Type 1 interferons in early rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a heterogeneous autoimmune disease predominantly causing synovial inflammation. Early and established RA exhibit both overlapping and distinct pathological processes. Early treatment improves clinical outcomes and delineating early disease pathology can inform novel therapeutic pathways. Type 1 interferons have diverse effects on immune function and relative exposure can be recorded using an interferon gene signature (IGS). The IGS is positive in 20-30% of established RA patients where it does not associate with disease activity but can predict response to some biological therapies. However, glucocorticoids and potentially other immunomodulatory therapies can modify the IGS.

I therefore examined the IGS in early, drug naïve RA focusing on prevalence, association with clinical phenotype, and impact on disease progression. I additionally attempted to identify the source of type 1 interferons and triggers for their production.

I demonstrated the IGS is increased in early RA, falls with treatment/time and appears to predict clinical response to initial therapies. In this latter capacity it out-performed baseline CRP, ESR and DAS-28. The IGS also positively associated with disease activity and IgM rheumatoid factor titres. The latter association was supported by an analysis of the B cell transcriptome of IGS+ early RA patients, where there was increased gene expression in pathways related to B cell activation; increased plasma cell differentiation; and propensity to produce IgM. Genes were also upregulated that are usually expressed in B cell malignancies, further emphasising a potentially pathologically activated state. Retroelements (SINE, LINE-1 and ERV), are putative triggers of type 1 interferons in autoimmunity. However early RA whole blood LINE-1 activity was comparable to healthy controls and was actually reduced in IGS+ patients. Furthermore pDCs showed uniformly reduced retroelement activity in early RA compared with healthy controls. Indeed there was differential retroelement expression across lymphocyte subsets in early RA; expression was highest in B and T cells and comparatively lower in DCs and monocytes. Finally despite being a major source of type 1 interferons, pDCs in IGS+ early RA patients had comparable interferon-α expression to other lymphocytes.

In conclusion I demonstrate that type 1 interferons may play a pathogenic role in early RA and disease progression - although their source and triggers remain unclear. Nonetheless I hypothesise that therapies that target type 1 interferons could have clinical benefit in early RA.

Dedication

I would like to dedicate this work to:

1) My husband. He knows why.

2) My parents. For instilling in me a belief anything was achievable.

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Declaration

The candidate (Faye A H Cooles) confirms that the work submitted is her own work and that appropriate credit has been given where reference has been made to the work of others.

The work in this thesis was performed from December 2013 to November 2016. All work was performed in the Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University.

No part of this thesis has been submitted for the award of any other degree.

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ACPA: anti-cyclic citrullinated peptides antibody

ACR: American college of Rheumatology

AGS: Aciardi-Goutieres syndrome

AHR: acyl hydrocarbon receptor

APC: antigen presenting cell

APRIL: a proliferation-inducing ligand

anti-CarP: anti-carbamylated protein antibodies

BAFF/BLYs: B-cell activating factor

BCR: B cell receptor

Blimp-1: B lymphocyte-induced maturation protein-1

BTLA: B- and T-lymphocyte attenuator

CCP: cyclic citrullinated peptides

CCR: C-C chemokine receptor

CD: cluster of differentiation

CFSE: carboxyfluorescein succinimidyl ester

cGAS: cyclic GMP-AMP synthase

CIA: collagen induced arthritis

CPG: 5'—C—phosphate—G—3'

CRP: C-reactive protein

CT: cycle threshold

CTLA-4: cytotoxic T lymphocyte-associated antigen-4

CVD: cardiovascular disease

DAMP: damage-associated molecular patterns

DAS-28: disease activity score-28

DC: dendritic cell

DLBCL: diffuse large B cell lymphoma

DMARD: disease modifying anti rheumatic medication

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DRBPs: double-stranded RNA-binding proteins

DNMT: DNA methyltransferase

dsRNA: double stranded RNA

ELISA: enzyme-linked immunosorbent assay

eRA: early rheumatoid arthritis

ERV: endogenous retrovirus

ESR: erythrocyte sedimentation rate

EULAR: European league against rheumatic diseases

FACS: fluorescence-activated cell sorting

FcRL4: Fc receptor-like protein 4

FCS: foetal calf serum

FDR: false discovery rate

FLS: fibroblast like synoviocytes

FOXP3: forkhead box P3

GCN2: general control nonderepressible 2

cDNA: complementary DNA (deoxyribonucleic acid)

gDNA: genomic DNA (deoxyribonucleic acid)

GATA3: GATA-binding protein 3

GLM: generalised linear model

GM-CSF, Granulocyte-macrophage colony-stimulating factor

HBSS: Hanks balanced salt solution

HC: healthy control

HCQ: hydroxychloroquine

HCV: hepatitis C virus

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HERV: human endogenous retrovirus

HIV: human immunodeficiency virus

HNP1: human neutrophil peptide 1

HPLC: high-performance liquid chromatography

ICOS: inducible T-Cell Co-Stimulator

ICOSL: inducible T-Cell Co-Stimulator Ligand

IDO: indoleamine-pyrrole 2,3-dioxygenase

IFN: interferon

Ig: immunoglobulin

IGS: interferon gene signature

IL: interleukin

ILF3: interleukin enhancer-binding factor 3

ILT7: immunoglobulin-like transcript 7

IRAK4, interleukin-1 receptor-associated kinase 4

IRF: interferon regulatory factor

IPA®: Ingenuity® Pathway Analysis

IPC: interferon producing cell

IRG: interferon response gene

IRF: interferon regulatory factor

ITAM: immunoreceptor tyrosine-based activation motif

ITIM: immunoreceptor tyrosine inhibitory motif

Lin: lineage

LINE-1: long interspersed nuclear element 1

LGP2: laboratory of genetics and physiology

LPS: lipopolysaccharide

LTR: long terminal repeat

MAPK: mitogen activated protein kinase

MAVS: mitochondrial antiviral-signalling protein

MDA-5: melanoma differentiation-associated protein 5

mDC: myeloid dendritic cell

MFI: median fluorescence intensity

MHC: major histocompatibility complex

MLR: mixed leukocyte reaction

MoDC: monocyte derived dendritic cell

mRNA: messenger RNA

MS: multiple sclerosis

MSD: Meso Scale Discovery

MxA: myxovirus-resistance protein A

MyD88: myeloid differentiation factor 88

NET: neutrophil extracellular trap

NF90: Nuclear Factor 90

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

ORF: open reading frame

PAMP: pathogen associated molecular patterns

PBMC: peripheral blood mononuclear cells

PBS: phosphate buffered saline

PCA: principal components analysis

PD1: programmed cell death protein 1

PDL1: Programmed death-ligand 1

PCR: polyermerase chain reaction

pDC: plasmacytoid dendritic cell

Poly IC: polyinosinic-polycytidylic acid

PRR: pattern recognition receptor

pSS: primary Sjogrens syndrome

PTP: protein tyrosine phosphatase

QC: quality control

RA: rheumatoid Arthritis

RANK-L: Receptor activator of nuclear factor kappa-B ligand

RF: rheumatoid Factor

RIG-1: retinoic acid-inducible gene-1

RMPI medium: Roswell Park Memorial Institute medium

RNA: ribonucleic acid

RNP: ribonucleoprotein

RNase H2: Ribonuclease H2

RORγt: retinoic acid receptor-related orphan receptor-γt

RT-qPCR: real time quantitative polymerase chain reaction

SAMHD1: SAM Domain and HD Domain 1

SINE: Short interspersed nuclear element

Sero-: seronegative

Sero+: seropositive

SF: synovial fluid

SJC: swollen joint count

SLE: systemic lupus erythematous

ssRNA: single stranded RNA

STAT: Signal Transducer and Activator of Transcription

STING: stimulator of interferon genes

SyK: spleen tyrosine kinase

TACI: transmembrane activator and CAML interactor

TBK1: TANK binding kinase 1

TBP: TATA-binding protein

TCR: T cell receptor

TGFβ: transforming growth factor beta

TJC: tender joint count

TLR: Toll like receptor

TNF: tumour necrosis factor

TREX1: Three Prime Repair Exonuclease 1

TTS: tryptophanyl-tRNA-synthetase

UTR: untranslated region

VAS: visual analogue scale

Chapter 1: Introduction

1.1 Rheumatoid arthritis

1.1.1 Rheumatoid arthritis overview

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown aetiology however it is thought to result from a breakdown in the immune regulation of autoreactive responses. RA has a prevalence of approximately 1% in the UK general population and commonly presents in those of working age. It is associated with significant morbidity and mortality; there is a 50% increased risk of death from cardiovascular causes and one third of patients will not be working 2 years after diagnosis (1). There is a predominance for women being affected, with a male: female ratio of 1:3, and smoking, as well as genetic background, increase the risk of developing RA (2).

Autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibody (ACPA or anti-CCP antibody), are often, but not always, detected in the circulation of RA patients. At diagnosis patients present classically with a symmetrical polyarthritis of the small joints of the hands and feet however they can also initially present with involvement of larger joints. Indeed there is marked heterogeneity in patient presentation, autoantibody status and clinical background. However in all affected joints there is an inflammatory infiltrate consisting of a wide range of immune cells, including B cells, T cells and dendritic cells (DCs). This results in excessive production of proinflammatory mediators, such as tumour necrosis factor α (TNF-α), interferon-γ, interleukin (IL)-1β, IL-6, and IL-17. In addition there is synovial hyper-proliferation culminating in damage to the cartilage and underlying bone (figure 1.1). This eventually causes joint deformity and ongoing pain.

Therapies for RA generally involve either global immune suppression or immunomodulation by targeted means, e.g. TNF-α inhibition. These treatments unfortunately do not work in all patients. As such there is a need for the development of novel therapeutics (3). As understanding about disease pathogenesis and heterogeneity improves, the therapeutic potential for novel targeting of pathogenic cellular subsets and signalling pathways can be explored.

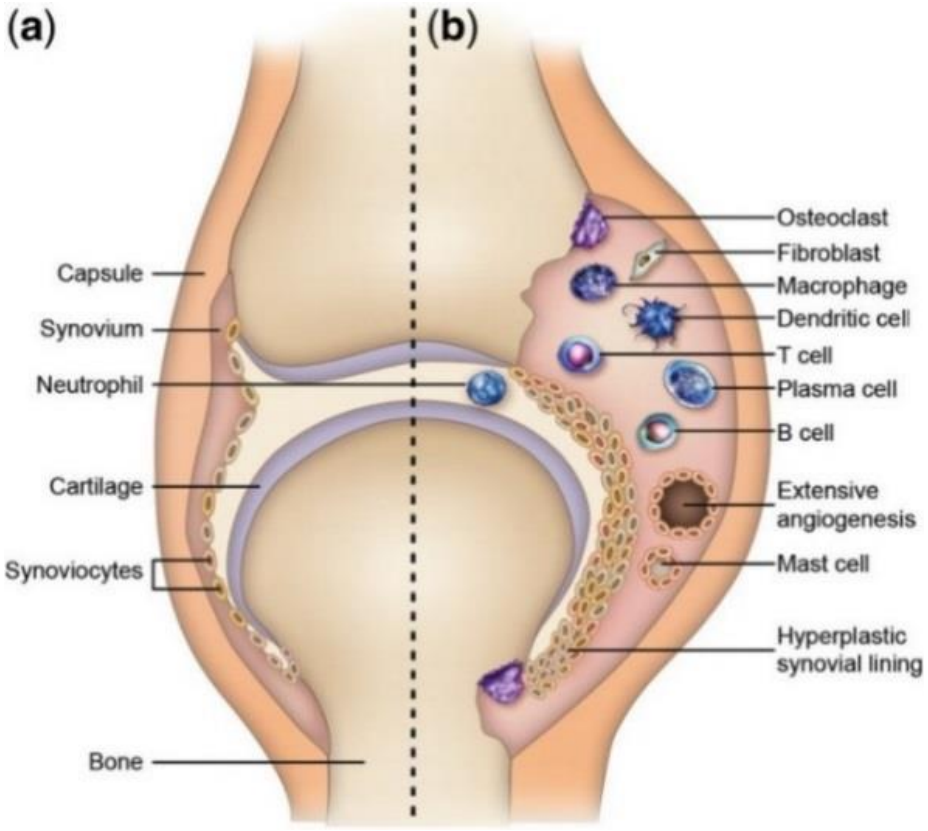


Figure 1.1. Schematic of a healthy joint and a rheumatoid arthritis joint

(a) A healthy joint **(b)** A RA joint with marked inflammatory cell infiltrate, thickened and inflamed synovium and bony erosions.

Image obtained from http://immunesystemrheumatoidarthritis.tumblr.com/ on 24/9/16.

1.1.2 Early rheumatoid arthritis

There is some suggestion that the pathological processes present in established RA may be distinct from those at disease onset (4-10). From a therapeutic perspective treating patients at this early stage has better long term outcomes in terms of patient morbidity, radiographic progression as well as disease control, the so called window of opportunity (10-20). In addition there is now an appreciation of the benefit of a "treat to target" approach in the management of early RA (21, 22). This involves rapid initiation, addition and escalation of disease-modifying anti-rheumatic drugs (DMARDs) in the early stages after diagnosis. The addition/modification of these therapies is based on disease activity as measured by conventional parameters, such as disease activity score-28 (DAS-28). This is a composite score which includes swollen and tender joint counts (SJC and TJC) as well as markers of inflammation, such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as well as a patient reported measure, visual analogue scale

(VAS). The aim of the "treat to target" regimen is to achieve rapid and sustained low disease activity as soon as a patient has been diagnosed.

From a research perspective there is therefore merit in identifying what are the prevalent pathological processes in early disease as these may provide therapeutic strategies with the potential for long term benefit. As expected DMARDs and glucocorticoids can modulate or mask the evidence of these processes (4, 23) and so examining a DMARD/glucocorticoid naïve population is likely to yield the most fruitful results.

In an early arthritis cohort, prior to onset of arthritis there will have been a breach of tolerance to self. The exact timing of when this happens is unclear however over recent years it is appreciated that patients develop RA associated autoantibodies, such as ACPA, many years prior to the onset of arthritis which may suggest that elements involving breach of tolerance are a relatively early event (24). These ACPA+ individuals are felt to be in a "pre-RA state" and for some this is associated with arthralgia, but no actual associated inflammation. However approximately 50% of these ACPA+ arthralgia patients will develop RA within 2 years (25). As such this population is being increasingly targeted for research into the early pathogenic processes driving the initiation and progression of RA.

1.2 Immune system overview

The role of the immune system is to protect the host from insults related to microbes or chemicals. The immune response has been artificially divided into innate immunity and adaptive or specific immunity and these are outlined below. A key element underpinning these functions is the recognition of self and non-self. This tolerance to self is a crucial factor in preventing inappropriate and damaging immune system activation. When there is breach in this tolerance the immune system can target and attack self organs/structures resulting in autoimmunity.

1.2.1 Innate immunity

Innate immunity is an evolutionarily ancient part of the host defense mechanism (26). It consists of barriers, such as epithelial mucosal membranes lining the oral mucosa and gut, the complement system as well as a group of immune cells. These cells consist of monocytes/macrophages, mast cells, neutrophils, eosinophils and natural killer (NK) cells (27).

The innate response is not antigen specific and a universal feature of these cells is the expression of immune recognition receptors which are triggered upon detection of pathogen-associated molecular patterns (PAMPs). These are patterns shared between pathogens, for instance bacterial lipopolysaccharide (LPS), carbohydrates, and double-stranded viral RNA**.** The best known of these receptors are the Toll like receptors (TLRs) which, when activated, promote rapid differentiation of short-lived effector cells whose main role is to eliminate any infection (28).

The innate system therefore provides a background level of defence against invading pathogens. For more severe infections it provides protection pending the activation of the adaptive immune system but in some instances the innate immune system can also instruct the adaptive immune system about the nature of a certain pathogenic challenge.

1.2.2 Adaptive immunity

Adaptive immunity is a complex system by which the body learns to recognize a pathogen's unique antigens and builds an antigen-specific response to destroy it. These responses are performed, under the direction of dendritic cells (DCs), by two main classes of lymphocytes called T cells and B cells. These subsets facilitate cell-mediated immune responses and antibody responses, respectively (29).

Dendritic cells

DCs are professional antigen-presenting cells (APC) and are central in the orchestration of the various forms of immunity and tolerance. They are a heterogeneous population of cells and there are currently 3 main subtypes in humans which are defined by the differential expression of surface proteins. These include in decreasing order of frequency myeloid dendritic cells (mDCs), plasmacytoid dendritic cells (pDCs) and CD141+ dendritic cells. They each have relatively different specialised functions however all are able to provide a link between the innate and adaptive immune system and effectively translate the innate signals of damage to promote a targeted anti-pathogen response (30). They exist in an immature state at sites of interaction with the environment such as the skin and mucosa and continuously sample their environment by micropinocytosis. This allows uptake of both self and non-self proteins which are subsequently degraded into peptides. These peptides are loaded onto major histocompatibility complex (MHC) molecules in a post golgi compartment and trafficked to the cell membrane where they are displayed on the cell surface. This process is known as antigen presentation (31).

