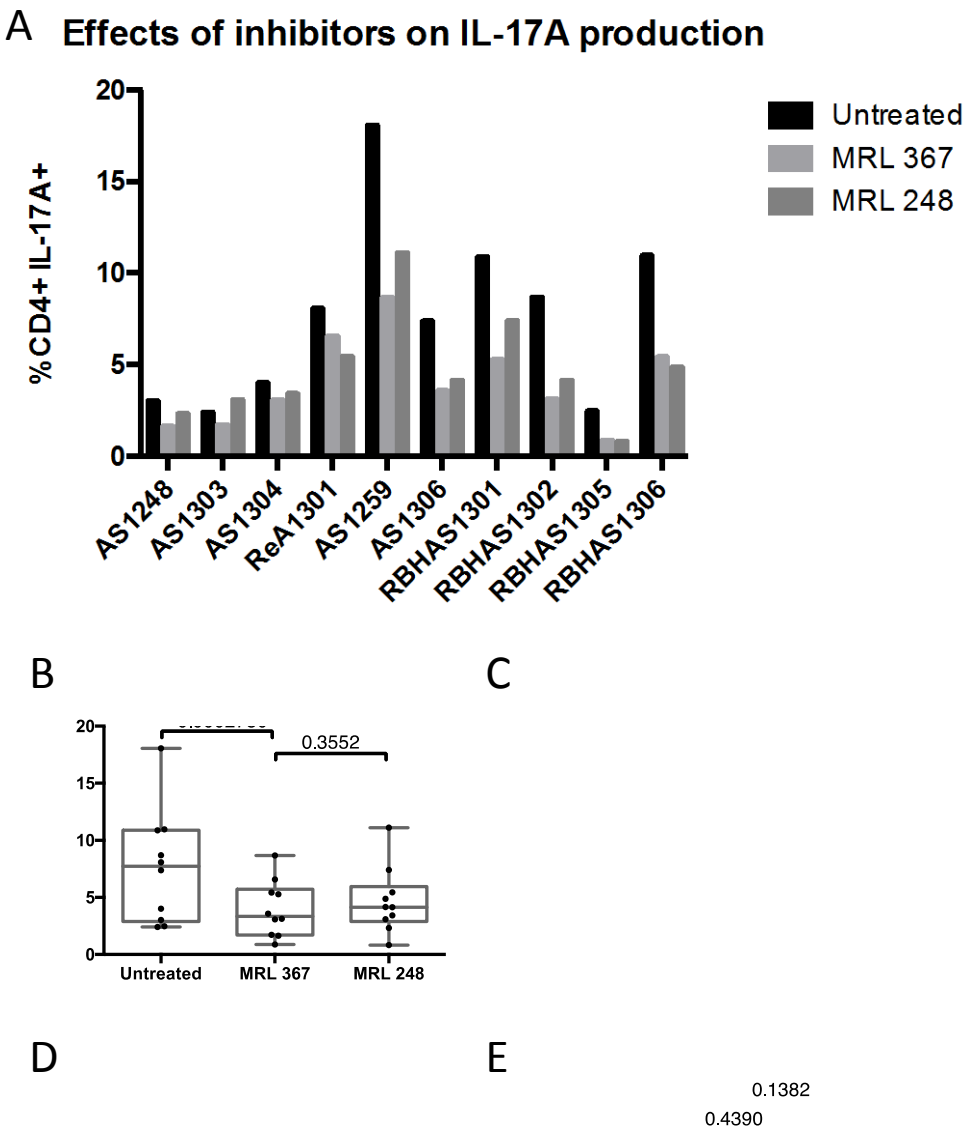


Figure 5.6. RORγt inhibitors MRL-367 and MRL-248 significantly suppress the percentage of Th17 cells in AS PBMCs without affecting cell viability.
Panel A shows the effects of MRL-367 and MRL-248 compared to control on the percentage of Th17 using SpA PBMCs from 10 donors cultured for 6 days in in 7 independent experiments. The mean data are summarised in Panel B where a statistically significant suppression of Th17 percentage is shown compared to controls. Panel C shows the same data as Panel B but standardised to the baseline level of Th17 for each donor. Panel D shows the amount of IL-17A in the culture supernatants measured by ELISA from one experiment. Panel D shows the viability of cells cultured in control conditions or with each inhibitor expressed as percentage of live cells at day 6 in the 10 donors. Statistical significance was determined using a one-way ANOVA with multiple comparisons.

5.3.5 Inhibition of RORγt is specific to Th17 cells

Figure 5.7 shows that RORγt inhibitors also suppressed the total percentage of IL-22 secreting cells but did not significantly alter the overall percentage of IFN-γ, TNF-α or GM-CSF positive cells. The inhibitors were tested in the same 10 individuals as shown figure 5.6 using multicolour intracellular flow cytometry.

However when the percentage of cells co-staining for IL-17A with IL-22, IFN-γ, TNF-α, or GM-CSF was analysed I observed a statistically significant suppression of double-positive populations with the inhibitor MRL-367. For MRL-248, IL-17A and IFN-γ, IL-22 and TNF-α co-staining cells were suppressed. IL-17A/GM-CSF double positive cells were significantly suppressed by MRL367 but not MRL248 (figure 5.8).

Figure 5.7

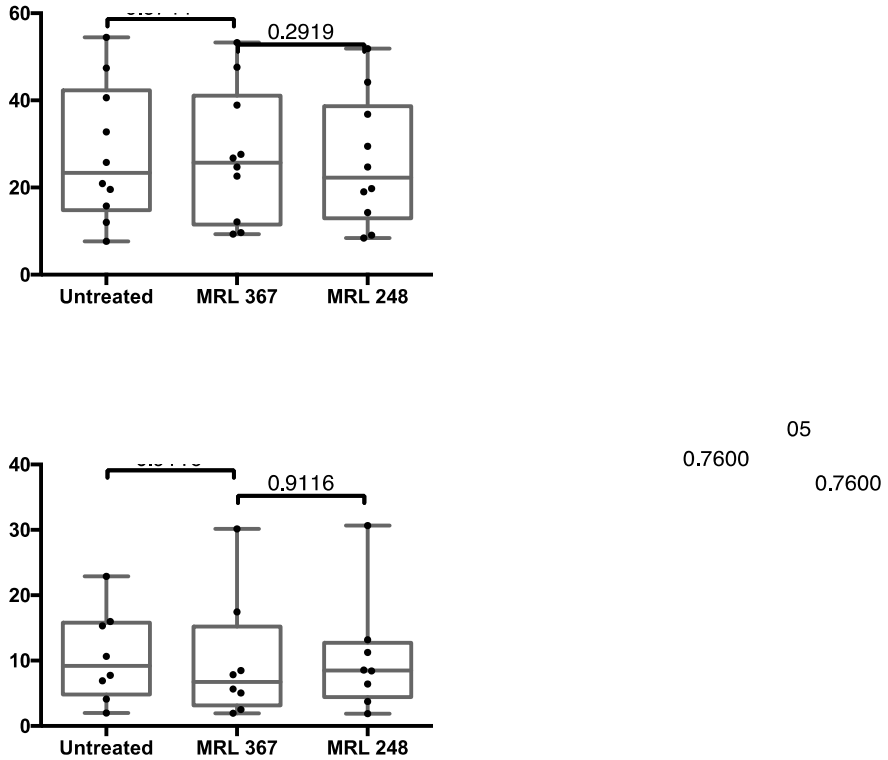


Figure 5.7. RORγt inhibitors MRL-367 and MRL-248 significantly reduce the total percentage of IL-22-positive cells but not IFN-γ, TNF-α or GM-CSF-positive cells.
PBMCs from SpA were cultured in the presence of inhibitors were stained for the intracellular cytokines IL-22 (n=5), IFN-γ (n=10), TNF-α (n=7) and GM-CSF (n=8). A statistically significant reduction in the percentage of CD4 cells positive for IL-22 was observed with both MRL-367 and MRL-248 at day 6 compared to control. There were no statistically significant differences observed for IFN-γ, TNF-α and GM-CSF. Statistical significance was determined using a one-way ANOVA with multiple comparisons.

Figure 5.8

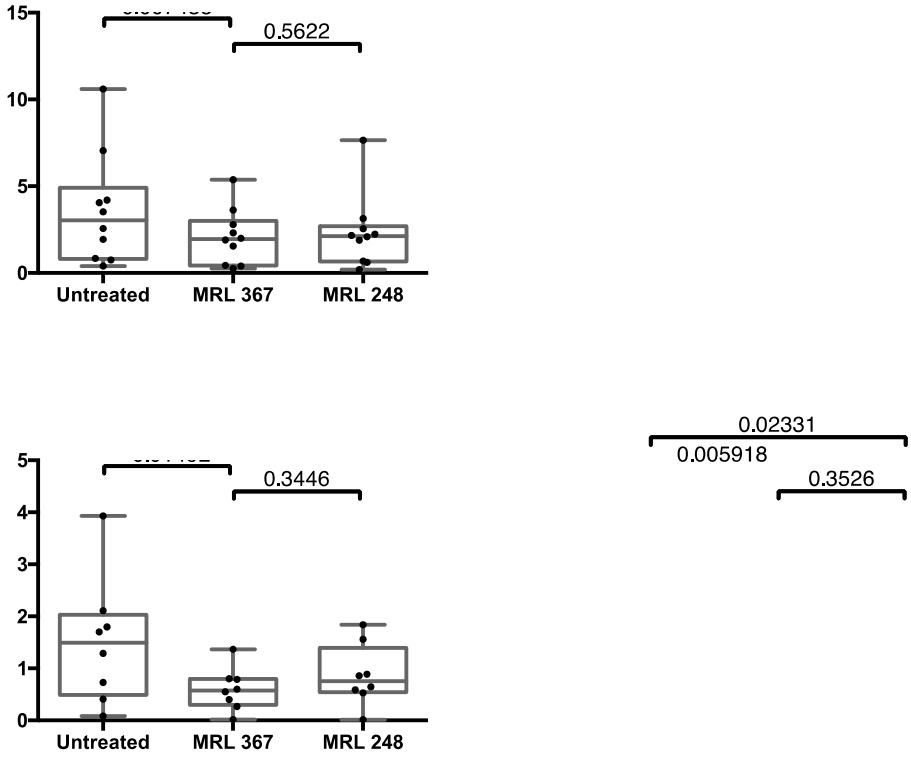


Figure 5.8. RORγt inhibitors MRL-367 and MRL-248 significantly reduce the percentage of other cytokines if co-expressed with IL-17A.
PBMCs from SpA were cultured in the presence of inhibitors or controls were stained for intracellular IL-17A in addition to IL-22 (n=5), IFN-γ (n=10), TNF-α (n=7) and GM-CSF (n=8). A statistically significant reduction in the percentage of CD4 cells positive for IL-17A and each of the four other cytokines was observed with MRL-367. MRL-248 led to a statistically significant reduction in the percentage of IL-17A co-staining with IFN-γ, IL-22 and TNF-α but not IL-17A/GM-CSF double positive cells. Statistical significance was determined using a one-way ANOVA with multiple comparisons.

5.3.6 RORγt inhibitors also suppress IL-17A production from CD8 cells

Within the same PBMC cultures I also looked at the effects of the two inhibitors on CD8-positive T cells. CD8 staining was only carried out in 4 individuals within the cohort of 10 patients. In those four individuals a statistically significant reduction in the number of IL-17A positive CD8 T cells was observed for the inhibitor MRL-248 compared to the untreated control (but not for MRL-367), (figure 5.9). There was no observable change in the percentage of IFN-γ positive cells.

Figure 5.9

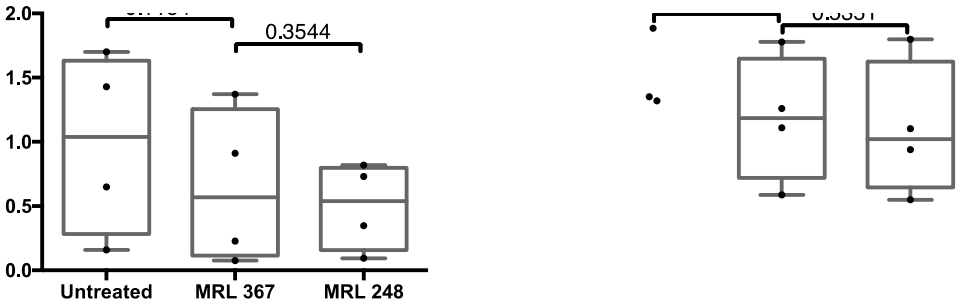


Figure 5.9. RORγt inhibitor MRL-248 suppresses the percentage of IL-17A positive CD8 cells but has no effect on IFN-γ.
SpA PBMCs from 4 donors were also stained for CD8 and the percentage of CD8 cells positive for IL-17A was cells determined (n=4). A statistically significant reduction in the percentage of CD8 cells positive for IL-17A was observed with the inhibitor MRL-248. There was no effect of either inhibitor on the percentage of CD8 cells positive for IFN-γ. Statistical significance was determined using a one-way ANOVA with multiple comparisons.

5.3.7 RORγt inhibitors suppress IL-17A secretion from synovial fluid cells but do not alter the percentage of IL-17A positive cells

Synovial fluid mononuclear cells from two SpA patients were cultured using the Th17 expansion assay. In these two individuals there was no clear effect of either inhibitor on the observed percentage of IL-17A positive cells using intracellular flow cytometry (figure 5.10A). However, both inhibitors significantly reduce the amount of secreted IL-17A present in the culture supernatants as measured by ELISA (figure 5.10B).

Figure 5.10 synovial fluid

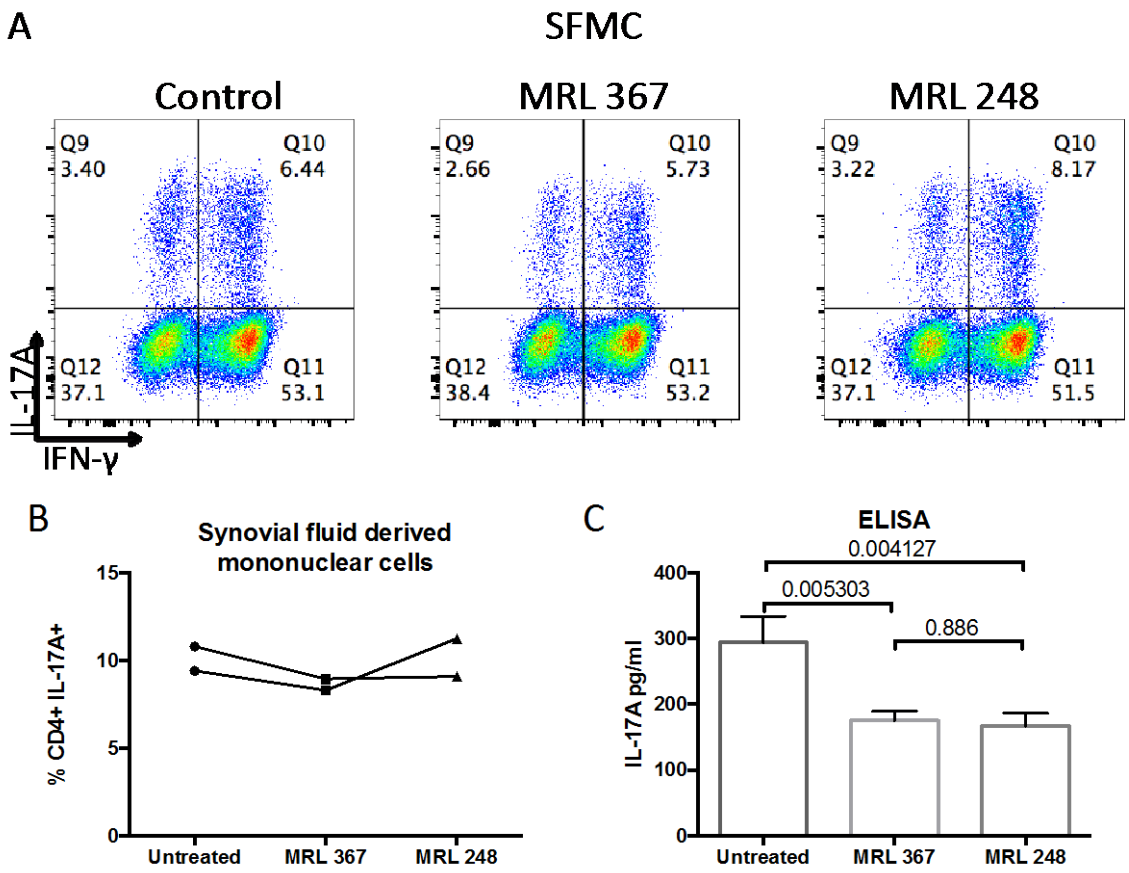


Figure 5.10. RORγt inhibitors MRL-367 and MRL-248 suppress the amount of secreted IL-17A produced by synovial fluid cells but do not reduce percentage of Th17 cells.
SpA SFMCs were cultured in the presence of the two RORγt inhibitors. A Representative flow cytometry plots of SFMC IL-17A and IFN-γ production with and without the two inhibitors. B Data from two donors shows no clear difference in the percentage Th17 cells with either inhibitor compared to control in the intracellular flow cytometry assay. C Statistically significant reduction in the amount of secreted IL-17A measured by ELISA was observed with both compounds. Statistical significance was determined using a one-way ANOVA with multiple comparisons.

5.4 Discussion

I show here that the two RORγt inhibitors MRL-367 and MRL-248 suppress in-vitrp Th17 expansion in the blood of patients with SpA without causing overall cell toxicity (although a directly toxic effect on CD4 cells cannot be ruled out). The suppression of Th17 responses is specific because the percentages of CD4 cells staining for Th17-associated intracellular cytokines IL-17A and IL-22 were significantly reduced in the presence of the inhibitors while the percentages of cells staining for other measured cytokines (IFN-γ, TNF-α and GM-CSF) were not. The results of this work, together with further work carried out by Jelle de Wit, have now been published in the Journal of Allergy and Clinical Immunology (de Wit & Al-Mossawi et al. 2015).