Innate immune system activation and release of cytokines is interpreted by DCs as a maturation signal. This causes a variety of effects which include migration of the DC to a draining lymph node and release of further proinflammatory cytokines. Maturation also increases stability of the peptide-MHC complex as well as triggering upregulation of co-stimulatory molecules, such as CD40 and CD86. DCs however can also be directly activated by exposure to pathogens as they also express pathogen recognition receptors (PRRs), such as TLRs, and thus respond to PAMPs. This interaction triggers intracellular signalling which results in activation of mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB) and interferon regulatory factor (IRF) pathways. This again leads to DC maturation, migration to lymphoid organs as well as upregulation of co-stimulatory molecules and release of proinflammatory cytokines (31, 32).

The upregulation of these co-stimulatory molecules is very important in the differentiation of self and maintenance of tolerance as MHC-peptide presentation in the absence of co-stimulation will not induce T cell activation. Immature DCs typically express low levels of MHC II and costimulatory molecules and therefore are inclined to promote T cell anergy and regulatory T (Treg) cell generation, which is important for the maintenance of immune homeostasis (33-35). This is also a feature of a physiological subset of DCs cells found *in vivo* which are termed regulatory DCs or tolerogenic DCs. Although these DCs are still able to present antigen to antigen-specific T cells they are resistant to maturation-inducing signal and downregulate expression of co-stimulatory molecules and proinflammatory cytokines. Simultaneously however they also upregulate the expression of inhibitory molecules such as programmed death ligand 1 (PDL1) and anti-inflammatory cytokines release, such as TGF-β (36). This results in T cell anergy and generation of Tregs as well as T cell apoptosis (37).

Therefore priming and activation of T cells following interaction with DCs requires both a MHC-peptide complex/T cell receptor (TCR) interaction as well as the engagement of co-stimulatory molecules, such as CD86 or CD40, with T cell expressed CD28 or CD40L, respectively. Notably MHC class I will activate naive CD8 cells whereas MHC class II leads to the activation of CD4 T cells (38). As well as cell-cell contact naive T cells are also exposed to inflammatory cytokines over this period which is an important element in activation and can skew downstream development (39) (see T cell section below).

PDCs are a relatively newly identified DC subset. They were initially described by pathologists in the 1950s as plasmacytoid T cells or plasmacytoid B cells based on their

morphology and expression of surface markers which included, CD4, HLA-DR, CD36 and CD68 (40). A few decades later in the 1980s a poorly defined cell subtype was identified that produced large amounts of type 1 interferon causing it to be categorised as a natural-type-1-interferon producing cell. From the work in the late 1990s of Nobel Prize winner Ralph Steinman and others these 2 cell subsets were shown to be the same and in fact were DCs, leading to the classification of pDCs that are known today (41). Identification in humans of the unique surface marker CD303 (BDCA-2), a novel type II transmembrane C-type lectin, has greatly aided in identification and subsequent investigation of pDCs and they are now generally defined (in humans) as being $CD303^+$, $HLA\text{-}DR^+$, $CD123^+$, $CD45RA^+$ and $CD11c^-$ (42). Even within the DC population pDCs are a rare subtype and make up only 0.3-0.5% of circulating PBMCs. Despite their low number they function as the main producers of interferon-α *in vivo* and have been shown to produce 1000 times more interferon-α than any other cell type (43). PDCs do not exist in isolation and recently it has been shown that upon stimulation their interferon-α production can be further amplified through interactions with B cells, NK cells as well as T cells (44). They can also function as APCs by the upregulation of HLA-DR and production of other pro-inflammatory cytokines, such as TNF-α and IL-6 (45). Furthermore TGF-β exposed pDCs can participate in skewing towards a Th17 response (46). Thus pDCs, by their production of type 1 interferons as well as their APC capacity, can provide a link between the innate and adaptive immune system in the fight against foreign invaders or in the promotion of an autoimmune phenotype (41).

Conversely pDCs have also been proposed to have tolerogenic potential (45). They express high levels of the immunomodulatory enzyme indoleamine-pyrrole 2,3-dioxygenase (IDO) (45, 47, 48). This is a rate limiting intracellular enzyme in the break-down of the essential amino acid tryptophan into kynurenine and this can have tolerising effects by a variety of ways (49). The localised depletion of tryptophan can activate the stress kinase general control nonderepressible 2 (GCN2) in developing $CD4^+$ T cells which, along with the generation of soluble tryptophan catabolites, promotes Treg differentiation (49, 50). In addition kynurenines have been recently identified as ligands for the aryl hydrocarbon receptor (AHR) and ligation of this receptor promotes Tregs differentiation as well as inhibiting Th17 differentiation (51-53). These effects are seen i*n vivo* and *in vitro* where pDCs skew naïve T cells towards a functional Treg phenotype via IDO (45, 47, 54, 55) as well as by increased expression of PDL1 and inducible T-cell co-stimulator ligand (ICOSL) (56-61). However what is the net effect of pDCs in human

disease with regards to these two, relatively conflicting, functional capacities is often unclear.

T cells

T cells are divided into two main classes, $CD4^+$ and $CD8^+$ but both start their development in the thymus from bone marrow derived progenitor cells. Here they undergo extensive positive and negative selection that ultimately results in naive $CD4^+$ and $CD8^+$ T cells displaying on their cell surface an extensive and comprehensive selection of unique TCRs (62). These TCRs recognise a wide range of foreign peptides displayed in conjunction with MHC molecules on the surface of APCs, the most efficient of which is the DC.

$CD4^+$ T cells can form a variety of diverse functional subsets depending on the signals they receive during their differentiation process. These include a growing diversity of T helper (Th) subclasses each with a distinct transcription factor resulting in distinctive cytokine production profiles and functions (38) (figure 1.2). Th1 cells produce, among others, pro-inflammatory cytokine interferon-γ and evoke cell-mediated immunity and phagocyte-dependent inflammation. An imbalance favouring Th1 response has conventionally been associated with the development of autoimmunity. Th2 cells, which produce IL-4, IL-10, and IL-13, evoke strong antibody responses (including those of the IgE class) and eosinophil accumulation, but inhibit several functions of phagocytic cells. When the balance favours Th2 response it classically promotes allergy (63). More recently Th17 cells were described which produce, amongst other cytokines, IL-17 which has been shown to facilitate anti-fungal responses, as well as having a major role inflammation and autoimmunity (64-66). Naive $CD4^+$ T cells can also differentiate into follicular helper T (Tfh) cells, which assist B cell responses, or Tregs, which are involved in homeostasis and tolerance.

Activation of naive $CD8^+$ T cells results in a highly cytotoxic cell subset. They traffic rapidly out of the lymphoid tissue into the peripheries to the site of infection where they kill any infected cells and release cytokines such as interferon-γ. This has earned them their alternative name, cytotoxic T cells (67).

Once primed and activated T cells undergo clonal expansion and acquire effector cell functions. This is followed by an eventual reduction in number and a formation of memory cell populations that are long lived and remain in the circulation and lymphoid

organs. Upon re-exposure to the same antigen the T cells are able to expand rapidly from the memory T cell pool demonstrating the existence of immune memory (68).