ROR inhibitors described previously include digoxin, SR1001, TMP778 and SR2211 (Huh et al. 2011; M. R. Chang et al. 2014; J. Yang et al. 2014). These suppress Th17 responses in murine Experimental Autoimmune Encephalomyelitis (EAE) and collagen- induced arthritis but there is less data on human disease. The RORγt inverse agonist TMP778 reduced both EAE and imiquimod-induced cutaneous inflammation in mice, and also inhibited Th17-associated gene expression in cells derived from patients with psoriasis (Skepner et al. 2014).

Digoxin and TMP778 specifically suppressed RORγt, whereas SR1001 also inhibited RORα (Huh et al. 2011; Solt et al. 2011; Skepner et al. 2014). MRL-248 and MRL-367 specifically suppress RORγt via binding to the ligand-binding domain, likely by inhibiting the interaction with co-factor steroid receptor co-activator 1. MRL-248 and MRL-367 are structurally comparable

and have similar suppressive effects on Th17 cells in SpA. Whereas digoxin, TMP778 and SR1001, which have a similar mechanism of action (Huh et al. 2011; Solt et al. 2011; Skepner et al. 2014), show IC_{50} potency in the μM range, MRL-248 and MRL-367 displayed IC_{50} values in the nM range (de Wit & Al-Mossawi et al. 2016).

In addition to reducing the percentage of Th17 cells after the one-week culture system, both inhibitors also significantly suppressed the secreted IL-17A in the culture supernatants measured by ELISA. The ELISA assay differs from the intracellular cytokine staining (ICS) assay. ELISA looks at the overall rate of IL-17A production in the culture system while ICS captures the cells capable of producing IL-17A upon stimulation with PMA and ionomycin. This difference in the two assays may explain the discrepancy observed in the results observed in the synovial fluid (where the inhibitors did not suppress the percentage of Th17 cells by ICS but significantly reduced the amount of secreted IL-17A in the culture supernatant). These findings were supported by further experiments (de Wit & Al-Mossawi et al. 2016) which showed that RORγt inhibition with MRL-367 and MRL-248 suppressed IL-17A production by ELISA in pre-sorted Th17 cells from healthy donors but not the overall percentage of Th17 cells by ICS. Taken together, these results suggest the RORγt transcription factor is required for the differentiation and expansion of Th17 cells and their ability to secrete IL-17A under culture conditions but RORγt is less important for the maintenance of the Th17 phenotpye in pre-committed cells. This may be relevant when considering the potential efficacy of this treatment in the clinic

The effect of the two inhibitors on IL-17A, and to a lesser extent IL-22, seems specific to these two cytokines in terms of inhibition of T cell phenotype. The overall percentage of CD4 cells expressing the other tested cytokines was unaltered. However the expression of TNF-α, IFN-γ and GM-CSF in combination with IL-17A was significantly suppressed. This is relevant because Th17s, especially those that co-express GM-CSF, are thought to have an important role in driving pathogenesis in autoimmune inflammatory disorders (El-Behi et al. 2011; Yosef et al. 2013). T helper differentiation from naïve CD4 cells involves several intracellular signalling cascades that are often regulated in a reciprocal manner (C. B. Wilson, Rowell, and Sekimata 2009). It is reassuring to find that in this assay of human Th17 cell expansion, I did not see reciprocal effects of RORγt expression on IFN-γ-secreting Th1 cells. In this set of experiments I did not test the reciprocal effects of RORγt inhibition on regulatory T cells (Tregs). Tregs and Th17 cells share common differentiation pathways (Ayyoub, Raffin, and Valmori 2012) and conversion of one cell type to another has been reported in mouse models (Gagliani et al. 2015; Komatsu et al. 2014). The role of RORγt inhibition on human Th17 conversion to Treg will be the subject of future work in the Bowness group.

This effect on the 'pathogenic' polyfunctional cells, with a reduction in the overall percentage of Th17 cells rather than complete abrogation of the Th17 response, may be beneficial if these compounds are taken forward into clinical trials. Complete neutralisation of IL-17A would have an impact on the protective role of this cytokine in the gut. This may explain the observation of worsening disease in Crohn's patients treated with anti-IL-17A monoclonal

antibodies (Hueber et al. 2012). I would argue that the maintenance of some physiological IL-17A may be a useful feature not just in Crohn's but also in other conditions where it may may reduce immunosuppressive complications.

My data also suggests that RORγt inhibitors play a role in driving type-17 immunity in CD8-positive cells. It is unclear what role IL-17A-producing CD8 cells play in the pathogenesis of autoimmune inflammatory diseases, but my work in chapter 3 and the work of others does suggest they are expanded in the disease context (Menon et al. 2014). Even though the number of experiments shown in figure 5.9 is low and does not meet statistical significance for MRL-367, the result is significant for MRL-248 in the 4 individuals. This work was replicated in the publication (de Wit & Al-Mossawi et al. 2016) where clear suppression of the percentage of IL-17A-producing CD8 cells was observed with both inhibitors.

A criticism of this work may be that the Th17 cells generated in the expansion assay do not represent true in-vivo Th17 cells. The exact mechanism and cytokine requirements for human in-vitro Th17 cell differentiation from naïve CD4 cells are a controversial topic and there is no consensus in the literature. Some authors have shown a requirement for TGF-β (L. Yang 2008; N. Manel, Unutmaz, and Littman 2008) while others have shown TGF-β to suppress Th17 responses (H. G. Evans et al. 2007). In addition the combination and concentrations of IL-6, IL-23, IL-1β and IL-21 differs in different publications (Evans et al. 2007; McGeachy et al. 2007; L. Yang 2008; Manel, Unutmaz, and Littman 2008; Ayyoub, Raffin, and Valmori 2012, 17; Annunziato et al.

2008). I would therefore argue that there is no ‘standard’ method of generating human Th17 cells in vitro and therefore any method which robustly and consistently increases the percentage of CD4 cells (figure 5.4) expressing intracellular IL-17A is valid. Also the expansion of human Th17 cells from the peripheral blood of patients with uveitis in the presence of IL-2 only has been previously published (Amadi-Obi et al. 2007). Finally, the observation that RORγt-inhibiting compounds limit this expansion further confirms that these cells are indeed Th17 cells which use RORγt as a transcription factor.

For this work I chose to use a whole PBMC system rather than a naïve T cell differentiation model, so that we might better mimic the in-vivo environment found at the inflammatory site, where the majority of cells secreting IL-17A are pre-differentiated and have a memory phenotype (Shen, Goodall, and Hill Gaston 2009). Testing the effects of RORγt inhibition on naïve CD4 polarisation towards Th17 was carried out in subsequent work and published in the paper resulting from this work (de Wit & Al-Mossawi et al. 2016).

Targeting the type 17 pathway using monoclonal antibodies against IL-17A (Baeten et al. 2013) or its cell surface receptor IL17RA (Mease et al. 2014) has been shown to be effective in AS and PsA respectively. In addition, ustekinumab, an antibody targeting the upstream IL-23/IL-12 shared subunit p40, has also been shown to be efficacious in AS (Poddubnyy et al. 2014), psoriasis (Griffiths et al. 2010) and PsA (McInnes et al. 2013). These results further highlight the role of this inflammatory pathway in immune mediated inflammatory disorders. The data presented in this chapter together with

subsequent work (de Wit & Al-Mossawi et al. 2016) would argue that RORγt may also be a viable therapeutic target, which can be blocked using small molecule inhibitors and would provide the basis for trials in patients with SpA and other immune-mediated inflammatory diseases (de Wit & Al-Mossawi et al. 2016).

Chapter 6: Overall discussion and future directions

6.1 GM-CSF secreting T cells likely comprise a distinct CD4 cell subset

The data presented in this thesis show for the first time an expansion of the percentage GM-CSF-positive CD4 cells in the peripheral blood of patients with SpA compared to healthy controls and RA disease controls. The increase in GM-CSF is not limited to CD4 cells but also occurs in CD8 and γδ T cells. Moreover, the expansion of the percentage of GM-CSF positive cells occurs both within the Th17 compartment (increase in IL-17A/GM-CSF double positive cells) and also independently of IFN-γ-producing Th1 cells and IL-17A producing Th17 cells. This would suggest that GM-CSF-producing T helper cells represent a unique subset within the T helper differentiation fates.

The existence of an independent subset of Th-GMCSF cells is supported by my RNA sequencing data, which show a unique expression profile for GM-CSF single positive cells and a unique clustering of upregulated genes. However, in the mouse models of GM-CSF-driven disease RORγt has been suggested as a driver of GM-CSF (Codarri et al. 2011). The RNA sequencing data show similar expression of RORC in GM-CSF single positive cells and IL-17A single positive cells supporting this view, but notably in the data presented in chapter 5 of this thesis inhibition of RORγt suppressed IL-17A but not GM-CSF (de Wit & Al-Mossawi et al. 2016). In order to confirm this finding, candidate master regulators of a GM-CSF programme in CD4 cells

must be validated at a functional level and this will be the subject of future work (see below).

The identification of this subset of cells in AS opens up a new therapeutic angle. Monoclonal antibodies targeting GM-CSF (Behrens et al. 2014) or its cell surface receptor (Burmester et al. 2013) are currently in clinical trials for RA and have so far shown efficacy and safety in phase 2 studies (Behrens et al. 2014; Burmester et al. 2013). The expansion of this subset of T cells in AS would argue for trials in this disease, However safety concerns regarding alveolar proteinosis (Kitamura et al. 1999) are important to take into account in any such trials. Moreover, in a recent study it has been shown that GM-CSF production from ILC3 subsets in mouse models of inflammatory bowel disease are important for maintaining Tregs via the GM-CSF-dependent production of retinoic acid by myeloid cells (Mortha et al. 2014). This further illustrates the complexity of the biology of GM-CSF in maintaining homeostasis in addition to its potential pathogenic roles. Therefore it would argue for a therapeutic strategy which would be able to specifically target polyfucntional T cells rather than wholesale blockade of a cytokine pathway.

6.2 Pathogenic Th17 cell phenotype is enhanced in AS

Several reports in the mouse literature have now confirmed a heterogeneity of cytokine production within the Th17 compartment (Gaublomme et al. 2015; Yosef et al. 2013). In particular Th17 cells co-producing GM-CSF have been shown to be pathogenic in the context of mouse models of inflammation (Codarri et al. 2011; El-Behi et al. 2011; Sheng et al. 2014). The data in

chapter 3 of this thesis show an expansion of such “pathogenic Th17” cells co-expressing IL-17A and GM-CSF in the blood and synovial fluid of patients with SpA. Transcriptional analysis of these double positive cells from healthy donor lymphocyte cones shows a higher expression of both RORC and TBX21 compared to single positive IL-17A producing cells. Moreover, RORγt inhibition does suppress this double-positive population in addition to IL-17A single positive cells (de Wit & Al-Mossawi et al. 2016). This would suggest that high RORγt expression may be one driver of pathogenic GM-CSF-producing Th17 cells and would support the mouse findings (Codarri et al. 2011).

In addition, my transcriptional analysis shows a unique cluster of activated genes in the double positive “pathogenic” cells, which distinguishes these cells from classic Th17 cells. Some of these genes may represent good targets for therapeutic manipulation but require functional validation first. In a recent study it was shown that blimp1 is a key driver of pathogenic Th17 cells in mice downstream of IL-23 (Jain et al. 2016) and that blimp1 directly binds to the promoter sites of both the csf2 and il17a genes in mice. Moreover the authors show that blimp2 knockout mice are protected from EAE (Jain et al. 2016). In the RNA sequencing data set generated in my study, the expression of PRDM1, the human gene for blimp1 was only seen to be differentially increased compared to CD45RA positive naïve cells in IFN-γ and GM-CSF single positive populations but not in IL-17A single positives or IL-17A/GM-CSF double positives. A difference in biology between mouse and humans may be one explanation. A second explanation may be that the IL-17A/GM-

CSF double positive cells sorted from lymphocyte cones are not representative of true “pathogenic” T cells from the site of inflammation. This highlights the need to validate the RNA sequencing data from ex-vivo T cells derived from human inflammatory sites. The idea of targeting the “pathogenic” component of the immune response while maintaining normal immunity would be hugely beneficial across the spectrum of inflammatory disease and the data showing the role of factors such as blimp1 in mice would suggest this may be a viable option in patients.

6.3 IL-7 promotes human T cell GM-CSF production

My data presented in chapter 3 of this thesis (figure 3.18 and figure 3.19) clearly show a role for IL-7 in driving an increase in the number of CD4 cells producing GM-CSF in vitro. This finding corroborates previous mouse data showing that IL-7, acting via STAT5, is a key cytokine in promoting GM-CSF-positive T cell expansion (Sheng et al. 2014). My data show the effect of IL-7 on CD4 T cells is specific to the GM-CSF single positive T cell subset only and does not lead to expansion of IL-17A/GM-CSF double positive cells, suggesting that other factors may driving the “pathogenic” polyfunctional CD4 subset. However the set of experiments were limited in number and an effect of IL-7 on other cytokines in a larger experiments cannot be ruled out. Moreover, the experiments using recombinant IL-7 (figure 3.18 and figure 3.19) do not take into account the effects of IL7R polymorphisms on T cell function, especially since polymorphisms in this receptor have been associated with AS (Cortes et al. 2013). Nevertheless, my data show a role

for IL-7 in promoting T cell GM-CSF and the enhanced concentrations of IL-7 seen in the joint may therefore be relevant (van Roon et al. 2005).

6.4 Exploring the functional role of IL7R polymorphisms

In this thesis I have sought to understand the functional effects of IL7R polymorphisms in the context of healthy donors rather than disease. The expression of this receptor is known to be predominantly on lymphoid cells, in particular CD4 and CD8 T cells where expression is essential for development and long term survival of memory responses (R. Mazzucchelli and Durum 2007). However, expression quantitative trait loci (eQTL) data sets show genotype-dependent differential expression of the IL7R gene in monocytes after LPS activation (Fairfax et al. 2014) but not in T cells (Raj et al. 2014). The data I have presented in this thesis confirm a genotypic effect of IL7R expression on monocytes after LPS or TNFα stimulation, but not on T cells after CD2/3/28 stimulation in healthy donors. The effects of IL-7 signalling on CD14 monocytes remain controversial and we still do not know how this differential expression will affect the activated monocyte pool in vivo. The existence of CD14 cells expressing the IL7R within ex-vivo isolated inflammatory synovial fluid would suggest a potential role for this pathway in disease pathogenesis. Moreover, my data do not exclude an effect of IL7R polymorphisms on signalling through that receptor in T cells or monocytes. Such an effect may be independent of surface expression levels.

My data raise the possibility of pursuing the IL-7 axis for therapeutic benefit. However, it should be borne in mind that blockade of this cytokine may have

severe detrimental effects since the knock-out murine studies show a severe immunodeficiency (Roifman et al. 2000). IL-7 signalling is thought to be important for the survival of the memory T cell pool (Bradley, Haynes, and Swain 2005), therefore neutralisation of this cytokine, even after thymic T cell development, may result in loss of existing memory responses. In the context of autoimmunity, this may be beneficial for serve cases as it may re-establish tolerance, especially since studies with anti-CD52 mediated depletion of mature CD4 cells by almetuzumab indicate the immune system has the capacity to re-establish the T cell pool from stem cells (Lundin et al. 2004).

6.5 Concluding remarks

Despite advances in treatments for AS, a great unmet clinical need remains. Genome Wide Association Studies have been very important in highlighting key aspects of biology (such as type 17 immunity in disease pathogenesis) but an understanding of these pathways at a functional level is crucial to avoid costly negative clinical trials. This thesis sought to answer a number of questions regarding the pathobiology of AS in order to identify new therapeutic targets in this disease. I show for the first time that GM-CSF may play an important role in “pathogenic” Th17 biology in AS. I also show that IL7R polymorphisms (also genetically associated with AS) have a functional effect via expression on monocytes. Finally, I show the potential of using small molecule RORγt inhibitors to target in-vitro type 17 responses in AS. I believe that this thesis has laid important groundwork and pre-clinical data to inform clinical trials in AS to target GM-CSF and RORγt. In addition, I have generated some important new questions which will hopefully be the subject of on-going research in the future.

6.6 Future directions

6.6.1 Validation of triple capture data at single cell level from patient-derived samples

The triple capture assay and subsequent RNA sequencing has generated an important transcriptional data set which, for the first time, provides an insight into the transcriptional regulation of human pathogenic Th17 cells. This data will require validation from patient-derived samples, especially from the site of inflammation, which I plan to do by single cell transcriptomic analysis. The final validation will be with knock-down experiments using siRNA or CRISPR technology of key transcriptional hubs to prove functional roles.

6.6.2 Effects of IL-7 signalling on monocyte transcriptional profile

I have shown the genotypic effects of IL7R polymorphisms on the expression of this receptor in activated monocytes. However, knowledge of IL-7 signalling on monocyte development remains lacking. A non-biased RNA sequencing approach to this pathway will be helpful to understand the effects of IL-7 biology in human monocytes and will hopefully yield further interesting results. This experiment has been carried out with isolated monocytes from 8 donors, treated with IL-7 alone, LPS alone or LPS plus IL-7 samples are currently awaiting sequencing.

References

Acosta-Rodriguez, E. V. 2007. “Surface Phenotype and Antigenic Specificity of Human Interleukin 17-Producing T Helper Memory Cells.” *Nature Immunol.* 8: 639–46. doi:10.1038/ni1467.

Acosta-Rodriguez, Eva V., Giorgio Napolitani, Antonio Lanzavecchia, and Federica Sallusto. 2007. “Interleukins 1beta and 6 but Not Transforming Growth Factor-Beta Are Essential for the Differentiation of Interleukin 17-Producing Human T Helper Cells.” *Nature Immunology* 8 (9): 942–49. doi:10.1038/ni1496.

Aggarwal, Sudeepta, Nico Ghilardi, Ming-Hong Xie, Frederic J. de Sauvage, and Austin L. Gurney. 2003. “Interleukin-23 Promotes a Distinct CD4 T Cell Activation State Characterized by the Production of Interleukin-17.” *Journal of Biological Chemistry* 278 (3): 1910–14. doi:10.1074/jbc.M207577200.

Akagawa, Kiyoko S., Iwao Komuro, Hiroko Kanazawa, Toshio Yamazaki, Keiko Mochida, and Fumio Kishi. 2006. “Functional Heterogeneity of Colony-Stimulating Factor-Induced Human Monocyte-Derived Macrophages.” *Respirology* 11 (January): S32–36. doi:10.1111/j.1440-1843.2006.00805.x.

Alderson, Mark, Teresa Tough, Steven F. Ziegler, and Kenneth Grabstein. 1991. “Interleukin 7 Induces Cytokine Secretion and Tumoricidal Activity by Human Peripheral Blood Monocytes.” *The Journal of Experimental Medicine* 173 (4): 923–30.

Allen, Rachel L., Chris A. O’Callaghan, Andrew J. McMichael, and Paul Bowness. 1999. “Cutting Edge: HLA-B27 Can Form a Novel β2-Microglobulin-Free Heavy Chain Homodimer Structure.” *The Journal of Immunology* 162 (9): 5045–48.

Al-Shami, Amin, Rosanne Spolski, John Kelly, Terry Fry, Pamela L. Schwartzberg, Akhilesh Pandey, Crystal L. Mackall, and Warren J. Leonard. 2004. “A Role for Thymic Stromal Lymphopoietin in CD4+ T Cell Development.” *The Journal of Experimental Medicine* 200 (2): 159–68. doi:10.1084/jem.20031975.

Amadi-Obi, Ahjoku, Cheng-Rong Yu, Xuebin Liu, Rashid M. Mahdi, Grace Levy Clarke, Robert B. Nussenblatt, Igal Gery, Yun Sang Lee, and Charles E. Egwuagu. 2007. “TH17 Cells Contribute to Uveitis and Scleritis and Are Expanded by IL-2 and Inhibited by IL-27/STAT1.” *Nature Medicine* 13 (6): 711–18. doi:10.1038/nm1585.

Amir, El-ad David, Kara L. Davis, Michelle D. Tadmor, Erin F. Simonds, Jacob H. Levine, Sean C. Bendall, Daniel K. Shenfeld, Smita Krishnaswamy, Garry P. Nolan, and Dana Pe’er. 2013. “viSNE Enables Visualization of High Dimensional Single-Cell Data and Reveals Phenotypic Heterogeneity of Leukemia.” *Nature Biotechnology* 31 (6): 545–52. doi:10.1038/nbt.2594.

Anders, Simon, Paul Theodor Pyl, and Wolfgang Huber. 2015. “HTSeq--a Python Framework to Work with High-Throughput Sequencing Data.” *Bioinformatics (Oxford, England)* 31 (2): 166–69. doi:10.1093/bioinformatics/btu638.

Andrianakos, Alexandros, Panagiotis Trontzas, Fotis Christoyannis, Petros Dantis, Costas Voudouris, Athanasios Georgountzos, George Kaziolas, et al. 2003.

"Prevalence of Rheumatic Diseases in Greece: A Cross-Sectional Population Based Epidemiological Study. The ESORDIG Study." *The Journal of Rheumatology* 30 (7): 1589–1601.

Annunziato, F., L. Cosmi, F. Liotta, E. Maggi, and S. Romagnani. 2008. "The Phenotype of Human Th17 Cells and Their Precursors, the Cytokines That Mediate Their Differentiation and the Role of Th17 Cells in Inflammation." *International Immunology* 20 (11): 1361–68. doi:10.1093/intimm/dxn106.

Annunziato, Francesco, Lorenzo Cosmi, Francesco Liotta, Enrico Maggi, and Sergio Romagnani. 2008. "The Phenotype of Human Th17 Cells and Their Precursors, the Cytokines That Mediate Their Differentiation and the Role of Th17 Cells in Inflammation." *International Immunology* 20 (11): 1361–68. doi:10.1093/intimm/dxn106.

Appel, Heiner, Wolfgang Kuon, Maren Kuhne, Peihua Wu, Stefanie Kuhlmann, Simon Kollnberger, Andreas Thiel, Paul Bowness, and Joachim Sieper. 2004. "Use of HLA-B27 Tetramers to Identify Low-Frequency Antigen-Specific T Cells in Chlamydia-Triggered Reactive Arthritis." *Arthritis Research & Therapy* 6 (6): R521. doi:10.1186/ar1221.

Artis, David, and Hergen Spits. 2015. "The Biology of Innate Lymphoid Cells." *Nature* 517 (7534): 293–301. doi:10.1038/nature14189.

Ayyoub, Maha, Caroline Raffin, and Danila Valmori. 2012. "Generation of Th17 from Human Naive CD4+ T Cells Preferentially Occurs from FOXP3+ Tregs upon Costimulation via CD28 or CD5." *Blood* 119 (20): 4810–12. doi:10.1182/blood-2012-02-409722.

Baeten, Dominique, Xenofon Baraliakos, Jürgen Braun, Joachim Sieper, Paul Emery, Désirée van der Heijde, Iain McInnes, et al. 2013. "Anti-Interleukin-17A Monoclonal Antibody Secukinumab in Treatment of Ankylosing Spondylitis: A Randomised, Double-Blind, Placebo-Controlled Trial." *The Lancet* 382 (9906): 1705–13. doi:10.1016/S0140-6736(13)61134-4.

Baeten, Dominique, Joachim Sieper, Jürgen Braun, Xenofon Baraliakos, Maxime Dougados, Paul Emery, Atul Deodhar, et al. 2015. "Secukinumab, an Interleukin-17A Inhibitor, in Ankylosing Spondylitis." *New England Journal of Medicine* 373 (26): 2534–48. doi:10.1056/NEJMoa1505066.

Battistone, M. J., B. J. Manaster, Domenic J. Reda, and Daniel O. Clegg. 1999. "The Prevalence of Sacroiliitis in Psoriatic Arthritis: New Perspectives from a Large, Multicenter Cohort." *Skeletal Radiology* 28 (4): 196–201. doi:10.1007/s002560050500.

Behrens, Frank, Paul P. Tak, Mikkel Østergaard, Rumen Stoilov, Piotr Wiland, Thomas W. Huizinga, Vadym Y. Berenfus, et al. 2014. "MOR103, a Human Monoclonal Antibody to Granulocyte–macrophage Colony-Stimulating Factor, in the Treatment of Patients with Moderate Rheumatoid Arthritis: Results of a Phase Ib/IIa Randomised, Double-Blind, Placebo-Controlled, Dose-Escalation Trial." *Annals of the Rheumatic Diseases*, February, annrheumdis-2013-204816. doi:10.1136/annrheumdis-2013-204816.

Bendall, Sean C., Erin F. Simonds, Peng Qiu, El-ad D. Amir, Peter O. Krutzik, Rachel Finck, Robert V. Bruggner, et al. 2011. "Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum." *Science* 332 (6030): 687–96. doi:10.1126/science.1198704.

Bettelli, E. 2006. “Reciprocal Developmental Pathways for the Generation of Pathogenic Effector TH17 and Regulatory T Cells.” *Nature* 441: 235–38. doi:10.1038/nature04753.

Biondo, Mark, Zeyad Nasa, Aiden Marshall, Ban Hock Toh, and Frank Alderuccio. 2001. “Local Transgenic Expression of Granulocyte Macrophage-Colony Stimulating Factor Initiates Autoimmunity.” *The Journal of Immunology* 166 (3): 2090–99. doi:10.4049/jimmunol.166.3.2090.

Bird, Lucy A., Chen Au Peh, Simon Kollnberger, Tim Elliott, Andrew J. McMichael, and Paul Bowness. 2003. “Lymphoblastoid Cells Express HLA-B27 Homodimers Both Intracellularly and at the Cell Surface Following Endosomal Recycling.” *European Journal of Immunology* 33 (3): 748–59. doi:10.1002/eji.200323678.

Bluestone, Jeffrey A., Charles R. Mackay, John J. O’Shea, and Brigitta Stockinger. 2009. “The Functional Plasticity of T Cell Subsets.” *Nature Reviews Immunology* 9 (11): 811–16. doi:10.1038/nri2654.

Bowes, John, Ashley Budu-Aggrey, Ulrike Huffmeier, Steffen Uebe, Kathryn Steel, Harry L. Hebert, Chris Wallace, et al. 2015. “Dense Genotyping of Immune-Related Susceptibility Loci Reveals New Insights into the Genetics of Psoriatic Arthritis.” *Nature Communications* 6 (February): 6046. doi:10.1038/ncomms7046.

Bowness, Paul. 2015. “Hla-B27.” *Annual Review of Immunology* 33 (1): 29–48. doi:10.1146/annurev-immunol-032414-112110.

Bowness, Paul, Anna Ridley, Jacqueline Shaw, Antoni T. Chan, Isabel Wong-Baeza, Myles Fleming, Fraser Cummings, Andrew McMichael, and Simon Kollnberger. 2011. “Th17 Cells Expressing KIR3DL2+ and Responsive to HLA-B27 Homodimers Are Increased in Ankylosing Spondylitis.” *The Journal of Immunology* 186 (4): 2672–80. doi:10.4049/jimmunol.1002653.

Bradley, Linda M., Laura Haynes, and Susan L. Swain. 2005. “IL-7: Maintaining T-Cell Memory and Achieving Homeostasis.” *Trends in Immunology* 26 (3): 172–76. doi:10.1016/j.it.2005.01.004.

Brakenhoff, Lianne K. P. M., Désirée M. van der Heijde, Daniel W. Hommes, Tom W. J. Huizinga, and Herma H. Fidder. 2010. “The Joint–gut Axis in Inflammatory Bowel Diseases.” *Journal of Crohn’s and Colitis* 4 (3): 257–68. doi:10.1016/j.crohns.2009.11.005.

Braun, J., R. van den Berg, X. Baraliakos, H. Boehm, R. Burgos-Vargas, E. Collantes-Estevez, H. Dagfinrud, et al. 2011. “2010 Update of the ASAS/EULAR Recommendations for the Management of Ankylosing Spondylitis.” *Annals of the Rheumatic Diseases* 70 (6): 896–904. doi:10.1136/ard.2011.151027.

Braun, J., J. Listing, and J. Sieper. 2005. “Reply.” *Arthritis & Rheumatism* 52 (12): 4049–50. doi:10.1002/art.21609.

Braun, Juergen, and Joachim Sieper. 2002. “Therapy of Ankylosing Spondylitis and Other Spondyloarthritides: Established Medical Treatment, Anti-TNF-α Therapy and Other Novel Approaches.” *Arthritis Research & Therapy* 4: 307. doi:10.1186/ar592.

Braun, Jürgen, Mathias Bollow, Gerold Remlinger, Ulrich Eggens, Martin Rudwaleit, Armin Distler, and Joachim Sieper. 1998. “Prevalence of Spondylarthropathies in HLA-B27 Positive and Negative Blood Donors.”

Arthritis & Rheumatism 41 (1): 58–67. doi:10.1002/1529-0131(199801)41:1<58::AID-ART8>3.0.CO;2-G.

Braun, JüRgen, Matthias Bollow, Lucia Neure, Eva Seipelt, Fikret Seyrekbasan, Hermann Herbst, Ulrich Eggens, Armin Distler, and Jochen Sieper. 1995. "Use of Immunohistologic and in Situ Hybridization Techniques in the Examination of Sacroiliac Joint Biopsy Specimens from Patients with Ankylosing Spondylitis." *Arthritis & Rheumatism* 38 (4): 499–505. doi:10.1002/art.1780380407.

Broughton, Sophie E., Tracy L. Nero, Urmi Dhagat, Winnie L. Kan, Timothy R. Hercus, Denis Tvorogov, Angel F. Lopez, and Michael W. Parker. 2015. "The Bc Receptor Family – Structural Insights and Their Functional Implications." *Cytokine*, ICIS Awardee Reviews, 74 (2): 247–58. doi:10.1016/j.cyto.2015.02.005.

Bruges-Armas, J., C. Lima, M. J. Peixoto, P. Santos, D. Mendonça, B. Martins Da Silva, G. Herrero-Beaumont, and A. Calin. 2002. "Prevalence of Spondyloarthritis in Terceira, Azores: A Population Based Study." *Annals of the Rheumatic Diseases* 61 (6): 551–53. doi:10.1136/ard.61.6.551.

Brugnera, Enrico, Avinash Bhandoola, Ricardo Cibotti, Qing Yu, Terry I Guinter, Yoshio Yamashita, Susan O Sharrow, and Alfred Singer. 2000. "Coreceptor Reversal in the Thymus: Signaled CD4+8+ Thymocytes Initially Terminate CD8 Transcription Even When Differentiating into CD8+ T Cells." *Immunity* 13 (1): 59–71. doi:10.1016/S1074-7613(00)00008-X.

Buonocore, Sofia, Philip P. Ahern, Holm H. Uhlig, Ivaylo I. Ivanov, Dan R. Littman, Kevin J. Maloy, and Fiona Powrie. 2010. "Innate Lymphoid Cells Drive Interleukin-23-Dependent Innate Intestinal Pathology." *Nature* 464 (7293): 1371–75. doi:10.1038/nature08949.

Burmester, Gerd R., Michael E. Weinblatt, Iain B. McInnes, Duncan Porter, Olga Barbarash, Mykola Vatutin, Istvan Szombati, et al. 2013. "Efficacy and Safety of Mavrilimumab in Subjects with Rheumatoid Arthritis." *Annals of the Rheumatic Diseases* 72 (9): 1445–52. doi:10.1136/annrheumdis-2012-202450.

Burton, P.R., D.G. Clayton, L.R. Cardon, N. Craddock, P. Deloukas, A. Duncanson, D.P. Kwiatkowski, et al. 2007. "Association Scan of 14,500 Nonsynonymous SNPs in Four Diseases Identifies Autoimmunity Variants." *Nature Genetics* 39 (11): 1329–37. doi:10.1038/ng.2007.17.

Campbell, Ian K., Annemarie van Nieuwenhuijze, Elodie Segura, Kristy O'Donnell, Elise Coghill, Mirja Hommel, Steve Gerondakis, José A. Villadangos, and Ian P. Wicks. 2011. "Differentiation of Inflammatory Dendritic Cells Is Mediated by NF-κB1–Dependent GM-CSF Production in CD4 T Cells." *The Journal of Immunology* 186 (9): 5468–77. doi:10.4049/jimmunol.1002923.

Carpino, Nick, William E. Thierfelder, Ming-shi Chang, Chris Saris, Steven J. Turner, Steven F. Ziegler, and James N. Ihle. 2004. "Absence of an Essential Role for Thymic Stromal Lymphopoietin Receptor in Murine B-Cell Development." *Molecular and Cellular Biology* 24 (6): 2584–92. doi:10.1128/MCB.24.6.2584-2592.2004.

Caux, C., B. Vanbervliet, C. Massacrier, C. Dezutter-Dambuyant, B. de Saint-Vis, C. Jacquet, K. Yoneda, S. Imamura, D. Schmitt, and J. Banchereau. 1996. "CD34+

Hematopoietic Progenitors from Human Cord Blood Differentiate along Two Independent Dendritic Cell Pathways in Response to GM-CSF+TNF Alpha." *The Journal of Experimental Medicine* 184 (2): 695–706. doi:10.1084/jem.184.2.695.

Cella, Marina, Anja Fuchs, William Vermi, Fabio Facchetti, Karel Otero, Jochen K. M. Lennerz, Jason M. Doherty, Jason C. Mills, and Marco Colonna. 2008. "A Human Natural Killer Cell Subset Provides an Innate Source of IL-22 for Mucosal Immunity." *Nature* 457 (7230): 722–25. doi:10.1038/nature07537.

Chan, A. T., S. D. Kollnberger, L. R. Wedderburn, and P. Bowness. 2005. "Expansion and Enhanced Survival of Natural Killer Cells Expressing the Killer Immunoglobulin-like Receptor KIR3DL2 in Spondylarthritis." *Arthritis & Rheumatism* 52 (11): 3586–3595. doi:10.1002/art.21395.

Chang, Mi Ra, Brent Lyda, Theodore M. Kamenecka, and Patrick R. Griffin. 2014. "Pharmacologic Repression of Retinoic Acid Receptor-Related Orphan Nuclear Receptor γ Is Therapeutic in the Collagen-Induced Arthritis Experimental Model." *Arthritis & Rheumatology (Hoboken, N.J.)* 66 (3): 579–88. doi:10.1002/art.38272.

Chang, Ya-Jen, Hye Young Kim, Lee A. Albacker, Nicole Baumgarth, Andrew N. J. McKenzie, Dirk E. Smith, Rosemarie H. DeKruyff, and Dale T. Umetsu. 2011. "Innate Lymphoid Cells Mediate Influenza-Induced Airway Hyper-Reactivity Independently of Adaptive Immunity." *Nature Immunology* 12 (7): 631–38. doi:10.1038/ni.2045.

Chen, Liye, Roman Fischer, Yanchun Peng, Emma Reeves, Kirsty McHugh, Nicola Ternette, Tomas Hanke, et al. 2014. "Critical Role of Endoplasmic Reticulum Aminopeptidase 1 in Determining the Length and Sequence of Peptides Bound and Presented by HLA–B27." *Arthritis & Rheumatology* 66 (2): 284–94. doi:10.1002/art.38249.