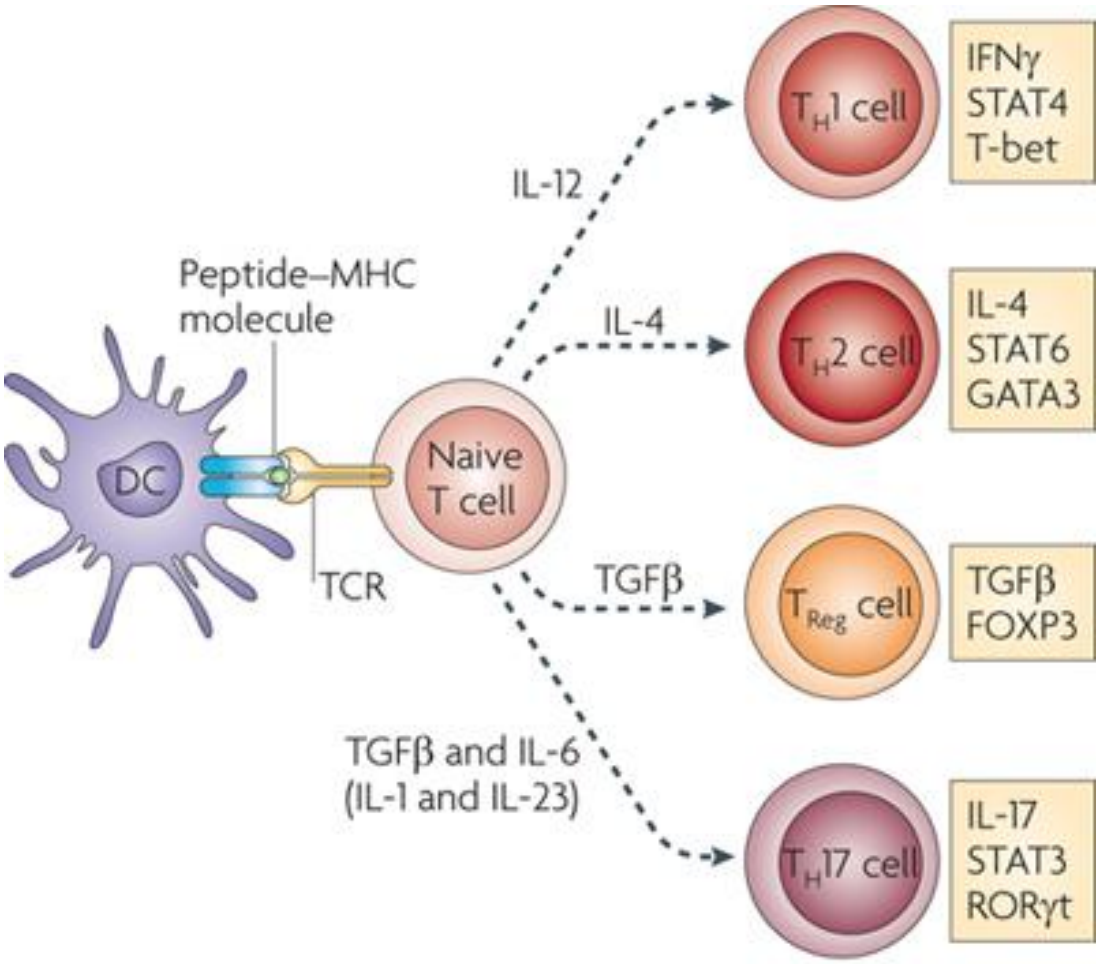


Figure 1.2 Dendritic cell and Naive T cell interactions

Dendritic cells (DCs) have the ability to skew T cell differentiation. Naïve $CD4^+$ T cells can be polarized into different effector T cell subsets; T helper 1 (T_H1), T_H2, T_H17 and regulatory T (Treg) cell. This process is controlled by distinct sets of transcription factors as well as the inflammatory cytokine milieu; IL-12 will promote T_H1 differentiation characterised by expression of the transcription factor T-bet and STAT4; IL-4 will promote T_H2 differentiation characterised by expression of the transcription factor GATA3 and STAT6; TGFβ will promote Treg differentiation characterised by expression of the transcription factor FOXP3; IL-6 and TGFβ will promote T_H17 differentiation characterized by expression of the transcription factors RORγt and STAT3, additionally IL-1 and IL-23 can promote and/or stabilize T_H17 cell differentiation and expansion.

IL, interleukin; STAT, signal transducer and activator of transcription; GATA3, GATA-binding protein 3; TGFβ, transforming growth factor-β; FOXP3, forkhead box P3; RORγt, retinoic acid receptor-related orphan receptor-γt; IFNγ, interferon-γ; TCR, T cell receptor Figure adapted from Zou *et al*, 2010 (65)

B cells

The other arm of the adaptive immune system is the B cell. These cells differentiate in the bone marrow before migrating to lymphoid organs and circulating in the blood. They require external signals for survival which maintains a stable homeostasis of the B cell pool. The B cells express a B cell receptor (BCR) which can sense soluble antigen as well as membrane bound antigen, e.g. provided by APCs. The BCR consists of membrane bound immunoglobulin (IgM) non-covalently bonded to a heterodimer composed of CD79a (Igα)/ CD79b (Igβ). The antigen specificity is determined by the variable domain of the IgM. Following the binding of the BCR to its cognate antigen tyrosine residues in the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) portion of CD79a and CD79b are phosphorylated and trigger downstream signaling pathways that trigger the first signal of B cell activation and cause migration to lymphoid tissue (69). The B cell can also present antigen in MHC class II complexes. This results in further costimulation by interactions with Tfh residing within the lymphoid tissue (70). In addition, B cells can respond to antigen independently of T cell help by activation of PRRs, such as TLR7, and after costimulation signals from other immune cells, such as DCs via B-cell activating factor (BAFF)–BAFF receptor interactions (71-73).

After activation the naïve B cell can differentiate into a short-lived IgM (or IgG) secreting plasma cell which occurs outside germinal centres. Alternatively within germinal centres the B cell can undergo class switch and differentiate into memory B cells or long-lived plasma cells that produce IgG, IgA or IgE antibodies (Figure 1.3) (68). Memory B cells remain in the circulation and in the secondary lymphoid organs after the infective insult has passed but are intrinsically programmed for enhanced antigen responses, in part due to their increased receptivity to antigen and to co-stimulation. Thus if the same antigen is ever encountered again they rapidly proliferate and differentiate into a high affinity antibody (IgG, IgA, IgE isotypes) producing plasmablast and plasma cell (68). Finally there is a further subset of B cells that has only been relatively recently identified termed regulatory B cells (Bregs). This subset has the ability to suppress or modulate T cell immune responses via a variety of mechanisms, including IL-10 and TGF-β production, as well as cell-cell contact (74).

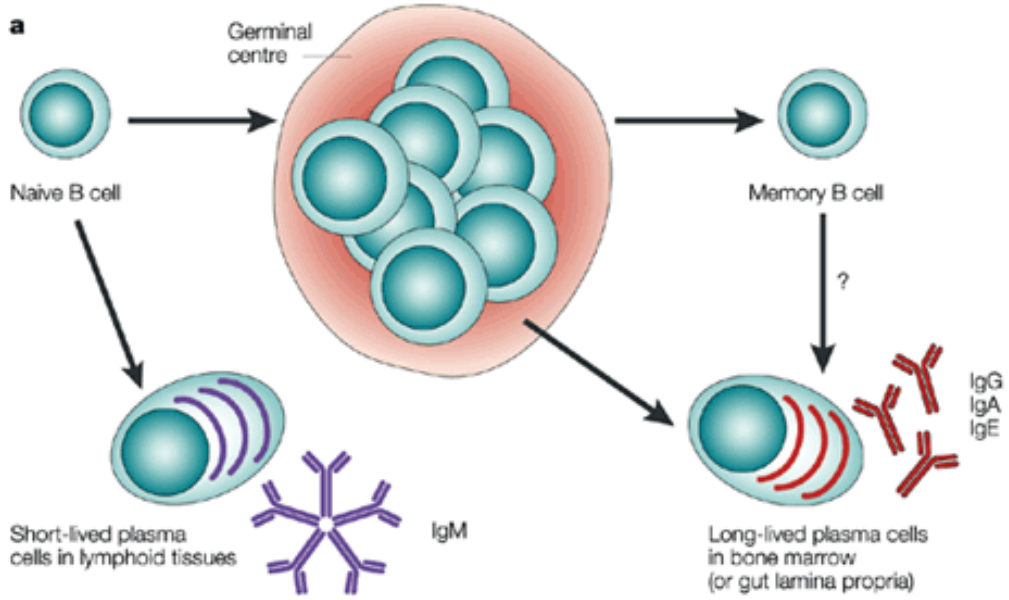


Figure 1.3 Naïve B cell differentiation pathways

Naïve B cells can be become activated by antigen and T cell help whereby they can differentiate into short lived plasma cells which reside in the lymphoid tissue. These are generated during the primary response and mainly generate IgM but can produce IgG as well. However upon activation naïve B cells can also seed into germinal centres. From there, often with the help of T follicular cells, they can either become 1) memory cells which are long lived and remain in the circulation, or 2) they can terminally differentiate into long lived plasma cells in the bone marrow which make mainly IgA, IgG and IgE. Finally there is some suggestion that upon encountering the same antigen again memory B cells can differentiate into terminally differentiated long lived plasma cells also. All arrows are driven by both antigen exposure and T cell help. Figure adapted from Gray, 2010 (68).

1.2.3 Evidence of immune dysfunction in RA

Rheumatoid arthritis is an autoimmune condition where there is dysregulated immune function and this can affect nearly all immune cells (10, 75-83). Understanding the pathogenic processes involved can both direct understanding of the disease as well as help identify new therapeutic targets.