Cho, Mi-La, Jung-Won Kang, Young-Mee Moon, Hyo-Jung Nam, Joo-Yeon Jhun, Seong-Beom Heo, Hyun-Tak Jin, et al. 2006. "STAT3 and NF-κB Signal Pathway Is Required for IL-23-Mediated IL-17 Production in Spontaneous Arthritis Animal Model IL-1 Receptor Antagonist-Deficient Mice." *The Journal of Immunology* 176 (9): 5652–61. doi:10.4049/jimmunol.176.9.5652.

Ciccia, Francesco, Antonina Accardo-Palumbo, Aroldo Rizzo, Giuliana Guggino, Stefania Raimondo, AnnaRita Giardina, Alessandra Cannizzaro, Robert A. Colbert, Riccardo Alessandro, and Giovanni Triolo. 2014. "Evidence That Autophagy, but Not the Unfolded Protein Response, Regulates the Expression of IL-23 in the Gut of Patients with Ankylosing Spondylitis and Subclinical Gut Inflammation." *Annals of the Rheumatic Diseases* 73 (8): 1566–74. doi:10.1136/annrheumdis-2012-202925.

Ciccia, Francesco, Giuliana Guggino, Aroldo Rizzo, Laura Saieva, Sergio Peralta, AnnaRita Giardina, Alessandra Cannizzaro, et al. 2015. "Type 3 Innate Lymphoid Cells Producing IL-17 and IL-22 Are Expanded in the Gut, in the Peripheral Blood, Synovial Fluid and Bone Marrow of Patients with Ankylosing Spondylitis." *Annals of the Rheumatic Diseases*, April, annrheumdis-2014-206323. doi:10.1136/annrheumdis-2014-206323.

Clegg, Daniel O., Domenic J. Reda, and Mazen Abdellatif. 1999. "Comparison of Sulfasalazine and Placebo for the Treatment of Axial and Peripheral Articular

Manifestations of the Seronegative Spondylarthropathies: A Department of Veterans Affairs Cooperative Study." *Arthritis & Rheumatism* 42 (11): 2325–29. doi:10.1002/1529-0131(199911)42:11<2325::AID-ANR10>3.0.CO;2-C.

Codarri, Laura, Gabor Gyülvészi, Vinko Tosevski, Lysann Hesske, Adriano Fontana, Laurent Magnenat, Tobias Suter, and Burkhard Becher. 2011. "RORγt Drives Production of the Cytokine GM-CSF in Helper T Cells, Which Is Essential for the Effector Phase of Autoimmune Neuroinflammation." *Nature Immunology* 12 (6): 560–67. doi:10.1038/ni.2027.

Collamer, Angelique N., and Daniel F. Battafarano. 2010. "Psoriatic Skin Lesions Induced by Tumor Necrosis Factor Antagonist Therapy: Clinical Features and Possible Immunopathogenesis." *Seminars in Arthritis and Rheumatism* 40 (3): 233–40. doi:10.1016/j.semarthrit.2010.04.003.

Cook, Andrew D, Emma L Braine, Ian K Campbell, Melissa J Rich, and John A Hamilton. 2001. "Blockade of Collagen-Induced Arthritis Post-Onset by Antibody to Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF): Requirement for GM-CSF in the Effector Phase of Disease." *Arthritis Research* 3 (5): 293–98.

Cornish, Ann L., Ian K. Campbell, Brent S. McKenzie, Simon Chatfield, and Ian P. Wicks. 2009. "G-CSF and GM-CSF as Therapeutic Targets in Rheumatoid Arthritis." *Nature Reviews Rheumatology* 5 (10): 554–59. doi:10.1038/nrrheum.2009.178.

Cortes, Adrian, Johanna Hadler, Jenny P Pointon, Philip C Robinson, Tugce Karaderi, Paul Leo, Katie Cremin, et al. 2013. "Identification of Multiple Risk Variants for Ankylosing Spondylitis through High-Density Genotyping of Immune-Related Loci." *Nature Genetics* 45 (7): 730–38. doi:10.1038/ng.2667.

Cosmi, Lorenzo, Raffaele De Palma, Veronica Santarlasci, Laura Maggi, Manuela Capone, Francesca Frosali, Gabriella Rodolico, et al. 2008. "Human Interleukin 17–producing Cells Originate from a CD161+CD4+ T Cell Precursor." *The Journal of Experimental Medicine* 205 (8): 1903–16. doi:10.1084/jem.20080397.

Costello, M.-E., F. Ciccia, D. Willner, N. Warrington, P.C. Robinson, B. Gardiner, M. Marshall, T.J. Kenna, G. Triolo, and M.A. Brown. 2015. "Brief Report: Intestinal Dysbiosis in Ankylosing Spondylitis." *Arthritis and Rheumatology* 67 (3): 686–91. doi:10.1002/art.38967.

Cowburn, Andrew S., Charlotte Summers, Benjamin J. Dunmore, Neda Farahi, Richard P. Hayhoe, Cristin G. Print, Simon J. Cook, and Edwin R. Chilvers. 2011. "Granulocyte/Macrophage Colony–Stimulating Factor Causes a Paradoxical Increase in the BH3-Only Pro-Apoptotic Protein Bim in Human Neutrophils." *American Journal of Respiratory Cell and Molecular Biology* 44 (6): 879–87. doi:10.1165/rcmb.2010-0101OC.

Cua, Daniel J., Jonathan Sherlock, Yi Chen, Craig A. Murphy, Barbara Joyce, Brian Seymour, Linda Lucian, et al. 2003. "Interleukin-23 rather than Interleukin-12 Is the Critical Cytokine for Autoimmune Inflammation of the Brain." *Nature* 421 (6924): 744–48. doi:10.1038/nature01355.

Cua, Daniel J., and Cristina M. Tato. 2010. "Innate IL-17-Producing Cells: The Sentinels of the Immune System." *Nat Rev Immunol* 10 (7): 479–89. doi:10.1038/nri2800.

Cupedo, Tom, Natasha K. Crellin, Natalie Papazian, Elwin J. Rombouts, Kees Weijer, Jane L. Grogan, Willem E. Fibbe, Jan J. Cornelissen, and Hergen Spits. 2009. "Human Fetal Lymphoid Tissue–inducer Cells Are Interleukin 17–producing Precursors to RORC+ CD127+ Natural Killer–like Cells." *Nature Immunology* 10 (1): 66–74. doi:10.1038/ni.1668.

Curran, Colleen S., and Paul J. Bertics. 2012. "Lactoferrin Regulates an Axis Involving CD11b and CD49d Integrins and the Chemokines MIP-1α and MCP-1 in GM-CSF-Treated Human Primary Eosinophils." *Journal of Interferon & Cytokine Research* 32 (10): 450–61. doi:10.1089/jir.2011.0111.

Dai, Xu-Ming, Gregory R. Ryan, Andrew J. Hapel, Melissa G. Dominguez, Robert G. Russell, Sara Kapp, Vonetta Sylvestre, and E. Richard Stanley. 2002. "Targeted Disruption of the Mouse Colony-Stimulating Factor 1 Receptor Gene Results in Osteopetrosis, Mononuclear Phagocyte Deficiency, Increased Primitive Progenitor Cell Frequencies, and Reproductive Defects." *Blood* 99 (1): 111–20. doi:10.1182/blood.V99.1.111.

Danoy, Patrick, Karena Pryce, Johanna Hadler, Linda A. Bradbury, Claire Farrar, Jennifer Pointon, Michael Ward, et al. 2010a. "Association of Variants at 1q32 and STAT3 with Ankylosing Spondylitis Suggests Genetic Overlap with Crohn's Disease." *PLoS Genet* 6 (12): e1001195. doi:10.1371/journal.pgen.1001195.

———. 2010b. "Association of Variants at 1q32 and STAT3 with Ankylosing Spondylitis Suggests Genetic Overlap with Crohn's Disease." *PLoS Genet* 6 (12): e1001195. doi:10.1371/journal.pgen.1001195.

De Vos, M., C. Cuvelier, H. Mielants, E. Veys, F. Barbier, and A. Elewaut. 1989. "Ileocolonoscopy in Seronegative Spondylarthropathy." *Gastroenterology* 96 (2 Pt 1): 339–44.

de Wit, Jelle, M. Hussein Al-Mossawi, Michael H. Hühn, Carolina V. Arancibia-Cárcamo, Karen Doig, Benjamin Kendrick, Roger Gundle, et al. 2016. "RORγt Inhibitors Suppress TH17 Responses in Inflammatory Arthritis and Inflammatory Bowel Disease." *The Journal of Allergy and Clinical Immunology* 137 (3): 960–63. doi:10.1016/j.jaci.2015.09.048.

Dehghan, Abbas, Josée Dupuis, Maja Barbalic, Joshua C. Bis, Gudny Eiriksdottir, Chen Lu, Niina Pellikka, et al. 2011. "Meta-Analysis of Genome-Wide Association Studies in >80 000 Subjects Identifies Multiple Loci for C-Reactive Protein Levels." *Circulation* 123 (7): 731–38. doi:10.1161/CIRCULATIONAHA.110.948570.

DeLay, Monica L., Matthew J. Turner, Erin I. Klenk, Judith A. Smith, Dawn P. Sowders, and Robert A. Colbert. 2009. "HLA-B27 Misfolding and the Unfolded Protein Response Augment Interleukin-23 Production and Are Associated with Th17 Activation in Transgenic Rats." *Arthritis and Rheumatism* 60 (9): 2633–43. doi:10.1002/art.24763.

Dick, Andrew D., Ilknur Tugal-Tutkun, Stephen Foster, Manfred Zierhut, S. H. Melissa Liew, Vladimir Bezlyak, and Sofia Androudi. 2013. "Secukinumab in the Treatment of Noninfectious Uveitis: Results of Three Randomized, Controlled Clinical Trials." *Ophthalmology* 120 (4): 777–87. doi:10.1016/j.ophtha.2012.09.040.

Döhring, C., D. Scheidegger, J. Samaridis, M. Cella, and M. Colonna. 1996. “A Human Killer Inhibitory Receptor Specific for HLA-A1,2.” *Journal of Immunology (Baltimore, Md.: 1950)* 156 (9): 3098–3101.

Dougados, Maxime, Sjef VAN DER Linden, Marjatta Leirisalo-Repo, Bernhard Huitfeldt, Roger Juhlin, Eric Veys, Henning Zeidler, et al. 1995. “Sulfasalazine in the Treatment of Spondylarthropathy.” *Arthritis & Rheumatism* 38 (5): 618–27. doi:10.1002/art.1780380507.

Drancourt, Michel, Claude Bollet, Antoine Carlioz, Rolland Martelin, Jean-Pierre Gayral, and Didier Raoult. 2000. “16S Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates.” *Journal of Clinical Microbiology* 38 (10): 3623–30.

Duhen, Thomas, Rebekka Geiger, David Jarrossay, Antonio Lanzavecchia, and Federica Sallusto. 2009. “Production of Interleukin 22 but Not Interleukin 17 by a Subset of Human Skin-Homing Memory T Cells.” *Nature Immunology* 10 (8): 857–63. doi:10.1038/ni.1767.

Durum, Scott K., Serge Candèias, Hiroshi Nakajima, Warren J. Leonard, Allison M. Baird, Leslie J. Berg, and Kathrin Muegge. 1998. “Interleukin 7 Receptor Control of T Cell Receptor γ Gene Rearrangement: Role of Receptor-Associated Chains and Locus Accessibility.” *The Journal of Experimental Medicine* 188 (12): 2233–41. doi:10.1084/jem.188.12.2233.

El-Behi, Mohamed, Bogoljub Ciric, Hong Dai, Yaping Yan, Melissa Cullimore, Farinaz Safavi, Guang-Xian Zhang, Bonnie N. Dittel, and Abdolmohamad Rostami. 2011. “The Encephalitogenicity of TH17 Cells Is Dependent on IL-1- and IL-23-Induced Production of the Cytokine GM-CSF.” *Nature Immunology* 12 (6): 568–75. doi:10.1038/ni.2031.

Eliakim, R., A. Karban, D. Markovits, E. Bardan, S. Bar-Meir, D. Abramowich, and E. Scapa. 2005. “Comparison of Capsule Endoscopy with Ileocolonoscopy for Detecting Small-Bowel Lesions in Patients with Seronegative Spondyloarthropathies.” *Endoscopy* 37 (12): 1165–69. doi:10.1055/s-2005-870559.

Eriksen, N H, F Espersen, V T Rosdahl, and K Jensen. 1995. “Carriage of Staphylococcus Aureus among 104 Healthy Persons during a 19-Month Period.” *Epidemiol Infect.* 115(1) (August): 51–60.

Evans, David M., Chris C. A. Spencer, Jennifer J. Pointon, Zhan Su, David Harvey, Grazyna Kochan, Udo Oppermann, et al. 2011. “Interaction between ERAP1 and HLA-B27 in Ankylosing Spondylitis Implicates Peptide Handling in the Mechanism for HLA-B27 in Disease Susceptibility.” *Nature Genetics* 43 (8): 761–67. doi:10.1038/ng.873.

Evans, H. G., T. Suddason, I. Jackson, L. S. Taams, and G. M. Lord. 2007. “Optimal Induction of T Helper 17 Cells in Humans Requires T Cell Receptor Ligation in the Context of Toll-like Receptor-Activated Monocytes.” *Proc. Natl Acad. Sci. USA* 104: 17034–39. doi:10.1073/pnas.0708426104.

Eyre, Steve, John Bowes, Dorothée Diogo, Annette Lee, Anne Barton, Paul Martin, Alexandra Zhernakova, et al. 2012. “High-Density Genetic Mapping Identifies New Susceptibility Loci for Rheumatoid Arthritis.” *Nature Genetics* 44 (12): 1336–40. doi:10.1038/ng.2462.

Fairfax, Benjamin P., Peter Humburg, Seiko Makino, Vivek Naranbhai, Daniel Wong, Evelyn Lau, Luke Jostins, et al. 2014. “Innate Immune Activity Conditions the Effect of Regulatory Variants upon Monocyte Gene Expression.” *Science (New York, N.Y.)* 343 (6175): 1246949. doi:10.1126/science.1246949.

Feldmann, Marc. 2002. “Development of Anti-TNF Therapy for Rheumatoid Arthritis.” *Nature Reviews Immunology* 2 (5): 364–71. doi:10.1038/nri802.

Feldtkeller, Ernst, Muhammad Khan, Désirée van der Heijde, Sjef van der Linden, and Jürgen Braun. 2003. “Age at Disease Onset and Diagnosis Delay in HLA-B27 Negative vs. Positive Patients with Ankylosing Spondylitis.” *Rheumatology International* 23 (2): 61–66. doi:10.1007/s00296-002-0237-4.

Fleetwood, Andrew J., Hang Dinh, Andrew D. Cook, Paul J. Hertzog, and John A. Hamilton. 2009. “GM-CSF- and M-CSF-Dependent Macrophage Phenotypes Display Differential Dependence on Type I Interferon Signaling.” *Journal of Leukocyte Biology* 86 (2): 411–21. doi:10.1189/jlb.1108702.

Franchimont, Denis, Jérôme Galon, Melanie S. Vacchio, Samuel Fan, Roberta Visconti, David M. Frucht, Vincent Geenen, George P. Chrousos, Jonathan D. Ashwell, and John J. O’Shea. 2002. “Positive Effects of Glucocorticoids on T Cell Function by up-Regulation of IL-7 Receptor Alpha.” *Journal of Immunology (Baltimore, Md.: 1950)* 168 (5): 2212–18.

Fry, Terry J., and Crystal L. Mackall. 2005. “The Many Faces of IL-7: From Lymphopoiesis to Peripheral T Cell Maintenance.” *Journal of Immunology (Baltimore, Md.: 1950)* 174 (11): 6571–76.

Fry, Terry J., Marcin Moniuszko, Stephen Creekmore, Susan J. Donohue, Daniel C. Douek, Steven Giardina, Toby T. Hecht, et al. 2003. “IL-7 Therapy Dramatically Alters Peripheral T-Cell Homeostasis in Normal and SIV-Infected Nonhuman Primates.” *Blood* 101 (6): 2294–99. doi:10.1182/blood-2002-07-2297.

Fuchs, Anja, William Vermi, Jacob S. Lee, Silvia Lonardi, Susan Gilfillan, Rodney D. Newberry, Marina Cella, and Marco Colonna. 2013. “Intraepithelial Type 1 Innate Lymphoid Cells Are a Unique Subset of IL-12- and IL-15-Responsive IFN-γ-Producing Cells.” *Immunity* 38 (4): 769–81. doi:10.1016/j.immuni.2013.02.010.

Funderburg, Nicholas, Angel A. Luciano, Wei Jiang, Benigno Rodriguez, Scott F. Sieg, and Michael M. Lederman. 2008. “Toll-Like Receptor Ligands Induce Human T Cell Activation and Death, a Model for HIV Pathogenesis.” *PLoS ONE* 3 (4): e1915. doi:10.1371/journal.pone.0001915.

Gagliani, Nicola, Maria Carolina Amezcua Vesely, Andrea Iseppon, Leonie Brockmann, Hao Xu, Noah W. Palm, Marcel R. de Zoete, et al. 2015. “Th17 Cells Transdifferentiate into Regulatory T Cells during Resolution of Inflammation.” *Nature* 523 (7559): 221–25. doi:10.1038/nature14452.

Garrett, S, Jenkinson T, Kennedy Lg, Whitelock H, Gaisford P, and Calin A. 1994. “A New Approach to Defining Disease Status in Ankylosing Spondylitis: The Bath Ankylosing Spondylitis Disease Activity Index.” *The Journal of Rheumatology* 21 (12): 2286–91.