Dendritic cells

Due to their ability to link both the innate and adaptive immune systems DCs are of interest in RA. Synovial tissue and fluid of RA patients has a marked infiltration of both immature and mature DCs which closely associate with T cells and B cell follicles. These DCs are highly activated with upregulation of MHC and costimulatory molecules. They are likely to also play a key role in promoting synovial inflammation by the production of large amounts of T cell attracting chemokines, as well as the pro-inflammatory cytokines IL-12 and IL-23 which promote T cell differentiation into pathogenic Th1 or Th17 cells, respectively (84).

A number of groups have shown that pDCs are not normally seen in the synovial compartment but migrate there in inflammatory settings, such as RA (85-88). Synovial fluid pDCs can activate T-cells *ex vivo* and have high CD303 expression suggesting interferon-α production (85-87, 89). Synovial fluid pDCs also have marked expression of myxovirus resistance protein A (MxA), a cytoplasmic protein induced by type 1 interferons suggesting active exposure to these cytokines intra-articularly (85, 89). Furthermore pDCs appear to cluster with B-cells within the rheumatoid synovium and there is a positive association between the number of pDCs in the joint and RF antibodies and ACPA titres (88). However the role of pDCs is likely to be complex and remains controversial as RA patients with inactive disease had higher levels of pDCs within the circulation which were able to skew T cells into IL-10 producing Tregs (54). In addition pDC depletion in a mouse model of RA actually caused a deterioration in phenotype with increased autoantibody generation (90). Thus it is still unclear if the net function of pDCs in RA is inflammatory or tolerogenic.

T cells

Over recent years T cells have been acknowledged as one of the main pathological cell subsets in RA. Some of the earliest evidence has been the infiltration of inflamed RA synovial tissue with T cells (91). There has also been success with therapies modulating

T cell function which includes the use of abatacept, a cytotoxic T lymphocyte-associated antigen-4 (CTLA4) fusion protein (92).

RA was historically considered a Th1 disease due to the clear association between interferon-γ and pathogenesis (93). Currently however there is evidence that an abnormal Th17 population is also present in RA. IL-17 is present at sites of inflammatory arthritis and in several animal models of arthritis inhibition of IL-17 limits inflammation and joint erosion. One proposed mechanism may be that IL-17 can amplify the inflammation induced by other cytokines, primarily TNF-α (94). However it is becoming apparent that Th2 cytokines are likely to be relevant also, particularly in early disease (80, 95). There is debate over the number of Tregs in RA with no clear consensus if there is a change in number, nonetheless there is some evidence that those that do exist also a have reduced functional capabilities making them less efficient at supressing inflammatory responses (75, 96-99).

B cells

As mentioned autoantibodies are frequently a hallmark of RA where there has been a breach of tolerance (100). This finding suggests B cells may be an important player in RA disease initiation and progression. The most frequently reported autoantibodies are RF and ACPA or anti-CCP, however the autoantigen(s) for these antibodies has not been defined with certainty (100). Autoantibodies are often present many years before the onset of symptoms (24) and ACPA antibodies present an independent risk factor for the development of RA (25, 100). These antibodies may have effects on clinical features; ACPA antibodies have been associated with increased bone erosions (101) and *in vitro* promote the differentiation of mononuclear cells to osteoclasts and therefore directly promote bone loss (102). Recently other antibodies have also been associated with RA and pre-RA states, for example anti-carbamylated protein antibodies (anti-CarP) (103). As well as their ability to produce autoantibodies, B cells can further contribute to disease pathogenesis by cytokine production, antigen presentation and T cell activation and have also been shown to be increased in the inflamed synovial compartment in RA, sometimes arranged in ectopic lymphoid follicles which appear to produce autoantibodies (83, 104, 105). There is also increasing evidence that Bregs are reduced in RA (106). The pathogenic role of B cells in RA has also be validated by the therapeutic success of B cell depleting therapies, such as rituximab which depletes CD20 expressing B cells (92).

1.3 Type 1 interferons

There is clear evidence of dysregulated cytokine production in RA and the importance of certain pathways, such as TNF-α or IL-6 has again been confirmed by the success of therapies that target them or their receptors (92). These cytokines have been further proposed as a mechanism whereby interaction between the innate and adaptive immune systems may occur and dysfunctional pathways entrenched (66). The work presented in this thesis focuses on one of these classes of cytokines, type 1 interferons.

Type 1 interferons are a family of cytokines first discovered over 50 years ago and were identified by their ability to inhibit or "interfere with" viral replication. Since their initial discovery it has emerged that, in humans, the type 1 interferon family is more extensive and complex than initially presumed. It consists of 13 functional interferon-α genes and single genes for interferon-β, interferon-κ, interferon-ε and interferon-ω (107). For the majority of this thesis I will focus on the effects of interferon-α. With the appropriate stimuli, usually during viral infections, all cell subsets can produce type 1 interferons. However DCs, and particularly pDCs, are the most prolific producers of type 1 interferons (43).

1.3.1 Type 1 interferon signalling

All members of the type 1 interferon family (interferon-α, -β, -κ, -ε and –ω) bind to a common heterodimer receptor consisting of 2 subunits, interferon-α receptor 1 and 2 (IFNAR1 and IFNAR2). Ligation of this receptor results in the activation of two receptor associated protein tyrosine kinases, Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). These phosphorylate signal transducer and activator of transcription (STAT) 1 and STAT2 which subsequently dimerise into a STAT1/STAT2 heterodimer. This binds to interferon regulatory factor 9 (IRF9) to form a trimer and it is this complex that binds to specific DNA target sequences within the nucleus celled interferon stimulated response elements (ISREs). Once bound these promote the transcription of interferon response genes (IRGs), such as MxA. There are over 2000 IRGs that can affect a wide range of signalling pathways generally associated with anti-viral response (108). Alternatively, the phosphorylated STAT proteins can form STAT1-STAT1 homodimers and these are able to bind to gamma-activated sequences (GASs) to induce pro-inflammatory genes. These are normally activated by type 2 interferons, such as interferon-γ, hence there can be an element of overlap between type 1 and type 2 interferon signalling (figure 1.4).

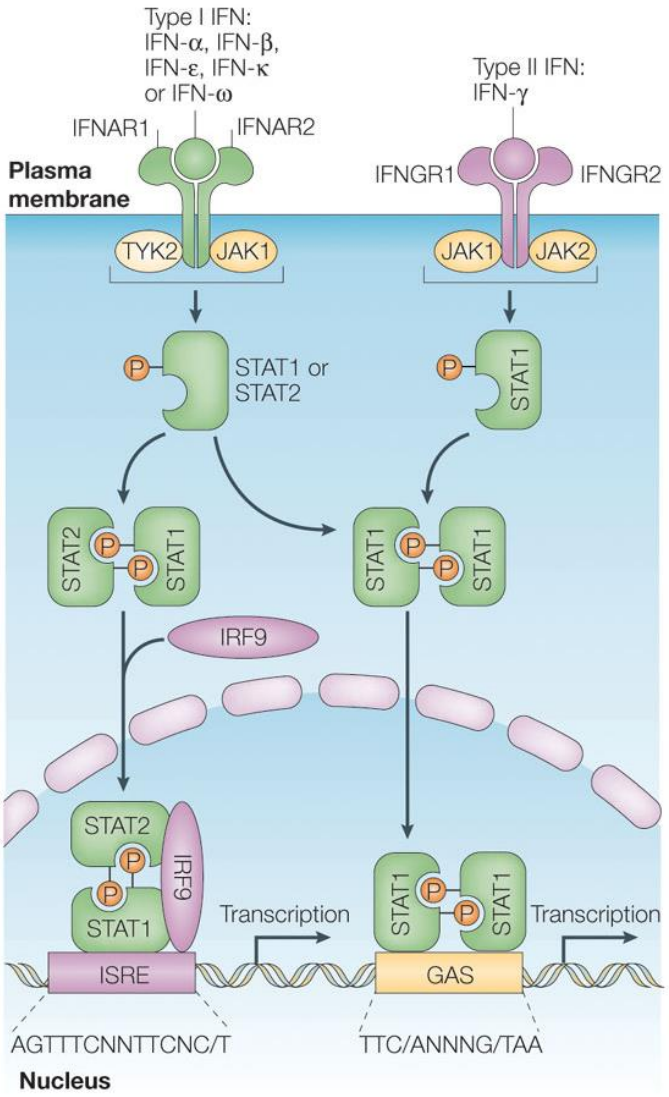


Figure 1.4 Interferon receptors and activation of classical JAK–STAT pathways by type I and type II interferons.