Gaublomme, Jellert T., Nir Yosef, Youjin Lee, Rona S. Gertner, Li V. Yang, Chuan Wu, Pier Paolo Pandolfi, et al. 2015. “Single-Cell Genomics Unveils Critical

Regulators of Th17 Cell Pathogenicity." *Cell* 163 (6): 1400–1412. doi:10.1016/j.cell.2015.11.009.

Gearing, D P, J A King, N M Gough, and N A Nicola. 1989. "Expression Cloning of a Receptor for Human Granulocyte-Macrophage Colony-Stimulating Factor." *The EMBO Journal* 8 (12): 3667–76.

Gellert, Martin. 2002. "V(D)J Recombination: RAG Proteins, Repair Factors, and Regulation." *Annual Review of Biochemistry* 71 (1): 101–32. doi:10.1146/annurev.biochem.71.090501.150203.

Genovese, Mark C., Patrick Durez, Hanno B. Richards, Jerzy Supronik, Eva Dokoupilova, Vadim Mazurov, Jacob A. Aelion, et al. 2013. "Efficacy and Safety of Secukinumab in Patients with Rheumatoid Arthritis: A Phase II, Dose-Finding, Double-Blind, Randomised, Placebo Controlled Study." *Annals of the Rheumatic Diseases* 72 (6): 863–69. doi:10.1136/annrheumdis-2012-201601.

Geremia, Alessandra, Carolina V Arancibia-Cárcamo, Myles P. P Fleming, Nigel Rust, Baljit Singh, Neil J Mortensen, Simon P. L Travis, and Fiona Powrie. 2011. "IL-23–responsive Innate Lymphoid Cells Are Increased in Inflammatory Bowel Disease." *The Journal of Experimental Medicine* 208 (6): 1127–33. doi:10.1084/jem.20101712.

Ghoreschi, Kamran, Arian Laurence, Xiang-Ping Yang, Cristina M. Tato, Mandy J. McGeachy, Joanne E. Konkel, Haydee L. Ramos, et al. 2010. "Generation of Pathogenic TH17 Cells in the Absence of TGF-[Bgr] Signalling." *Nature* 467 (7318): 967–71. doi:10.1038/nature09447.

Goodall, Jane C., Changxin Wu, Yongsheng Zhang, Louise McNeill, Lou Ellis, Vladimir Saudek, and J. S. Hill Gaston. 2010. "Endoplasmic Reticulum Stress-Induced Transcription Factor, CHOP, Is Crucial for Dendritic Cell IL-23 Expression." *Proceedings of the National Academy of Sciences of the United States of America* 107 (41): 17698–703. doi:10.1073/pnas.1011736107.

Gordon, Siamon, and Fernando O. Martinez. 2010. "Alternative Activation of Macrophages: Mechanism and Functions." *Immunity* 32 (5): 593–604. doi:10.1016/j.immuni.2010.05.007.

Gregory, Simon G., Silke Schmidt, Puneet Seth, Jorge R. Oksenberg, John Hart, Angela Prokop, Stacy J. Caillier, et al. 2007. "Interleukin 7 Receptor α Chain (IL7R) Shows Allelic and Functional Association with Multiple Sclerosis." *Nature Genetics* 39 (9): 1083–91. doi:10.1038/ng2103.

Greven, D. E. A., E. S. Cohen, D. M. Gerlag, J. Campbell, J. Woods, N. Davis, A. van Nieuwenhuijze, et al. 2014. "Preclinical Characterisation of the GM-CSF Receptor as a Therapeutic Target in Rheumatoid Arthritis." *Annals of the Rheumatic Diseases*, June, annrheumdis-2014-205234. doi:10.1136/annrheumdis-2014-205234.

Grice, Elizabeth A., and Julia A. Segre. 2012. "The Human Microbiome: Our Second Genome." *Annual Review of Genomics and Human Genetics* 13: 151–70. doi:10.1146/annurev-genom-090711-163814.

Griffiths, Christopher E.M., Bruce E. Strober, Peter van de Kerkhof, Vincent Ho, Roseanne Fidelus-Gort, Newman Yeilding, Cynthia Guzzo, et al. 2010. "Comparison of Ustekinumab and Etanercept for Moderate-to-Severe

Psoriasis." *New England Journal of Medicine* 362 (2): 118–28. doi:10.1056/NEJMoa0810652.

Griseri, Thibault, Isabelle C. Arnold, Claire Pearson, Thomas Krausgruber, Chris Schiering, Fanny Franchini, Julie Schulthess, Brent S. McKenzie, Paul R. Crocker, and Fiona Powrie. 2015. "Granulocyte Macrophage Colony-Stimulating Factor-Activated Eosinophils Promote Interleukin-23 Driven Chronic Colitis." *Immunity* 43 (1): 187–99. doi:10.1016/j.immuni.2015.07.008.

Guimond, Martin, Rachelle G. Veenstra, David J. Grindler, Hua Zhang, Yongzhi Cui, Ryan D. Murphy, Su Young Kim, et al. 2009. "Interleukin 7 Signaling in Dendritic Cells Regulates the Homeostatic Proliferation and Niche Size of CD4+ T Cells." *Nature Immunology* 10 (2): 149–57. doi:10.1038/ni.1695.

Guthridge, M. A., and A. F. Lopez. 2007. "Phosphotyrosine/phosphoserine Binary Switches: A New Paradigm for the Regulation of PI3K Signalling and Growth Factor Pleiotropy?" *Biochemical Society Transactions* 35 (2): 250–52. doi:10.1042/BST0350250.

Hamilton, John A. 2002. "GM-CSF in Inflammation and Autoimmunity." *Trends in Immunology* 23 (8): 403–8. doi:10.1016/S1471-4906(02)02260-3.

Hamilton, John A., and Adrian Achuthan. 2013. "Colony Stimulating Factors and Myeloid Cell Biology in Health and Disease." *Trends in Immunology* 34 (2): 81–89. doi:10.1016/j.it.2012.08.006.

Harrington, Laurie E., Robin D. Hatton, Paul R. Mangan, Henrietta Turner, Theresa L. Murphy, Kenneth M. Murphy, and Casey T. Weaver. 2005. "Interleukin 17-Producing CD4+ Effector T Cells Develop via a Lineage Distinct from the T Helper Type 1 and 2 Lineages." *Nature Immunology* 6 (11): 1123–32. doi:10.1038/ni1254.

Hartgring, Sarita A. Y., Joel A. G. van Roon, Marion Wenting-van Wijk, Kim M. G. Jacobs, Zalima N. Jahangier, Cynthia R. Willis, Johannes W. J. Bijlsma, and Floris P. J. G. Lafeber. 2009. "Elevated Expression of Interleukin-7 Receptor in Inflamed Joints Mediates Interleukin-7-Induced Immune Activation in Rheumatoid Arthritis." *Arthritis and Rheumatism* 60 (9): 2595–2605. doi:10.1002/art.24754.

Hartmann, G, and A M Krieg. 1999. "CpG DNA and LPS Induce Distinct Patterns of Activation in Human Monocytes." *Gene Therapy* 6 (5): 893–903. doi:10.1038/sj.gt.3300880.

He, Y. W., and T. R. Malek. 1996. "Interleukin-7 Receptor Alpha Is Essential for the Development of Gamma Delta + T Cells, but Not Natural Killer Cells." *The Journal of Experimental Medicine* 184 (1): 289–93.

Henes, Joerg C., Marius Horger, Ilhan Guenaydin, Lothar Kanz, and Ina Koetter. 2010. "Mixed Response to Tocilizumab for Ankylosing Spondylitis." *Annals of the Rheumatic Diseases*, June, annrheumdis126706. doi:10.1136/ard.2009.126706.

Hirata, Yoshihiro, Laia Egea, Sara M. Dann, Lars Eckmann, and Martin F. Kagnoff. 2010. "GM-CSF-Facilitated Dendritic Cell Recruitment and Survival Govern the Intestinal Mucosal Response to a Mouse Enteric Bacterial Pathogen." *Cell Host & Microbe* 7 (2): 151–63. doi:10.1016/j.chom.2010.01.006.

Hogquist, Kristin A. 2001. “Signal Strength in Thymic Selection and Lineage Commitment.” *Current Opinion in Immunology* 13 (2): 225–31. doi:10.1016/S0952-7915(00)00208-9.

Huang, Pengxiang, Vikas Chandra, and Fraydoon Rastinejad. 2010. “Structural Overview of the Nuclear Receptor Superfamily: Insights into Physiology and Therapeutics.” *Annual Review of Physiology* 72 (1): 247–72. doi:10.1146/annurev-physiol-021909-135917.

Hueber, Wolfgang, Bruce E. Sands, Steve Lewitzky, Marc Vandemeulebroecke, Walter Reinisch, Peter D. R. Higgins, Jan Wehkamp, et al. 2012. “Secukinumab, a Human Anti-IL-17A Monoclonal Antibody, for Moderate to Severe Crohn’s Disease: Unexpected Results of a Randomised, Double-Blind Placebo-Controlled Trial.” *Gut*, May. doi:10.1136/gutjnl-2011-301668.

Huh, Jun R., Monica W. L. Leung, Pengxiang Huang, Daniel A. Ryan, Michael R. Krout, Raghu R. V. Malapaka, Jonathan Chow, et al. 2011. “Digoxin and Its Derivatives Suppress TH17 Cell Differentiation by Antagonizing RORγt Activity.” *Nature* 472 (7344): 486–90. doi:10.1038/nature09978.

Huh, Jun R., and Dan R. Littman. 2012. “Small Molecule Inhibitors of RORγt: Targeting Th17 Cells and Other Applications.” *European Journal of Immunology* 42 (9): 2232–37. doi:10.1002/eji.201242740.

Huster, Katharina M., Verena Busch, Matthias Schiemann, Kathrin Linkemann, Kristen M. Kerksiek, Hermann Wagner, and Dirk H. Busch. 2004. “Selective Expression of IL-7 Receptor on Memory T Cells Identifies Early CD40L-Dependent Generation of Distinct CD8+ Memory T Cell Subsets.” *Proceedings of the National Academy of Sciences of the United States of America* 101 (15): 5610–15. doi:10.1073/pnas.0308054101.

Ivanov, Ivaylo I., Brent S. McKenzie, Liang Zhou, Carlos E. Tadokoro, Alice Lepelley, Juan J. Lafaille, Daniel J. Cua, and Dan R. Littman. 2006. “The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells.” *Cell* 126 (6): 1121–33. doi:10.1016/j.cell.2006.07.035.

Jacques, P., and D. Elewaut. 2008. “Joint Expedition: Linking Gut Inflammation to Arthritis.” *Mucosal Immunology* 1 (5): 364–71. doi:10.1038/mi.2008.24.

Jain, Renu, Yi Chen, Yuka Kanno, Barbara Joyce-Shaikh, Golnaz Vahedi, Kiyoshi Hirahara, Wendy M. Blumenschein, et al. 2016. “Interleukin-23-Induced Transcription Factor Blimp-1 Promotes Pathogenicity of T Helper 17 Cells.” *Immunity* 44 (1): 131–42. doi:10.1016/j.immuni.2015.11.009.

Jandus, Camilla, Gilles Bioley, Jean-Paul Rivals, Jean Dudler, Daniel Speiser, and Pedro Romero. 2008. “Increased Numbers of Circulating Polyfunctional Th17 Memory Cells in Patients with Seronegative Spondylarthritides.” *Arthritis & Rheumatism* 58 (8): 2307–17. doi:10.1002/art.23655.

Jetten, Anton M. 2009. “Retinoid-Related Orphan Receptors (RORs): Critical Roles in Development, Immunity, Circadian Rhythm, and Cellular Metabolism.” *Nuclear Receptor Signaling* 7: e003. doi:10.1621/nrs.07003.

Joshi, Shweta, Alok R. Singh, Muamera Zulcic, Lei Bao, Karen Messer, Trey Ideker, Janusz Dutkowski, and Donald L. Durden. 2014. “Rac2 Controls Tumor Growth, Metastasis and M1-M2 Macrophage Differentiation In Vivo.” *PLoS ONE* 9 (4): e95893. doi:10.1371/journal.pone.0095893.

Kateete, David P, Cyrus N Kimani, Fred A Katabazi, Alfred Okeng, Moses S Okee, Ann Nanteza, Moses L Joloba, and Florence C Najjuka. 2010. “Identification of Staphylococcus Aureus: DNase and Mannitol Salt Agar Improve the Efficiency of the Tube Coagulase Test.” *Annals of Clinical Microbiology and Antimicrobials* 9 (August): 23. doi:10.1186/1476-0711-9-23.

Kenna, Tony J., Stuart I. Davidson, Ran Duan, Linda A. Bradbury, Janelle McFarlane, Malcolm Smith, Helen Weedon, et al. 2011. “Enrichment of Circulating IL-17-Secreting IL-23 Receptor-Positive Γδ T Cells in Patients with Active Ankylosing Spondylitis.” *Arthritis & Rheumatism*, n/a-n/a. doi:10.1002/art.33507.

Khameneh, Hanif Javanmard, Siti Aminah Bte Mohammad Isa, Lin Min, Fam Wee Nih, and Christiane Ruedl. 2011. “GM-CSF Signalling Boosts Dramatically IL-1Production.” *PLoS ONE* 6 (7): e23025. doi:10.1371/journal.pone.0023025.

Kiessling, R., Eva Klein, and H. Wigzell. 1975. “„Natural” Killer Cells in the Mouse. I. Cytotoxic Cells with Specificity for Mouse Moloney Leukemia Cells. Specificity and Distribution according to Genotype.” *European Journal of Immunology* 5 (2): 112–17. doi:10.1002/eji.1830050208.

Kim, Daehwan, Geo Pertea, Cole Trapnell, Harold Pimentel, Ryan Kelley, and Steven L. Salzberg. 2013. “TopHat2: Accurate Alignment of Transcriptomes in the Presence of Insertions, Deletions and Gene Fusions.” *Genome Biology* 14 (4): R36. doi:10.1186/gb-2013-14-4-r36.

King, Irah L., Mark A. Kroenke, and Benjamin M. Segal. 2010. “GM-CSF–dependent, CD103+ Dermal Dendritic Cells Play a Critical Role in Th Effector Cell Differentiation after Subcutaneous Immunization.” *The Journal of Experimental Medicine* 207 (5): 953–61. doi:10.1084/jem.20091844.

Kitamura, Takayuki, Naohiko Tanaka, Junichi Watanabe, Uchida, Shiro Kanegasaki, Yoshitsugu Yamada, and Koh Nakata. 1999. “Idiopathic Pulmonary Alveolar Proteinosis as an Autoimmune Disease with Neutralizing Antibody against Granulocyte/Macrophage Colony-Stimulating Factor.” *The Journal of Experimental Medicine* 190 (6): 875–80. doi:10.1084/jem.190.6.875.

Kleinewietfeld, Markus, Arndt Manzel, Jens Titze, Heda Kvakan, Nir Yosef, Ralf A. Linker, Dominik N. Muller, and David A. Hafler. 2013. “Sodium Chloride Drives Autoimmune Disease by the Induction of Pathogenic TH17 Cells.” *Nature* 496 (7446): 518–22. doi:10.1038/nature11868.

Kollnberger, Simon, Lucy Bird, Mei-Yi Sun, Christelle Retiere, Veronique M. Braud, Andrew McMichael, and Paul Bowness. 2002. “Cell-Surface Expression and Immune Receptor Recognition of HLA–B27 Homodimers.” *Arthritis & Rheumatism* 46 (11): 2972–2982. doi:10.1002/art.10605.

Komatsu, Noriko, Kazuo Okamoto, Shinichiro Sawa, Tomoki Nakashima, Masatsugu Oh-hora, Tatsuhiko Kodama, Sakae Tanaka, Jeffrey A. Bluestone, and Hiroshi Takayanagi. 2014. “Pathogenic Conversion of Foxp3+ T Cells into TH17 Cells in Autoimmune Arthritis.” *Nature Medicine* 20 (1): 62–68. doi:10.1038/nm.3432.

Kurebayashi, S., E. Ueda, M. Sakaue, D. D. Patel, A. Medvedev, F. Zhang, and A. M. Jetten. 2000. “Retinoid-Related Orphan Receptor Gamma (RORgamma) Is Essential for Lymphoid Organogenesis and Controls Apoptosis during Thymopoiesis.” *Proceedings of the National Academy of Sciences of the United States of America* 97 (18): 10132–37.

Laggner, U., P. Di Meglio, G. K. Perera, C. Hundhausen, K. E. Lacy, N. Ali, C. H. Smith, A. C. Hayday, B. J. Nickoloff, and F. O. Nestle. 2011. “Identification of a Novel Proinflammatory Human Skin-Homing Vgamma9Vdelta2 T Cell Subset with a Potential Role in Psoriasis.” *J Immunol* 187 (September): 2783–93. doi:10.4049/jimmunol.1100804.

Lang, Richard A., Donald Metcalf, R. Andrew Cuthbertson, Ian Lyons, Ed Stanley, Anne Kelso, George Kannourakis, et al. 1987. “Transgenic Mice Expressing a Hemopoietic Growth Factor Gene (GM-CSF) Develop Accumulations of Macrophages, Blindness, and a Fatal Syndrome of Tissue Damage.” *Cell* 51 (4): 675–86. doi:10.1016/0092-8674(87)90136-X.

Lanier, Lewis L. 2005. “Nk Cell Recognition.” *Annual Review of Immunology* 23 (1): 225–74. doi:10.1146/annurev.immunol.23.021704.115526.

Lee, Eun Bong, Roy Fleischmann, Stephen Hall, Bethanie Wilkinson, John D. Bradley, David Gruben, Tamas Koncz, et al. 2014. “Tofacitinib versus Methotrexate in Rheumatoid Arthritis.” *New England Journal of Medicine* 370 (25): 2377–86. doi:10.1056/NEJMoa1310476.