Type I interferons (interferon-α, -β, -κ, -ε and –ω subtypes) bind a common type 1 interferon receptor (IFNAR) made up of two subunits IFNAR1 and IFNAR. These subunits associate with TYK2 and JAK1 respectively. TYK2 and JAK1 activation results in phosphorylation of STAT2 and STAT1; this leads to the formation of STAT1-STAT2-IRF9 complexes which translocate to the nucleus where they bind DNA interferon stimulated response elements (IRSEs) and initiate transcription of interferon response genes (IRGs). Type 2 interferon (interferon-γ only) binds the type II interferon receptor which is made up of IFNGR1 and IFNGR2. These subunits associate with JAK1 and JAK2 respectively. Both type I and II interferons can also induce STAT1-STAT1 homodimer formation. These translocate to the nucleus and bind GAS elements which are also present in the promotor regions of certain IRGs. Thus there can be a cross over between type 1 and type 2 interferon signalling.

STAT, signal transducer and activator of transcription; IRF, interferon regulatory factor; TYK2, tyrosine kinase 2; JAK, Janus kinase. Figure adapted from Platanias, 2005 (107)

1.3.2 Sensors that trigger type 1 interferon production

The production of type 1 interferons occurs predominantly following the encounter of PAMPS which are associated with foreign bacteria/viruses, such as viral RNA or bacterial DNA. These are detected by PRRs and can help discriminate between self and non-self since PAMPs are widely expressed in pathogens but not in host/human cells. PPRs that detect nucleic acids can be broadly grouped into TLRs, which monitor the contents of endosomal compartments, and cytosolic receptors, such as the retinoic acid-inducible gene-I-like receptors (RLRs), which monitor nucleic acids within the cytoplasm.

PDCs express the endosomally located TLR7 and TLR9 which detect single stranded RNA (ssRNA) and DNA hypomethylated CpG motifs, respectively. ssRNA is found in viruses and hypomethylated CpG is mainly found in bacterial DNA. TLR9 CpG ligands can be divided into CpG-A and CpG-B and, due to their intrinsic structure and the resultant speed of intracellular transport, CpG-A and CpG-B have different effects on pDCs. They both bind TLR9 which triggers signal transduction through the adaptor protein myeloid differentiation factor 88 (MyD88). However due to the phosphodiesterase backbone seen in CpG-A it is trapped for longer in the endosome. This predominantly induces the nuclear translocation of IRF7 and the subsequent rapid and potent transcription of interferon-α. CPG-B is more rapidly trafficked through the endosome and subsequently, via activation of MyD88-NFκB, predominantly causes the upregulation of co-stimulatory molecules and antigen presentation markers, such as CD86, HLA-DR, as well as the release of other inflammatory cytokines such as IL-6 and TNF-α (109). TLR7 signalling causes increase of type 1 interferons by MyD88 associated pathways but also causes pDC maturation (110).

The effect of TLR signalling can be modulated by ligation of certain pDC surface markers. Immunoglobulin-like transcript 7 (ILT7) is selectively expressed on human pDCs and crosslinking of ILT7 induces an ITAM-mediated signalling cascade which negatively regulates TLR7/9-induced type I interferon and cytokine responses. *In vivo* the physiological ligand for ILT7 is bone marrow stromal cell antigen 2 (BST2) which is expressed on B cells and pDCs upon exposure to type 1 interferons, thereby potentially functioning as a negative feedback mechanism (111). CD303 ligation is another important negative feedback mechanism on the production of type 1 interferons. CD303 signalling causes intracellular calcium mobilisation and protein tyrosine kinase phosphorylation, such as spleen tyrosine kinase (SyK). This reduces IRF7 nuclear translocation and can also decrease NFkB signalling, thus reducing type 1 interferon production and other pro-

inflammatory cytokine production (42, 112-114). More recently death receptor 6 (DR6) has been identified as being able to modulate pDC production of type 1 interferons although what relevance this has *in vivo* is unclear (115).

Other intracellular PRRs important in the induction of type 1 interferons are the cytoplasmic RLRs. This family consists of retinoic acid-inducible gene-I (RIG-I/DDX58), melanoma differentiation associated gene-5 (MDA5/IFIH1) and 'laboratory of genetics and physiology' (LGP2/ DHX58). These receptors detect structural features of viral RNA that are distinct from host RNA and initiate a potent antiviral response. RIG-1 is reported to detect short dsRNA and MDA-5 long dsRNA within the cytoplasm. Upon activation these RLRs bind to MAVS, a mitochondrial transmembrane protein that acts as an adaptor between the RLR sensors and TANK binding kinase 1 (TBK1) and the transcription factors IRF3 and IRF7. This ultimately promotes the production of type 1 interferons (116-118). Intracellular receptors for DNA include stimulator of interferon genes (STING), another transmembrane receptor, which also functions to link cytosolic DNA to a TBK1/IRF3/IRF7 antiviral response; thus stimulating type 1 interferons production (116, 119). STING expression is further amplified by exposure to type 1 interferons (120). In addition STING can also be activated by cyclic dinucleotides produced by bacteria as secondary messengers (121). Other DNA receptors include IFI16 and it is hypothesised that many more are still to be discovered (122).

Despite their overlapping function certain aspects are quite distinct between these PRRs. For example TLR are differentially expressed across cell types (i.e. pDCs only express 7 and 9) and are predominantly found in leukocytes, whereas cytosolic PRRs are expressed much more broadly and are present in most cells (116, 117). They may also have distinct relative importance in some cell subsets, for example one study suggested that in mice pDCs preferentially use the TLR system for viral detection (123) and, unlike monocytes, express constitutively low levels of RIG-1 (124). Other distinctions are that when pathogen-derived nucleotides interact with endosomally expressed TLRs following endocytosis this process does not require cellular infection. Alternatively, cytosolic PRRs detect nucleic acids that reach the cytosol, which typically indicates that the cell has been infected. This means TLR-expressing cells respond to pathogens independently of cellular infection, whereas cytosolic PRRs act as general cell-autonomous sensors of infection. Nonetheless taken together these two groups of PRRs can facilitate the recognition of viral/bacterial nucleic acids in multiple scenarios of infection and initiate a rapid anti-viral/bacterial type 1 interferon response.

1.3.3 Effect of type 1 interferons on immune cells

Type 1 interferons have the potential to enhance the survival, proliferation and effector functions of numerous immune cell types, many of which have implications for autoimmunity.

Dendritic cells

DCs exposed to type 1 interferons become activated with upregulated HLA-DR expression and increased expression of co-stimulatory molecules such as CD40, CD80 and CD86. In predisposed individuals this can lead to the efficient presentation of self-antigen to previously non proliferative and low-affinity autoreactive T cells, leading to induction of autoimmunity (125). Type 1 interferon signaling can also increase DC survival and this may also contribute to autoimmunity as, in SLE susceptible mice, apoptosis resistant DCs can induce a lupus like syndrome (126). Interferon-α, along with GM-CSF, can also cause the differentiation of monocytes into functional DCs which have an activated phenotype and can efficiently stimulate T cells (127-129). Finally, interferon-α upregulates pDC, mDC and monocyte expression of TLR7 and IRF leading to an increased responsiveness of these cells to any immunostimulatory TLR7 ligands, which would further augment interferon-α synthesis (130).

T cells

Type 1 interferons can also affect T cells. They can skew naïve $CD4^+$ T cells towards a Th1 phenotype, which results in increased interferon-γ production, as well as promoting the generation of T-follicular helper cells that are efficient at activating B cells and promoting antibody production (131). Regulatory T cell immunosuppressive function is also impaired by high interferon-α levels (132). Type 1 interferons can promote T cell survival and promote the cytolytic activity of $CD8^+$ cytotoxic T cells, as well as prolonging the proliferation and expansion of antigen specific $CD8^+$ T cells (133-137). Furthermore type I interferons can inhibit B- and T-cell lymphopoiesis. This would effectively limit any dilution of expanded antigen-specific clones which would be potentially beneficial in the response to foreign antigens, but harmful in autoimmunity (138).

B cells

Type 1 interferons can directly affect B cells in a number of ways. They can lower the BCR signaling threshold, increase TLR7 expression as well as upregulate cell surface

molecules such as CD69, CD86 and HLA-DR that affect B-cell function and survival (139, 140). Type 1 interferons further promote longevity by upregulating BCL2 which makes the B cells relatively resistant to apoptosis (141). Finally type I interferon exposure also positively affects the B-cell response to IL-6, which is an important cytokine involved both in B-cell survival and differentiation (142). Autoantibody production may be further affected by interferon-α due to it enhancing BAFF release from other immune cells, particularly DCs or monocytes. This promotes the survival, differentiation and activation of (auto)reactive B cells as well as promoting differentiation into plasmablasts and immunoglobulin class-switched cells (143-146). B cells may also positively feedback into the production of type 1 interferons by autoantibody containing immune complexes binding to FcγRIIa (CD32) on pDCs and subsequent internalization of the immune complex. These can contain self-antigen in the form of nuclear material, particularly in SLE, which can trigger intracellular nucleic acid sensing pathways to promote further pDC interferon-α release (147-151) (figure 1.5).