Lee, R. W. J., and A. D. Dick. 2012. “Current Concepts and Future Directions in the Pathogenesis and Treatment of Non-Infectious Intraocular Inflammation.” *Eye* 26 (1): 17–28. doi:10.1038/eye.2011.255.

Lee, Youjin, Amit Awasthi, Nir Yosef, Francisco J. Quintana, Sheng Xiao, Anneli Peters, Chuan Wu, et al. 2012. “Induction and Molecular Signature of Pathogenic TH17 Cells.” *Nature Immunology*. doi:10.1038/ni.2416.

Leijten, Emmerik F. A., Tessa S. van Kempen, Marianne Boes, Jocea M. R. Michels-van Amelsfort, Dirkjan Hijnen, Sarita A. Y. Hartgring, Joel A. G. van Roon, Mark H. Wenink, and Timothy R. D. J. Radstake. 2015. “Brief Report: Enrichment of Activated Group 3 Innate Lymphoid Cells in Psoriatic Arthritis Synovial Fluid.” *Arthritis & Rheumatology* 67 (10): 2673–78. doi:10.1002/art.39261.

Leonard, Warren J. 2001. “Cytokines and Immunodeficiency Diseases.” *Nature Reviews Immunology* 1 (3): 200–208. doi:10.1038/35105066.

Levin, S. D., R. M. Koelling, S. L. Friend, D. E. Isaksen, S. F. Ziegler, R. M. Perlmutter, and A. G. Farr. 1999. “Thymic Stromal Lymphopoietin: A Cytokine That Promotes the Development of IgM+ B Cells in Vitro and Signals via a Novel Mechanism.” *Journal of Immunology (Baltimore, Md.: 1950)* 162 (2): 677–83.

Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, Richard Durbin, and 1000 Genome Project Data Processing Subgroup. 2009. “The Sequence Alignment/Map Format and SAMtools.” *Bioinformatics (Oxford, England)* 25 (16): 2078–79. doi:10.1093/bioinformatics/btp352.

Li, Wen Qing, Qiong Jiang, Eiman Aleem, Philipp Kaldis, Annette R. Khaled, and Scott K. Durum. 2006. “IL-7 Promotes T Cell Proliferation through Destabilization of p27Kip1.” *The Journal of Experimental Medicine* 203 (3): 573–82. doi:10.1084/jem.20051520.

Linden, Sjef Van Der, Hans A. Valkenburg, and Arnold Cats. 1984. “Evaluation of Diagnostic Criteria for Ankylosing Spondylitis.” *Arthritis & Rheumatism* 27 (4): 361–68. doi:10.1002/art.1780270401.

Lindqvist, Ulla R. C., Gerd-Marie Alenius, Tomas Husmark, Elke Theander, Gunilla Holmström, Per T. Larsson, and Psoriatic Arthritis Group of the Society for

Rheumatology. 2008. “The Swedish Early Psoriatic Arthritis Register-- 2-Year Followup: A Comparison with Early Rheumatoid Arthritis.” *The Journal of Rheumatology* 35 (4): 668–73.

Liu, L.y., H. Wang, J. J. Xenakis, and L. A. Spencer. 2015. “Notch Signaling Mediates Granulocyte-Macrophage Colony-Stimulating Factor Priming-Induced Transendothelial Migration of Human Eosinophils.” *Allergy* 70 (7): 805–12. doi:10.1111/all.12624.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. “Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2.” *Genome Biology* 15 (12): 550. doi:10.1186/s13059-014-0550-8.

Lukens, John R., Maggie J. Barr, David D. Chaplin, Hongbo Chi, and Thirumala-Devi Kanneganti. 2012. “Inflammasome-Derived IL-1β Regulates the Production of GM-CSF by CD4+ T Cells and Γδ T Cells.” *The Journal of Immunology* 188 (7): 3107–15. doi:10.4049/jimmunol.1103308.

Lundin, J., A. Porwit-MacDonald, E. D. Rossmann, C. Karlsson, P. Edman, M. R. Rezvany, E. Kimby, A. Österborg, and H. Mellstedt. 2004. “Cellular Immune Reconstitution after Subcutaneous Alemtuzumab (Anti-CD52 Monoclonal Antibody, CAMPATH-1H) Treatment as First-Line Therapy for B-Cell Chronic Lymphocytic Leukaemia.” *Leukemia* 18 (3): 484–90. doi:10.1038/sj.leu.2403258.

Ma, Cindy S., Gary Y. J. Chew, Nicholas Simpson, Archana Priyadarshi, Melanie Wong, Bodo Grimbacher, David A. Fulcher, Stuart G. Tangye, and Matthew C. Cook. 2008. “Deficiency of Th17 Cells in Hyper IgE Syndrome due to Mutations in STAT3.” *The Journal of Experimental Medicine* 205 (7): 1551–57. doi:10.1084/jem.20080218.

Machado, Pedro. 2013. “Editorial: Anti–Tumor Necrosis Factor and New Bone Formation in Ankylosing Spondylitis: The Controversy Continues.” *Arthritis & Rheumatism* 65 (10): 2537–40. doi:10.1002/art.38068.

Mackall, Crystal L., Terry J. Fry, and Ronald E. Gress. 2011. “Harnessing the Biology of IL-7 for Therapeutic Application.” *Nature Reviews Immunology* 11 (5): 330–42. doi:10.1038/nri2970.

Maggi, Laura, Veronica Santarlasci, Manuela Capone, Anna Peired, Francesca Frosali, Sarah Q. Crome, Valentina Querci, et al. 2010. “CD161 Is a Marker of All Human IL-17-Producing T-Cell Subsets and Is Induced by RORC.” *European Journal of Immunology* 40 (8): 2174–2181. doi:10.1002/eji.200940257.

Magri, Giuliana, Michio Miyajima, Sabrina Bascones, Arthur Mortha, Irene Puga, Linda Cassis, Carolina M. Barra, et al. 2014. “Innate Lymphoid Cells Integrate Stromal and Immunological Signals to Enhance Antibody Production by Splenic Marginal Zone B Cells.” *Nature Immunology* 15 (4): 354–64. doi:10.1038/ni.2830.

Maini, R. N., P. C. Taylor, J. Szechinski, K. Pavelka, J. Bröll, G. Balint, P. Emery, et al. 2006. “Double-Blind Randomized Controlled Clinical Trial of the Interleukin-6 Receptor Antagonist, Tocilizumab, in European Patients with Rheumatoid Arthritis Who Had an Incomplete Response to Methotrexate.” *Arthritis & Rheumatism* 54 (9): 2817–29. doi:10.1002/art.22033.

Manel, N., D. Unutmaz, and D. R. Littman. 2008. “The Differentiation of Human T(H)-17 Cells Requires Transforming Growth Factor-Beta and Induction of the Nuclear Receptor RORgamma.” *Nat. Immunol*, May.

Manel, Nicolas, Derya Unutmaz, and Dan R Littman. 2008. “The Differentiation of Human TH-17 Cells Requires Transforming Growth Factor-β and Induction of the Nuclear Receptor RORγt.” *Nature Immunology* 9 (6): 641–49. doi:10.1038/ni.1610.

Mangan, P. R. 2006. “Transforming Growth Factor-Beta Induces Development of the TH17 Lineage.” *Nature* 441: 231–34. doi:10.1038/nature04754.

Martinez, Fernando O., Siamon Gordon, Massimo Locati, and Alberto Mantovani. 2006. “Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression.” *The Journal of Immunology* 177 (10): 7303–11. doi:10.4049/jimmunol.177.10.7303.

Matsuguchi, Tetsuya, Michael B. Lilly, and Andrew S. Kraft. 1998. “Cytoplasmic Domains of the Human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptor β Chain (Hβc) Responsible for Human GM-CSF-Induced Myeloid Cell Differentiation.” *Journal of Biological Chemistry* 273 (31): 19411–18. doi:10.1074/jbc.273.31.19411.

Mazzucchelli, Renata, and Scott K. Durum. 2007. “Interleukin-7 Receptor Expression: Intelligent Design.” *Nature Reviews Immunology* 7 (2): 144–54. doi:10.1038/nri2023.

Mazzucchelli, Renata I., Agostino Riva, and Scott K. Durum. 2012. “The Human IL-7 Receptor Gene: Deletions, Polymorphisms and Mutations.” *Seminars in Immunology*, IL-7 in the Immune System: Development and Function, 24 (3): 225–30. doi:10.1016/j.smim.2012.02.007.

McGeachy, Mandy J., Kristian S. Bak-Jensen, Yi Chen, Cristina M. Tato, Wendy Blumenschein, Terrill McClanahan, and Daniel J. Cua. 2007. “TGF-β and IL-6 Drive the Production of IL-17 and IL-10 by T Cells and Restrain TH-17 Cell–mediated Pathology.” *Nature Immunology* 8 (12): 1390–97. doi:10.1038/ni1539.

McGeachy, Mandy J, Yi Chen, Cristina M Tato, Arian Laurence, Barbara Joyce-Shaikh, Wendy M Blumenschein, Terrill K McClanahan, John J O’Shea, and Daniel J Cua. 2009. “The Interleukin 23 Receptor Is Essential for the Terminal Differentiation of Interleukin 17-Producing Effector T Helper Cells in Vivo.” *Nat Immunol* 10 (3): 314–24. doi:10.1038/ni.1698.

McGonagle, Dennis, Wayne Gibbon, and Paul Emery. 1998. “Classification of Inflammatory Arthritis by Enthesitis.” *The Lancet* 352 (9134): 1137–40. doi:10.1016/S0140-6736(97)12004-9.

McInnes, Iain B, Arthur Kavanaugh, Alice B Gottlieb, Lluís Puig, Proton Rahman, Christopher Ritchlin, Carrie Brodmerkel, et al. 2013. “Efficacy and Safety of Ustekinumab in Patients with Active Psoriatic Arthritis: 1 Year Results of the Phase 3, Multicentre, Double-Blind, Placebo-Controlled PSUMMIT 1 Trial.” *The Lancet* 382 (9894): 780–89. doi:10.1016/S0140-6736(13)60594-2.

Mear, J. P., K. L. Schreiber, C. Münz, X. Zhu, S. Stevanović, H. G. Rammensee, S. L. Rowland-Jones, and R. A. Colbert. 1999. “Misfolding of HLA-B27 as a Result of Its B Pocket Suggests a Novel Mechanism for Its Role in Susceptibility to

Spondyloarthropathies." *Journal of Immunology (Baltimore, Md.: 1950)* 163 (12): 6665–70.

Mease, Philip J., Mark C. Genovese, Maria W. Greenwald, Christopher T. Ritchlin, André D. Beaulieu, Atul Deodhar, Richard Newmark, JingYuan Feng, Ngozi Erondu, and Ajay Nirula. 2014. "Brodalumab, an Anti-IL17RA Monoclonal Antibody, in Psoriatic Arthritis." *New England Journal of Medicine* 370 (24): 2295–2306. doi:10.1056/NEJMoa1315231.

Mebius, Reina E, Paul Rennert, and Irving L Weissman. 1997. "Developing Lymph Nodes Collect CD4+CD3− LTβ+ Cells That Can Differentiate to APC, NK Cells, and Follicular Cells but Not T or B Cells." *Immunity* 7 (4): 493–504. doi:10.1016/S1074-7613(00)80371-4.

Mells, George F., James A. B. Floyd, Katherine I. Morley, Heather J. Cordell, Christopher S. Franklin, So-Youn Shin, Michael A. Heneghan, et al. 2011. "Genome-Wide Association Study Identifies 12 New Susceptibility Loci for Primary Biliary Cirrhosis." *Nature Genetics* 43 (4): 329–32. doi:10.1038/ng.789.

Melton, Andrew C., Jennifer Melrose, Liisa Alajoki, Sylvie Privat, Hannah Cho, Naomi Brown, Ana Marija Plavec, et al. 2013. "Regulation of IL-17A Production Is Distinct from IL-17F in a Primary Human Cell Co-Culture Model of T Cell-Mediated B Cell Activation." *PLoS ONE* 8 (3): e58966. doi:10.1371/journal.pone.0058966.

Menon, Bina, Nicola J. Gullick, Gina J. Walter, Megha Rajasekhar, Toby Garrood, Hayley G. Evans, Leonie S. Taams, and Bruce W. Kirkham. 2014. "Interleukin-17+CD8+ T Cells Are Enriched in the Joints of Patients with Psoriatic Arthritis and Correlate with Disease Activity and Joint Damage Progression." *Arthritis & Rheumatology (Hoboken, N.J.)* 66 (5): 1272–81. doi:10.1002/art.38376.

Metcalf, Donald. 2008. "Hematopoietic Cytokines." *Blood* 111 (2): 485–91. doi:10.1182/blood-2007-03-079681.

Milner, Joshua D., Jason M. Brenchley, Arian Laurence, Alexandra F. Freeman, Brenna J. Hill, Kevin M. Elias, Yuka Kanno, et al. 2008. "Impaired T(H)17 Cell Differentiation in Subjects with Autosomal Dominant Hyper-IgE Syndrome." *Nature* 452 (7188): 773–76. doi:10.1038/nature06764.

Miossec, P., T. Korn, and V. K Kuchroo. 2009. "Interleukin-17 and Type 17 Helper T Cells." *The New England Journal of Medicine* 361 (9): 888.

Mjösberg, Jenny M., Sara Trifari, Natasha K. Crellin, Charlotte P. Peters, Cornelis M. van Drunen, Berber Piet, Wytske J. Fokkens, Tom Cupedo, and Hergen Spits. 2011. "Human IL-25- and IL-33-Responsive Type 2 Innate Lymphoid Cells Are Defined by Expression of CRTH2 and CD161." *Nature Immunology* 12 (11): 1055–62. doi:10.1038/ni.2104.

Monticelli, Laurel A., Gregory F. Sonnenberg, Michael C. Abt, Theresa Alenghat, Carly G. K. Ziegler, Travis A. Doering, Jill M. Angelosanto, et al. 2011. "Innate Lymphoid Cells Promote Lung-Tissue Homeostasis after Infection with Influenza Virus." *Nature Immunology* 12 (11): 1045–54. doi:10.1038/ni.2131.

Mortha, Arthur, Aleksey Chudnovskiy, Daigo Hashimoto, Milena Bogunovic, Sean P. Spencer, Yasmine Belkaid, and Miriam Merad. 2014. "Microbiota-Dependent Crosstalk between Macrophages and ILC3 Promotes Intestinal Homeostasis." *Science (New York, N.Y.)* 343 (6178): 1249288. doi:10.1126/science.1249288.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. "Two Types of Murine Helper T Cell Clone. I. Definition according to Profiles of Lymphokine Activities and Secreted Proteins." *Journal of Immunology (Baltimore, Md.: 1950)* 136 (7): 2348–57.

Murphy, Craig A., Claire L. Langrish, Yi Chen, Wendy Blumenschein, Terrill McClanahan, Robert A. Kastelein, Jonathon D. Sedgwick, and Daniel J. Cua. 2003. "Divergent pro- and Antiinflammatory Roles for IL-23 and IL-12 in Joint Autoimmune Inflammation." *The Journal of Experimental Medicine* 198 (12): 1951–57. doi:10.1084/jem.20030896.

Murphy, Kenneth. 2011. *Janeway's Immunobiology*. 8th ed. http://www.garlandscience.com/product/isbn/9780815342434.

Naik, Shalin H., Donald Metcalf, Annemarie van Nieuwenhuijze, Ian Wicks, Li Wu, Meredith O'Keeffe, and Ken Shortman. 2006. "Intrasplenic Steady-State Dendritic Cell Precursors That Are Distinct from Monocytes." *Nature Immunology* 7 (6): 663–71. doi:10.1038/ni1340.

Nair, Rajan P., Kristina Callis Duffin, Cynthia Helms, Jun Ding, Philip E. Stuart, David Goldgar, Johann E. Gudjonsson, et al. 2009. "Genome-Wide Scan Reveals Association of Psoriasis with IL-23 and NF-κB Pathways." *Nature Genetics* 41 (2): 199–204. doi:10.1038/ng.311.

Namen, A. E., S. Lupton, K. Hjerrild, J. Wignall, D. Y. Mochizuki, A. Schmierer, B. Mosley, C. J. March, D. Urdal, and S. Gillis. 1988. "Stimulation of B-Cell Progenitors by Cloned Murine Interleukin-7." *Nature* 333 (6173): 571–73. doi:10.1038/333571a0.

Nistala, K., S. Adams, H. Cambrook, S. Ursu, B. Olivito, W. de Jager, J. G. Evans, R. Cimaz, M. Bajaj-Elliott, and L. R. Wedderburn. 2010. "Th17 Plasticity in Human Autoimmune Arthritis Is Driven by the Inflammatory Environment." *Proceedings of the National Academy of Sciences* 107 (33): 14751–56. doi:10.1073/pnas.1003852107.

Noster, Rebecca, René Riedel, Mir-Farzin Mashreghi, Helena Radbruch, Lutz Harms, Claudia Haftmann, Hyun-Dong Chang, Andreas Radbruch, and Christina E. Zielinski. 2014. "IL-17 and GM-CSF Expression Are Antagonistically Regulated by Human T Helper Cells." *Science Translational Medicine* 6 (241): 241ra80. doi:10.1126/scitranslmed.3008706.

Okuda, Keiko, Rosemary Foster, and James D. Griffin. 1999. "Signaling Domains of the Bc Chain of the GM-CSF/IL-3/IL-5 Receptor." *Annals of the New York Academy of Sciences* 872 (1): 305–13. doi:10.1111/j.1749-6632.1999.tb08474.x.

Oppmann, B. 2000. "Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12." *Immunity* 13: 715–25. doi:10.1016/S1074-7613(00)00070-4.

Orchard, T. R., S. Thiyagaraja, K. I. Welsh, B. P. Wordsworth, J. S. Hill Gaston, and D. P. Jewell. 2000. "Clinical Phenotype Is Related to HLA Genotype in the Peripheral Arthropathies of Inflammatory Bowel Disease." *Gastroenterology* 118 (2): 274–78.

Pallard, Caroline, Alexander P. A Stegmann, Titia van Kleffens, Fiona Smart, Ashok Venkitaraman, and Hergen Spits. 1999. "Distinct Roles of the Phosphatidylinositol 3-Kinase and STAT5 Pathways in IL-7-Mediated

Development of Human Thymocyte Precursors." *Immunity* 10 (5): 525–35. doi:10.1016/S1074-7613(00)80052-7.

Parham, C. 2002. "A Receptor for the Heterodimeric Cytokine IL-23 Is Composed of IL-12R[bgr]1 and a Novel Cytokine Receptor Subunit, IL-23R." *J. Immunol.* 168: 5699–5708.

Park, H. 2005. "A Distinct Lineage of CD4 T Cells Regulates Tissue Inflammation by Producing Interleukin 17." *Nature Immunol.* 6: 1133–41. doi:10.1038/ni1261.

Park, Jung-Hyun, Stanley Adoro, Terry Guinter, Batu Erman, Amala S. Alag, Marta Catalfamo, Motoko Y. Kimura, et al. 2010. "Signaling by Intrathymic Cytokines, Not T Cell Antigen Receptors, Specifies CD8 Lineage Choice and Promotes the Differentiation of Cytotoxic-Lineage T Cells." *Nature Immunology* 11 (3): 257–64. doi:10.1038/ni.1840.

Park, Jung-Hyun, Qing Yu, Batu Erman, Jacob S. Appelbaum, Diego Montoya-Durango, H. Leighton Grimes, and Alfred Singer. 2004. "Suppression of IL7Rα Transcription by IL-7 and Other Prosurvival Cytokines: A Novel Mechanism for Maximizing IL-7-Dependent T Cell Survival." *Immunity* 21 (2): 289–302. doi:10.1016/j.immuni.2004.07.016.

Pathan, Ejaz, Sonya Abraham, Elizabeth Van Rossen, Robin Withrington, Andrew Keat, Peter J. Charles, Erin Paterson, Muslima Chowdhury, Catherine McClinton, and Peter C. Taylor. 2012. "Efficacy and Safety of Apremilast, an Oral Phosphodiesterase 4 Inhibitor, in Ankylosing Spondylitis." *Annals of the Rheumatic Diseases*, September. doi:10.1136/annrheumdis-2012-201915.

Pesenacker, Anne M., David Bending, Simona Ursu, Qiong Wu, Kiran Nistala, and Lucy R. Wedderburn. 2013. "CD161 Defines the Subset of FoxP3+ T Cells Capable of Producing Proinflammatory Cytokines." *Blood* 121 (14): 2647–58. doi:10.1182/blood-2012-08-443473.

Pickens, Sarah R., Nathan D. Chamberlain, Michael V. Volin, Richard M. Pope, Nicholas E. Talarico, Arthur M. Mandelin, and Shiva Shahrara. 2011. "Characterization of Interleukin-7 and Interleukin-7 Receptor in the Pathogenesis of Rheumatoid Arthritis." *Arthritis and Rheumatism* 63 (10): 2884–93. doi:10.1002/art.30493.

Piper, Christopher, Anne M. Pesenacker, David Bending, Balathas Thirugnanabalan, Hemlata Varsani, Lucy R. Wedderburn, and Kiran Nistala. 2014. "Brief Report: T Cell Expression of Granulocyte–Macrophage Colony-Stimulating Factor in Juvenile Arthritis Is Contingent Upon Th17 Plasticity." *Arthritis & Rheumatology* 66 (7): 1955–60. doi:10.1002/art.38647.

Plum, J., M. De Smedt, G. Leclercq, B. Verhasselt, and B. Vandekerckhove. 1996. "Interleukin-7 Is a Critical Growth Factor in Early Human T-Cell Development." *Blood* 88 (11): 4239–45.

Poddubnyy, Denis, Kay-Geert A. Hermann, Johanna Callhoff, Joachim Listing, and Joachim Sieper. 2014. "Ustekinumab for the Treatment of Patients with Active Ankylosing Spondylitis: Results of a 28-Week, Prospective, Open-Label, Proof-of-Concept Study (TOPAS)." *Annals of the Rheumatic Diseases* 73 (5): 817–23. doi:10.1136/annrheumdis-2013-204248.

Praet, Liesbet Van, Peggy Jacques, Filip Van den Bosch, and Dirk Elewaut. 2012. "The Transition of Acute to Chronic Bowel Inflammation in Spondyloarthritis." *Nature Reviews Rheumatology* 8 (5): 288–95. doi:10.1038/nrrheum.2012.42.

Price, April E., Hong-Erh Liang, Brandon M. Sullivan, R. Lee Reinhardt, Chris J. Eisley, David J. Erle, and Richard M. Locksley. 2010. “Systemically Dispersed Innate IL-13–expressing Cells in Type 2 Immunity.” *Proceedings of the National Academy of Sciences* 107 (25): 11489–94. doi:10.1073/pnas.1003988107.

Proal, Amy D., Paul J. Albert, and Trevor G. Marshall. 2013. “The Human Microbiome and Autoimmunity.” *Current Opinion in Rheumatology* 25 (2): 234–40. doi:10.1097/BOR.0b013e32835cedbf.

Puel, Anne, Steven F. Ziegler, Rebecca H. Buckley, and Warren J. Leonard. 1998. “Defective IL7R Expression in T-B+NK + Severe Combined Immunodeficiency.” *Nature Genetics* 20 (4): 394–97. doi:10.1038/3877.

Punzi, Leonardo, Lorenzo Calò, and Mario Plebani. 2002. “Clinical Significance of Cytokine Determination in Synovial Fluid.” *Critical Reviews in Clinical Laboratory Sciences* 39 (1): 63–88. doi:10.1080/10408360290795448.

Purvis, Harriet A., Jeroen N. Stoop, Jelena Mann, Steven Woods, Anne E. Kozijn, Sophie Hambleton, John H. Robinson, John D. Isaacs, Amy E. Anderson, and Catharien M. U. Hilkens. 2010. “Low-Strength T-Cell Activation Promotes Th17 Responses.” *Blood* 116 (23): 4829–37. doi:10.1182/blood-2010-03-272153.

Raj, Towfique, Katie Rothamel, Sara Mostafavi, Chun Ye, Mark N. Lee, Joseph M. Replogle, Ting Feng, et al. 2014. “Polarization of the Effects of Autoimmune and Neurodegenerative Risk Alleles in Leukocytes.” *Science* 344 (6183): 519–23. doi:10.1126/science.1249547.

Rasouli, Javad, Bogoljub Ciric, Jaime Imitola, Patricia Gonnella, Daniel Hwang, Kedar Mahajan, Elisabeth R. Mari, et al. 2015. “Expression of GM-CSF in T Cells Is Increased in Multiple Sclerosis and Suppressed by IFN-β Therapy.” *The Journal of Immunology* 194 (11): 5085–93. doi:10.4049/jimmunol.1403243.

Rast, Jonathan P., L. Courtney Smith, Mariano Loza-Coll, Taku Hibino, and Gary W. Litman. 2006. “Genomic Insights into the Immune System of the Sea Urchin.” *Science (New York, N.Y.)* 314 (5801): 952–56. doi:10.1126/science.1134301.

Rath, H.C., H.H. Herfarth, J.S. Ikeda, W.B. Grenther, T.E. Hamm Jr., E. Balish, J.D. Taurog, R.E. Hammer, K.H. Wilson, and R.B. Sartor. 1996. “Normal Luminal Bacteria, Especially Bacteroides Species, Mediate Chronic Colitis, Gastritis, and Arthritis in HLA-B27/human β2 Microglobulin Transgenic Rats.” *Journal of Clinical Investigation* 98 (4): 945–53.

Rehaume, L.M., S. Mondot, D. Aguirre De Cárcer, J. Velasco, H. Benham, S.Z. Hasnain, J. Bowman, et al. 2014. “ZAP-70 Genotype Disrupts the Relationship between Microbiota and Host, Leading to Spondyloarthritis and Ileitis in SKG Mice.” *Arthritis and Rheumatology* 66 (10): 2780–92. doi:10.1002/art.38773.

Remtoula, Natacha, Armand Bensussan, and Anne Marie-Cardine. 2008. “Cutting Edge: Selective Expression of Inhibitory or Activating Killer Cell Ig-Like Receptors in Circulating CD4+ T Lymphocytes.” *The Journal of Immunology* 180 (5): 2767–71. doi:10.4049/jimmunol.180.5.2767.

Reveille, J.D., A.-M. Sims, P. Danoy, D.M. Evans, P. Leo, J.J. Pointon, R. Jin, et al. 2010. “Genome-Wide Association Study of Ankylosing Spondylitis Identifies Non-MHC Susceptibility Loci.” *Nature Genetics* 42 (2): 123–27. doi:10.1038/ng.513.

Ridley, Anna, Hiroko Hatano, Isabel Wong-Baeza, Jacqueline Shaw, Katherine K. Matthews, Hussein Al-Mossawi, Kristin Ladell, David A. Price, Paul Bowness, and Simon Kollnberger. 2015. "Activation-Induced KIR3DL2 Binding to HLA-B27 Licenses Pathogenic T Cell Differentiation in Spondyloarthritis." *Arthritis & Rheumatology*, November. doi:10.1002/art.39515.

Rihl, Markus, Herbert Kellner, Wolfgang Kellner, Christian Barthel, David T. Y. Yu, Paul P. Tak, Henning Zeidler, and Dominique Baeten. 2008. "Identification of Interleukin-7 as a Candidate Disease Mediator in Spondylarthritis." *Arthritis and Rheumatism* 58 (11): 3430–35. doi:10.1002/art.23998.

Robinson, Philip C., and Matthew A. Brown. 2014. "Genetics of Ankylosing Spondylitis." *Molecular Immunology*, The pathogenesis of ankylosing spondylitis: HLA-B27 and beyond, 57 (1): 2–11. doi:10.1016/j.molimm.2013.06.013.

Rogers, Paul R., and Michael Croft. 1999. "Peptide Dose, Affinity, and Time of Differentiation Can Contribute to the Th1/Th2 Cytokine Balance." *The Journal of Immunology* 163 (3): 1205–13.

Roifman, Chaim M., Junyan Zhang, David Chitayat, and Nigel Sharfe. 2000. "A Partial Deficiency of Interleukin-7Rα Is Sufficient to Abrogate T-Cell Development and Cause Severe Combined Immunodeficiency." *Blood* 96 (8): 2803–7.

Rudwaleit, M., D. van der Heijde, R. Landewé, N. Akkoc, J. Brandt, C. T. Chou, M. Dougados, et al. 2010. "The Assessment of SpondyloArthritis International Society Classification Criteria for Peripheral Spondyloarthritis and for Spondyloarthritis in General." *Annals of the Rheumatic Diseases*, November, annrheumdis133645. doi:10.1136/ard.2010.133645.

Rudwaleit, M., A. G. Jurik, K.-G. A. Hermann, R. Landewé, D. van der Heijde, X. Baraliakos, H. Marzo-Ortega, M. Østergaard, J. Braun, and J. Sieper. 2009. "Defining Active Sacroiliitis on Magnetic Resonance Imaging (MRI) for Classification of Axial Spondyloarthritis: A Consensual Approach by the ASAS/OMERACT MRI Group." *Annals of the Rheumatic Diseases* 68 (10): 1520–27. doi:10.1136/ard.2009.110767.

Rudwaleit, M., R. Landewé, D. van der Heijde, J. Listing, J. Brandt, J. Braun, R. Burgos-Vargas, et al. 2009. "The Development of Assessment of SpondyloArthritis International Society Classification Criteria for Axial Spondyloarthritis (Part I): Classification of Paper Patients by Expert Opinion Including Uncertainty Appraisal." *Annals of the Rheumatic Diseases* 68 (6): 770–76. doi:10.1136/ard.2009.108217.

Rudwaleit, Martin, Anke Metter, Joachim Listing, Joachim Sieper, and Jürgen Braun. 2006. "Inflammatory Back Pain in Ankylosing Spondylitis: A Reassessment of the Clinical History for Application as Classification and Diagnostic Criteria." *Arthritis & Rheumatism* 54 (2): 569–78. doi:10.1002/art.21619.

Ruof, J., and G. Stucki. 1999. "Validity Aspects of Erythrocyte Sedimentation Rate and C-Reactive Protein in Ankylosing Spondylitis: A Literature Review." *The Journal of Rheumatology* 26 (4): 966–70.

Sakaguchi, Noriko, Takeshi Takahashi, Hiroshi Hata, Takashi Nomura, Tomoyuki Tagami, Sayuri Yamazaki, Toshiko Sakihama, et al. 2003. "Altered Thymic T-Cell Selection due to a Mutation of the ZAP-70 Gene Causes Autoimmune Arthritis in Mice." *Nature* 426 (6965): 454–60. doi:10.1038/nature02119.

Salimi, Maryam, Jillian L. Barlow, Sean P. Saunders, Luzheng Xue, Danuta Gutowska-Owsiak, Xinwen Wang, Li-Chieh Huang, et al. 2013. “A Role for IL-25 and IL-33–driven Type-2 Innate Lymphoid Cells in Atopic Dermatitis.” *The Journal of Experimental Medicine* 210 (13): 2939–50. doi:10.1084/jem.20130351.

Santori, Fabio R., Pengxiang Huang, Serge A. van de Pavert, Eugene F. Douglass Jr., David J. Leaver, Brad A. Haubrich, Rok Keber, et al. 2015. “Identification of Natural RORγ Ligands That Regulate the Development of Lymphoid Cells.” *Cell Metabolism* 21 (2): 286–97. doi:10.1016/j.cmet.2015.01.004.

Saraux, A, F Guillemin, P Guggenbuhl, C Roux, P Fardellone, E Le Bihan, A Cantagrel, et al. 2005. “Prevalence of Spondyloarthropathies in France: 2001.” *Annals of the Rheumatic Diseases* 64 (10): 1431–35. doi:10.1136/ard.2004.029207.

Sarkar, S., L. A. Cooney, and D. A. Fox. 2010. “The Role of T Helper Type 17 Cells in Inflammatory Arthritis.” *Clinical & Experimental Immunology* 159 (3): 225–37. doi:10.1111/j.1365-2249.2009.04016.x.

Scher, Jose U., Andrew Sczesnak, Randy S. Longman, Nicola Segata, Carles Ubeda, Craig Bielski, Tim Rostron, et al. 2013. “Expansion of Intestinal Prevotella Copri Correlates with Enhanced Susceptibility to Arthritis.” *eLife* 2 (May). doi:10.7554/eLife.01202.

Schlosstein, L., P. I. Terasaki, R. Bluestone, and C. M. Pearson. 1973. “High Association of an HL-A Antigen, W27, with Ankylosing Spondylitis.” *The New England Journal of Medicine* 288 (14): 704–6. doi:10.1056/NEJM197304052881403.

Schluns, Kimberly S., William C. Kieper, Stephen C. Jameson, and Leo Lefrançois. 2000. “Interleukin-7 Mediates the Homeostasis of Naïve and Memory CD8 T Cells in Vivo.” *Nature Immunology* 1 (5): 426–32. doi:10.1038/80868.

Schwartz, D.A. 2001. “The Role of TLR4 in Endotoxin Responsiveness in Humans.” *Journal of Endotoxin Research* 7 (5): 389–93. doi:10.1179/096805101101532972.

Seddon, Benedict, Peter Tomlinson, and Rose Zamoyska. 2003. “Interleukin 7 and T Cell Receptor Signals Regulate Homeostasis of CD4 Memory Cells.” *Nature Immunology* 4 (7): 680–86. doi:10.1038/ni946.

Segura, Elodie, and Sebastian Amigorena. 2013. “Inflammatory Dendritic Cells in Mice and Humans.” *Trends in Immunology* 34 (9): 440–45. doi:10.1016/j.it.2013.06.001.

Segura, Elodie, Maxime Touzot, Armelle Bohineust, Antonio Cappuccio, Gilles Chiocchia, Anne Hosmalin, Marc Dalod, Vassili Soumelis, and Sebastian Amigorena. 2013. “Human Inflammatory Dendritic Cells Induce Th17 Cell Differentiation.” *Immunity* 38 (2): 336–48. doi:10.1016/j.immuni.2012.10.018.

Shen, Hui, Jane C. Goodall, and J. S. Hill Gaston. 2009. “Frequency and Phenotype of Peripheral Blood Th17 Cells in Ankylosing Spondylitis and Rheumatoid Arthritis.” *Arthritis & Rheumatism* 60 (6): 1647–56. doi:10.1002/art.24568.

Sheng, Wanqiang, Fan Yang, Yi Zhou, Henry Yang, Pey Yng Low, David Michael Kemeny, Patrick Tan, et al. 2014. “STAT5 Programs a Distinct Subset of GM-CSF-Producing T Helper Cells That Is Essential for Autoimmune Neuroinflammation.” *Cell Research* 24 (12): 1387–1402. doi:10.1038/cr.2014.154.

Sherlock, Jonathan P, Barbara Joyce-Shaikh, Scott P Turner, Cheng-Chi Chao, Manjiri Sathe, Jeff Grein, Daniel M Gorman, et al. 2012. “IL-23 Induces Spondyloarthropathy by Acting on ROR-Γt(+) CD3(+)CD4(-)CD8(-) Entheseal Resident T Cells.” *Nature Medicine* 18 (7): 1069–76. doi:10.1038/nm.2817.

Shibata, Yoko, Pierre-Yves Berclaz, Zissis C Chroneos, Mitsuhiro Yoshida, Jeffrey A Whitsett, and Bruce C Trapnell. 2001. “GM-CSF Regulates Alveolar Macrophage Differentiation and Innate Immunity in the Lung through PU.1.” *Immunity* 15 (4): 557–67. doi:10.1016/S1074-7613(01)00218-7.