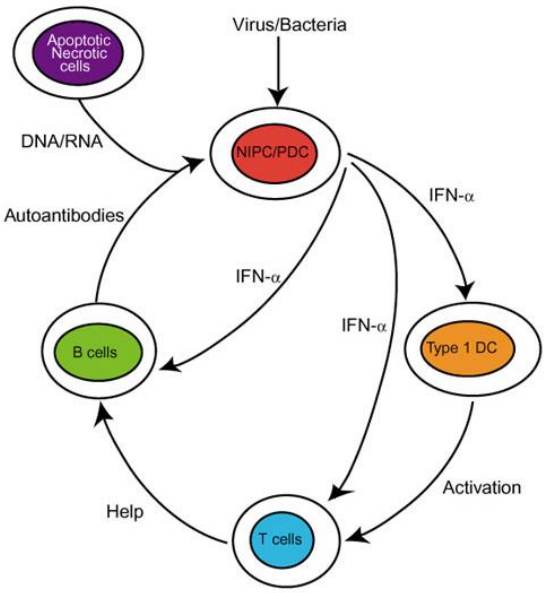


Figure 1.5 Schematic demonstrating the diverse effects of interferon-α on immune cells

Plasmacytoid dendritic cells (pDCs or natural interferon producing cells, NIPCs) are activated to release interferon-α (IFN-α) on interaction with either viruses/bacteria or with autoantibody containing immune complexes formed with necrotic or apoptotic cell material. IFN-α can cause type 1 DC (or myeloid dendritic cell) maturation which promotes T cell activation. T cells can also be directly affected by IFN-α and the end product of both these effects is B cell help. B cells are also directly activated by IFN-α but the additional T cell help can promote the generation of antibodies, some of which can be autoantibodies. These can form further immune complexes which promote further IFN-α release from pDCs.

1.3.4 Type 1 interferons in autoimmunity

As previously highlighted many of the effects induced on exposure of cells to type 1 interferons have relevance to autoimmunity. These effects have been investigated in a number of autoimmune conditions such as primary Sjogrens syndrome (pSS), dermatomyositis but most research has been done in systemic lupus erythematous (SLE) (152, 153). I will therefore mainly present evidence relating to type 1 interferons and SLE. SLE is a chronic autoimmune condition affecting the skin, joints, and internal organs. In the 1970s it was first noticed that patients with SLE had increased levels of interferon-α in their sera (78, 154). There have been a number of gene expression studies showing activation of the type 1 interferon pathway in SLE and in cross-sectional studies this was associated with disease severity as well as propensity to develop renal involvement (155-159). Furthermore interferon-α inducible chemokines (e.g. CXCL10, CCL2 and CCL19) have a clear temporal association with disease and could predict future disease flares (160). Finally trials interferon-α signalling inhibition have had some success ameliorating disease and autoantibody titres (157, 161, 162).

In vivo type 1 interferon activity is predominantly measured by expression of IRGs that are upregulated upon exposure to type 1 interferons. This is known as an interferon gene signature (IGS) and the majority of SLE patients have evidence of an IGS (108, 130, 155). There is increasing appreciation however that, in SLE at least, there is overlap between the effects of type 1, type 2 (interferon-γ) and indeed type 3 interferons (interferon-λ), in the generation of an IGS (163, 164). Nonetheless sufficient evidence as outlined above links type 1 interferons to disease pathology and SLE is acknowledged as a prototype disease where type 1 interferons play a clear pathogenic role (157).

1.4 Type 1 interferons in RA

In spite of the body of evidence that links interferon-α to autoimmunity, this cytokine has received relatively little attention in RA. However there is nonetheless an emerging appreciation that type 1 interferons may be implicated in the pathogenesis of RA.

1.4.1 Type 1 interferon associated genes and RA susceptibility

The most common polymorphism associated with increased RA risk is in the *HLA-DRB1* allele, termed the "shared epitope." This is a five amino acid sequence motif in residues 70–74 of the HLA-DRβ chain and is carried by the vast majority of RA patients (165). However increased availability of techniques that interrogate the genome has identified other gene polymorphisms that increase RA susceptibility. Among those identified a

considerable number are involved in the regulation or production of type 1 interferons (166). Three polymorphisms that increase susceptibility have been identified in IRF5 and these effects are most marked in those that have the shared epitope (167-170). There are also copy number variations of IRF1 which increase RA susceptibility (171), and *in vivo* RA mouse models lacking IRF1 had a reduced frequency and severity of disease (172). There have also been reports of associations with downstream signalling of type 1 interferons with STAT4, STAT1 and STAT2 all being implicated (173, 174)**.** Upstream signalling is also implicated with polymorphisms in IFI1H, a RNA cytoplasmic sensor for viral infection, as well as TLR genes (TLR2, TLR4, TLR8 and TLR9) increasing disease risk (175). In SLE these polymorphisms were associated with increased interferon activity suggesting there may also be a functional consequence of these polymorphisms in RA (176).

1.4.2 Type 1 interferons in early and established RA

There is evidence from both mouse work and clinical studies that type 1 interferons may be important early on in disease. In mice intra-articular type 1 interferon injections induce inflammatory arthritis (177) and inhibition of interferon-α signalling pathways by a variety of means prevented the development of inflammatory arthritis in susceptible animal models (172, 177-179). In humans interferon-α administered as an anti-viral therapy in some patients triggered a symmetrical polyarthritis consistent with RA and some even developed RA associated autoantibodies (180). Some have suggested that infections, known to produce a surge in circulating interferon-α, may promote disease flares in RA, although this is not been conclusively proven (181). Finally in ACPA positive arthralgia patients, an at risk group for developing RA, an IGS predicted the development of RA (182, 183).

There is an IGS reported in a subset of established RA patients, normally quoted to be present in between 20-30% (155, 184-186). This signature is also reported in synovial membrane which supports the involvement of type 1 interferons in target tissue (153). Nonetheless, contrary to what is seen in SLE, in established RA there is no published association between the IGS and disease activity, although notably for all these studies patients were on DMARDs (186, 187). One study did report an association between serum interferon-α and DAS-28 in established RA but this has not been replicated (188). There is currently conflicting evidence to support the hypothesis that the IGS in RA is the same "signature" as that seen in SLE. Some studies report the transcriptomics are the same (5, 189), whereas others suggest they are distinct with RA displaying a lower type 1

interferon involvement with a further emphasis on JAK/STAT pathways, chemokines and chemokine receptors (153, 185, 190, 191). This suggests the type 1 interferon associated pathology between these two conditions may be distinct. In addition the type 1 interferon family may be more complex than previously appreciated as the different type 1 interferons do not have a universal effect on RA pathophysiology. Interferon-β, a type 1 interferon encoded by a single gene, appears to be distinct from the interferon-α subtypes despite both cytokines binding to IFNAR (figure 1.4). Interferon-β has mainly anti-inflammatory properties and, following its successful use in the treatment of multiple sclerosis (MS) (192), investigations into a potential therapeutic role in RA were commenced. RA is frequently characterised by bony erosions and *in vitro* interferon-β plays an important role in bone homeostasis (193, 194). Interferon-β also downregulates the *in vitro* production of proinflammatory cytokines, such as IL-6 and TNF-α, from lymphocytes, macrophages and fibroblast like synoviocytes (FLS) as well as reducing the release of matrix metalloproteinases and GM-CSF from RA FLS (193, 195). These effects are also seen *in vivo* where interferon-β administration was able to ameliorate or prevent disease in animal models (196-198). Furthermore there was a modest response to interferon-β therapy in a phase 1 trial for the treatment of RA, however this was not reproduced in a double blinded phase 2 study (197, 199).

Despite the above caveats the IGS does have clinical relevance in RA as it can affect the response to certain biological therapies. The most extensively investigated of these has been rituximab, an anti-CD20 monoclonal antibody which depletes B cells. There is convincing evidence that a high IGS predicts, and is associated with, a poorer response to rituximab (23, 186, 200-202). Conversely however a high IGS predicts a better response to tocilizumab, an IL-6 targeting antibody (203), and, although there has been some variability between studies, a similar effect has been reported with infliximab, an anti-TNF-α antibody (204-206). There has been no investigation whether the IGS affects response to non-biological therapies, such as methotrexate or other standard DMARDs. Nonetheless the IGS is currently emerging as an intriguing biomarker in the treatment of RA.