Shiomi, Aoi, Takashi Usui, Yuki Ishikawa, Masakazu Shimizu, Kosaku Murakami, and Tsuneyo Mimori. 2014. “GM-CSF but Not IL-17 Is Critical for the Development of Severe Interstitial Lung Disease in SKG Mice.” *The Journal of Immunology* 193 (2): 849–59. doi:10.4049/jimmunol.1303255.

Sieper, J., A. Koenig, S. Baumgartner, C. Wishneski, J. Foehl, B. Vlahos, and B. Freundlich. 2010. “Analysis of Uveitis Rates across All Etanercept Ankylosing Spondylitis Clinical Trials.” *Annals of the Rheumatic Diseases* 69 (1): 226–29. doi:10.1136/ard.2008.103192.

Sieper, Joachim, Benjamin Porter-Brown, Liz Thompson, Olivier Harari, and Maxime Dougados. 2014. “Assessment of Short-Term Symptomatic Efficacy of Tocilizumab in Ankylosing Spondylitis: Results of Randomised, Placebo-Controlled Trials.” *Annals of the Rheumatic Diseases* 73 (1): 95–100. doi:10.1136/annrheumdis-2013-203559.

Skepner, Jill, Radha Ramesh, Mark Trocha, Darby Schmidt, Erkan Baloglu, Mercedes Lobera, Thaddeus Carlson, et al. 2014. “Pharmacologic Inhibition of RORγt Regulates Th17 Signature Gene Expression and Suppresses Cutaneous Inflammation in Vivo.” *Journal of Immunology (Baltimore, Md.: 1950)* 192 (6): 2564–75. doi:10.4049/jimmunol.1302190.

Skou, Jens Chr. 1957. “The Influence of Some Cations on an Adenosine Triphosphatase from Peripheral Nerves.” *Biochimica et Biophysica Acta* 23 (January): 394–401. doi:10.1016/0006-3002(57)90343-8.

Smith, Janine A., Darby J. S. Thompson, Scott M. Whitcup, Eric Suhler, Grace Clarke, Susan Smith, Michael Robinson, Jonghyeon Kim, and Karyl S. Barron. 2005. “A Randomized, Placebo-Controlled, Double-Masked Clinical Trial of Etanercept for the Treatment of Uveitis Associated with Juvenile Idiopathic Arthritis.” *Arthritis Care & Research* 53 (1): 18–23. doi:10.1002/art.20904.

Smolen, Josef S., Jürgen Braun, Maxime Dougados, Paul Emery, Oliver FitzGerald, Philip Helliwell, Arthur Kavanaugh, et al. 2014. “Treating Spondyloarthritis, Including Ankylosing Spondylitis and Psoriatic Arthritis, to Target: Recommendations of an International Task Force.” *Annals of the Rheumatic Diseases* 73 (1): 6–16. doi:10.1136/annrheumdis-2013-203419.

Solt, Laura A., Naresh Kumar, Philippe Nuhant, Yongjun Wang, Janelle L. Lauer, Jin Liu, Monica A. Istrate, et al. 2011. “Suppression of TH17 Differentiation and Autoimmunity by a Synthetic ROR Ligand.” *Nature* 472 (7344): 491–94. doi:10.1038/nature10075.

Sonnenberg, Gregory F., Laurel A. Monticelli, Theresa Alenghat, Thomas C. Fung, Natalie A. Hutnick, Jun Kunisawa, Naoko Shibata, et al. 2012. “Innate Lymphoid Cells Promote Anatomical Containment of Lymphoid-Resident

Commensal Bacteria." *Science* 336 (6086): 1321–25. doi:10.1126/science.1222551.

Soroosh, Pejman, Jiejun Wu, Xiaohua Xue, Jiao Song, Steven W. Sutton, Marciano Sablad, Jingxue Yu, et al. 2014. "Oxysterols Are Agonist Ligands of RORγt and Drive Th17 Cell Differentiation." *Proceedings of the National Academy of Sciences of the United States of America* 111 (33): 12163–68. doi:10.1073/pnas.1322807111.

Soumelis, Vassili, Pedro A. Reche, Holger Kanzler, Wei Yuan, Gina Edward, Bernhart Homey, Michel Gilliet, et al. 2002. "Human Epithelial Cells Trigger Dendritic Cell–mediated Allergic Inflammation by Producing TSLP." *Nature Immunology* 3 (7): 673–80. doi:10.1038/ni805.

Spits, Hergen, David Artis, Marco Colonna, Andreas Diefenbach, James P. Di Santo, Gerard Eberl, Shigeo Koyasu, et al. 2013. "Innate Lymphoid Cells — a Proposal for Uniform Nomenclature." *Nature Reviews Immunology* 13 (2): 145–49. doi:10.1038/nri3365.

Spits, Hergen, and Tom Cupedo. 2012. "Innate Lymphoid Cells: Emerging Insights in Development, Lineage Relationships, and Function." *Annual Review of Immunology* 30 (1): 647–75. doi:10.1146/annurev-immunol-020711-075053.

Stanley, E., G. J. Lieschke, D. Grail, D. Metcalf, G. Hodgson, J. A. Gall, D. W. Maher, J. Cebon, V. Sinickas, and A. R. Dunn. 1994. "Granulocyte/macrophage Colony-Stimulating Factor-Deficient Mice Show No Major Perturbation of Hematopoiesis but Develop a Characteristic Pulmonary Pathology." *Proceedings of the National Academy of Sciences* 91 (12): 5592–96.

Stehlin-Gaon, Catherine, Dominica Willmann, Denis Zeyer, Sarah Sanglier, Alain Van Dorsselaer, Jean-Paul Renaud, Dino Moras, and Roland Schüle. 2003. "All-Trans Retinoic Acid Is a Ligand for the Orphan Nuclear Receptor ROR Beta." *Nature Structural Biology* 10 (10): 820–25. doi:10.1038/nsb979.

Stolwijk, Carmen, Ivette Essers, Astrid van Tubergen, Annelies Boonen, Marloes T. Bazelier, Marie L. De Bruin, and Frank de Vries. 2015. "The Epidemiology of Extra-Articular Manifestations in Ankylosing Spondylitis: A Population-Based Matched Cohort Study." *Annals of the Rheumatic Diseases* 74 (7): 1373–78. doi:10.1136/annrheumdis-2014-205253.

Tam, Lai-Shan, Jieruo Gu, and David Yu. 2010. "Pathogenesis of Ankylosing Spondylitis." *Nature Reviews Rheumatology* 6 (7): 399–405. doi:10.1038/nrrheum.2010.79.

Taurog, Joel D., Martha L. Dorris, Nimman Satumtira, Tri M. Tran, Rohit Sharma, Ralf Dressel, Jens van den Brandt, and Holger M. Reichardt. 2009. "Spondylarthritis in HLA-B27/human beta2-Microglobulin-Transgenic Rats Is Not Prevented by Lack of CD8." *Arthritis and Rheumatism* 60 (7): 1977–84. doi:10.1002/art.24599.

Tillack, Cornelia, Laura Maximiliane Ehmann, Matthias Friedrich, Rüdiger P. Laubender, Pavol Papay, Harald Vogelsang, Johannes Stallhofer, et al. 2014. "Anti-TNF Antibody-Induced Psoriasiform Skin Lesions in Patients with Inflammatory Bowel Disease Are Characterised by Interferon-γ-Expressing Th1 Cells and IL-17A/IL-22-Expressing Th17 Cells and Respond to Anti-IL-12/IL-23 Antibody Treatment." *Gut* 63 (4): 567–77. doi:10.1136/gutjnl-2012-302853.

Timothy K. Starr, Stephen C. Jameson, and and Kristin A. Hogquist. 2003. “Positive and Negative Selection of T Cells.” *Annual Review of Immunology* 21 (1): 139–76. doi:10.1146/annurev.immunol.21.120601.141107.

Tonozuka, Y., K. Fujio, T. Sugiyama, T. Nosaka, M. Hirai, and T. Kitamura. 2001. “Molecular Cloning of a Human Novel Type I Cytokine Receptor Related to δ1/TSLPR.” *Cytogenetic and Genome Research* 93 (1–2): 23–25. doi:10.1159/000056941.

Uchida, Kanji, Koh Nakata, Takuji Suzuki, Maurizio Luisetti, Masato Watanabe, Diana E. Koch, Carrie A. Stevens, et al. 2009. “Granulocyte/macrophage–colony-Stimulating Factor Autoantibodies and Myeloid Cell Immune Functions in Healthy Subjects.” *Blood* 113 (11): 2547–56. doi:10.1182/blood-2008-05-155689.

Valmori, D., C. Raffin, I. Raimbaud, and M. Ayyoub. 2010. “Human RORgamma-T+ TH17 Cells Preferentially Differentiate from Naive FOXP3+Treg in the Presence of Lineage-Specific Polarizing Factors.” *Proceedings of the National Academy of Sciences* 107 (45): 19402–7. doi:10.1073/pnas.1008247107.

van der Heijde, Désirée, Atul Deodhar, J. Wei, Drescher Edit, and Keith Kanik,. 2016. “Tofacitinib in Patients with Ankylosing Spondylitis: A Phase 2, 16-Week, Randomized, Placebo-Controlled, Dose-Ranging Study.” *ACR Meeting Abstracts*. Accessed January 13. http://acrabstracts.org/abstract/tofacitinib-in-patients-with-ankylosing-spondylitis-a-phase-2-16-week-randomized-placebo-controlled-dose-ranging-study/.

van Nieuwenhuijze, Annemarie, Marije Koenders, Debbie Roeleveld, Matthew A. Sleeman, Wim van den Berg, and Ian P. Wicks. 2013. “GM-CSF as a Therapeutic Target in Inflammatory Diseases.” *Molecular Immunology* 56 (4): 675–82. doi:10.1016/j.molimm.2013.05.002.

van Roon, Joel A. G., Marieke C. Verweij, Marion Wenting-van Wijk, Kim M. G. Jacobs, Johannes W. J. Bijlsma, and Floris P. J. G. Lafeber. 2005. “Increased Intraarticular Interleukin-7 in Rheumatoid Arthritis Patients Stimulates Cell Contact-Dependent Activation of CD4(+) T Cells and Macrophages.” *Arthritis and Rheumatism* 52 (6): 1700–1710. doi:10.1002/art.21045.

Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. “TGFbeta in the Context of an Inflammatory Cytokine Milieu Supports de Novo Differentiation of IL-17-Producing T Cells.” *Immunity* 24: 179–89. doi:10.1016/j.immuni.2006.01.001.

Walker, Lucy J., Yu-Hoi Kang, Matthew O. Smith, Hannah Tharmalingham, Narayan Ramamurthy, Vicki M. Fleming, Natasha Sahgal, et al. 2012. “Human MAIT and CD8αα Cells Develop from a Pool of Type-17 Precommitted CD8+ T Cells.” *Blood* 119 (2): 422–33. doi:10.1182/blood-2011-05-353789.

Wang, Chao, Nir Yosef, Jellert Gaublomme, Chuan Wu, Youjin Lee, Clary B. Clish, Jim Kaminski, et al. 2015. “CD5L/AIM Regulates Lipid Biosynthesis and Restrains Th17 Cell Pathogenicity.” *Cell* 163 (6): 1413–27. doi:10.1016/j.cell.2015.10.068.

Wendling, Daniel, Julien Paccou, Jean-Marie Berthelot, René-Marc Flipo, Séverine Guillaume-Czitrom, Clément Prati, Emmanuelle Dernis, Guillaume Direz, Véronique Ferrazzi, and Jean-Michel Ristori. 2011. “New Onset of Uveitis During Anti-Tumor Necrosis Factor Treatment for Rheumatic Diseases.”

Seminars in Arthritis and Rheumatism 41 (3): 503–10. doi:10.1016/j.semarthrit.2011.05.005.

Wilson, Christopher B., Emily Rowell, and Masayuki Sekimata. 2009. "Epigenetic Control of T-Helper-Cell Differentiation." *Nature Reviews Immunology* 9 (2): 91–105. doi:10.1038/nri2487.

Wilson, N. J. 2007. "Development, Cytokine Profile and Function of Human Interleukin 17-Producing Helper T Cells." *Nature Immunol.* 8: 950–57. doi:10.1038/ni1497.

Wong, Chun Kwok, Kin Mang Lau, Iris Hiu Shuen Chan, Shuiqing Hu, Yvonne Yi On Lam, Angela On Kei Choi, and Christopher Wai Kei Lam. 2013. "MicroRNA-21* Regulates the Prosurvival Effect of GM-CSF on Human Eosinophils." *Immunobiology* 218 (2): 255–62. doi:10.1016/j.imbio.2012.05.019.

Wright, Helen L., Huw B. Thomas, Robert J. Moots, and Steven W. Edwards. 2013. "RNA-Seq Reveals Activation of Both Common and Cytokine-Specific Pathways Following Neutrophil Priming." *PLoS ONE* 8 (3): e58598. doi:10.1371/journal.pone.0058598.

Wu, Chuan, Nir Yosef, Theresa Thalhamer, Chen Zhu, Sheng Xiao, Yasuhiro Kishi, Aviv Regev, and Vijay K. Kuchroo. 2013. "Induction of Pathogenic TH17 Cells by Inducible Salt-Sensing Kinase SGK1." *Nature* 496 (7446): 513–17. doi:10.1038/nature11984.

Xiao, Sheng, Nir Yosef, Jianfei Yang, Yonghui Wang, Ling Zhou, Chen Zhu, Chuan Wu, et al. 2014. "Small-Molecule RORγt Antagonists Inhibit T Helper 17 Cell Transcriptional Network by Divergent Mechanisms." *Immunity* 40 (4): 477–89. doi:10.1016/j.immuni.2014.04.004.

Xu, Yuekang, Yifan Zhan, Andrew M. Lew, Shalin H. Naik, and Michael H. Kershaw. 2007. "Differential Development of Murine Dendritic Cells by GM-CSF versus Flt3 Ligand Has Implications for Inflammation and Trafficking." *The Journal of Immunology* 179 (11): 7577–84. doi:10.4049/jimmunol.179.11.7577.

Yang, Jianfei, Mark S. Sundrud, Jill Skepner, and Tetsuya Yamagata. 2014. "Targeting Th17 Cells in Autoimmune Diseases." *Trends in Pharmacological Sciences* 35 (10): 493–500. doi:10.1016/j.tips.2014.07.006.

Yang, L. 2008. "IL-21 and TGF-[Bgr] Are Required for Differentiation of Human TH17 Cells." *Nature*, May.

Yang, Li, David E. Anderson, Clare Baecher-Allan, William D. Hastings, Estelle Bettelli, Mohamed Oukka, Vijay K. Kuchroo, and David A. Hafler. 2008. "IL-21 and TGF-β Are Required for Differentiation of Human TH17 Cells." *Nature* 454 (7202): 350–52. doi:10.1038/nature07021.

Yeoman, H., D. R. Clark, and D. DeLuca. 1996. "Development of CD4 and CD8 Single Positive T Cells in Human Thymus Organ Culture: IL-7 Promotes Human T Cell Production by Supporting Immature T Cells." *Developmental and Comparative Immunology* 20 (4): 241–63.

Yong, K. L., P. M. Rowles, K. G. Patterson, and D. C. Linch. 1992. "Granulocyte-Macrophage Colony-Stimulating Factor Induces Neutrophil Adhesion to Pulmonary Vascular Endothelium in Vivo: Role of Beta 2 Integrins." *Blood* 80 (6): 1565–75.

Yosef, Nir, Alex K. Shalek, Jellert T. Gaublomme, Hulin Jin, Youjin Lee, Amit Awasthi, Chuan Wu, et al. 2013. “Dynamic Regulatory Network Controlling TH17 Cell Differentiation.” *Nature* 496 (7446): 461–68. doi:10.1038/nature11981.

Yousefi, S., C. Mihalache, E. Kozlowski, I. Schmid, and H. U. Simon. 2009. “Viable Neutrophils Release Mitochondrial DNA to Form Neutrophil Extracellular Traps.” *Cell Death & Differentiation* 16 (11): 1438–44. doi:10.1038/cdd.2009.96.

Zeboulon, N., M. Dougados, and L. Gossec. 2008. “Prevalence and Characteristics of Uveitis in the Spondyloarthropathies: A Systematic Literature Review.” *Annals of the Rheumatic Diseases* 67 (7): 955–59. doi:10.1136/ard.2007.075754.

Zenatti, Priscila P., Daniel Ribeiro, Wenqing Li, Linda Zuurbier, Milene C. Silva, Maddalena Paganin, Julia Tritapoe, et al. 2011. “Oncogenic IL7R Gain-of-Function Mutations in Childhood T-Cell Acute Lymphoblastic Leukemia.” *Nature Genetics* 43 (10): 932–39. doi:10.1038/ng.924.

Zhan, Yifan, Yuekang Xu, and Andrew M. Lew. 2012. “The Regulation of the Development and Function of Dendritic Cell Subsets by GM-CSF: More than a Hematopoietic Growth Factor.” *Molecular Immunology* 52 (1): 30–37. doi:10.1016/j.molimm.2012.04.009.

Zhou, Liang, Ivaylo I. Ivanov, Rosanne Spolski, Roy Min, Kevin Shenderov, Takeshi Egawa, David E. Levy, Warren J. Leonard, and Dan R. Littman. 2007. “IL-6 Programs TH-17 Cell Differentiation by Promoting Sequential Engagement of the IL-21 and IL-23 Pathways.” *Nature Immunology* 8 (9): 967–74. doi:10.1038/ni1488.

Zhu, Jinfang, and William E. Paul. 2008. “CD4 T Cells: Fates, Functions, and Faults.” *Blood* 112 (5): 1557–69. doi:10.1182/blood-2008-05-078154.