What may be driving this production of type 1 interferons in RA is not clear. A study using a lymphotoxin-beta receptor-immunoglobulin fusion protein that blocks the lymphotoxin-αβ/LIGHT axis demonstrated a fall in the IGS in RA following its administration suggesting that this pathway may be involved, although to date it is not clear how (187). Another mechanism may be related to PRR signalling. TLR transcripts

are increased in early RA synovial tissue and in active disease (207, 208). Triggers of these pathways, such as microbial products or endogenous cellular breakdown products, are also found in the RA joint (209-212). Therefore, although PRR signalling has not been linked to the production of type 1 interferons in RA, it is conceivable that they may be implicated.

1.5 Retrotransposons

1.5.1 Retrotransposon biology

DNA has been traditionally categorised into protein coding sections (genes) which make up approximately 2% of the human genome, and non-protein coding sections which have been termed "junk DNA". However, it is increasingly appreciated that these non-protein encoding sections are not truly "junk" and in fact have transcriptional activity and potential regulatory relevance (213). A substantial portion of the human genome is derived from ancient transposable elements, such as retroviruses, that have been incorporated into the genome. These transposable elements were first described in the 1950s and have been divided into two separate major classes: DNA transposons or RNA retrotransposons/retroelements which replicate via an RNA intermediate (214) These make up approximately 3% and 40% of the human genome, respectively. The majority of these transposable elements no longer retain the ability to replicate, however some of the RNA retrotransposons do and this subset is the focus of a part of my work (121).

RNA retrotransposons replicate through what has been described as a "copy and paste" mechanism whereby they insert new copies of themselves into the genome. A strand of mRNA is generated and is developed into a double stranded DNA product which is then inserted into a unique region of the genome, often separate from the area of origin (121, 215). Retrotransposons/retroelements can be divided broadly into two main classes (figure 1.6). The first of which are able to encode all proteins required for this replication and insertion process. These include endogenous retroviruses (ERVs), often detected as long terminal repeats (LTRs), and long interspersed nuclear element 1 (LINE-1). LTR/ERVs make up approximately 8% of the human genome and are more clearly identified as relics of ancient retroviruses that have been incorporated into the genome. Their replication cycle and organisation is similar to that seen in exogenous retroviruses, such as human immunodeficiency virus (HIV), and they contain the *pol* gene encoding reverse transcriptase. They also encode additional proteins, *gag* and *env* but generally, although transcriptionally active, do not expand through new integration (216, 217).

LINE-1 are smaller than ERVs, approximately 6kb in length, and encode 2 proteins, ORF1 and ORF2 that coordinate replication through target-primed reverse transcription. ORF2 can generate a "nick" in the genomic DNA allowing for retroelement insertion as well as facilitating ORF-2 dependent reverse transcription into c-DNA at the site of insertion (215, 218). The final class of retroelements are termed non-autonomous retroelements. These are short interspersed nuclear elements (SINEs), the most common of which are known as "Alu". These are quite small at only 300bp but make up approximately 11% of the human genome (215). Due to their smaller size they do not encode the machinery necessary for their retrotransposition into the genome. However they are highly active and hijack the LINE-1 reverse transcription to facilitate their insertion/replication (218).

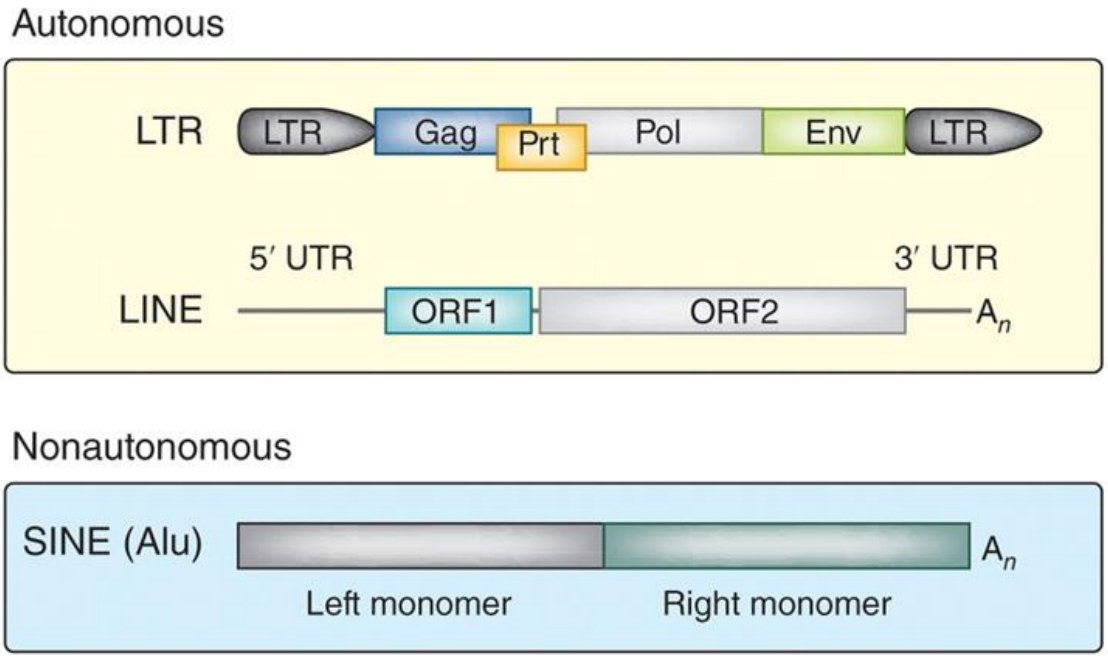


Figure 1.6 Types of retroelements in a mammalian genome

The types of retroelements in a mammalian genome are shown. Autonomous retroelements include endogenous retroviruses (ERV) - detected as long terminal repeats (LTRs) - as well as long interspersed nuclear element 1 (LINE-1). These both encode all proteins necessary for their replication and retrotransposition. Non-autonomous retroelements, such as short interspersed nuclear element 1 (SINE), the most common of which is Alu, are much smaller and hijack LINE-1 machinery to facilitate their mobility.

Gag, group-specific antigen; Prt, protease; Pol polymerase; Env, envelope; UTR untranslated region. Figure adapted from Volkman *et al.*, 2014 (121).

1.5.2 Retrotransposon processing and degradation

The retrotransposition of retroelements generates viral nucleic acid within the cell as both RNA and c-DNA but RNA-DNA hybrids can also form. This means that cytosolic RNA and DNA sensing pathways, such as the RLRs and STING (see section 1.3.2) can be triggered resulting in the release of type 1 interferons.

Research performed in the infectious diseases field on HIV, an exogenous retrovirus, as well as on so called type 1 interferonopathies, such as Aicardi–Goutières syndrome (AGS) has greatly aided understanding about intrinsic cellular mechanisms that have evolved to modulate retroviral replication (121). These mechanisms include a series of enzymes that can modulate both LINE-1 and ERV replication. The best characterised of these is three prime repair exonuclease 1 (Trex1), a human 3' repair exonuclease. This enzyme metabolises the DNA products of reverse transcription and therefore can block retrotransposition. This also prevents subsequent activation of cytosolic DNA sensing pathways, such as STING (219). Mutations in Trex1 which inhibit its function have been identified in AGS, as well as in some forms of familial chilblain SLE. These mutations cause a marked interferon-α response resulting in a severe autoimmune phenotype (220, 221). Other key enzymes include SAM Domain and HD Domain 1 (SAMHD1), which degrades cellular dNTPs thus preventing reverse transcription, and ribonuclease H2 (RNase H2), which degrades the RNA of any RNA-DNA hybrids and has also been proposed to degrade viral RNA in the context of reverse transcription. Again mutations in both these enzymes have been associated with the development of AGS (222, 223). These pathways are depicted in figure 1.7.

Additional pathways relating to nucleic acid sensing are also important. The enzyme cyclic GMP-AMP synthase (cGAS) can bind to immunostimulatory DNA, such as that formed during the process of retrotransposition, and promotes the formation of cyclic GMP-AMP. This can bind directly to STING and trigger downstream inflammatory responses (224). It is increasingly appreciated that the interferon response to DNA viruses, HIV and other retroviruses is mediated by the cGAS-cyclic GMP-AMP-STING pathway. However other pathways are also able to detect cytosolic DNA, for example IFI16 recognised HIV infection in monocytes (225) and undeniably it is likely that many potential DNA sensors are still currently unknown (122).

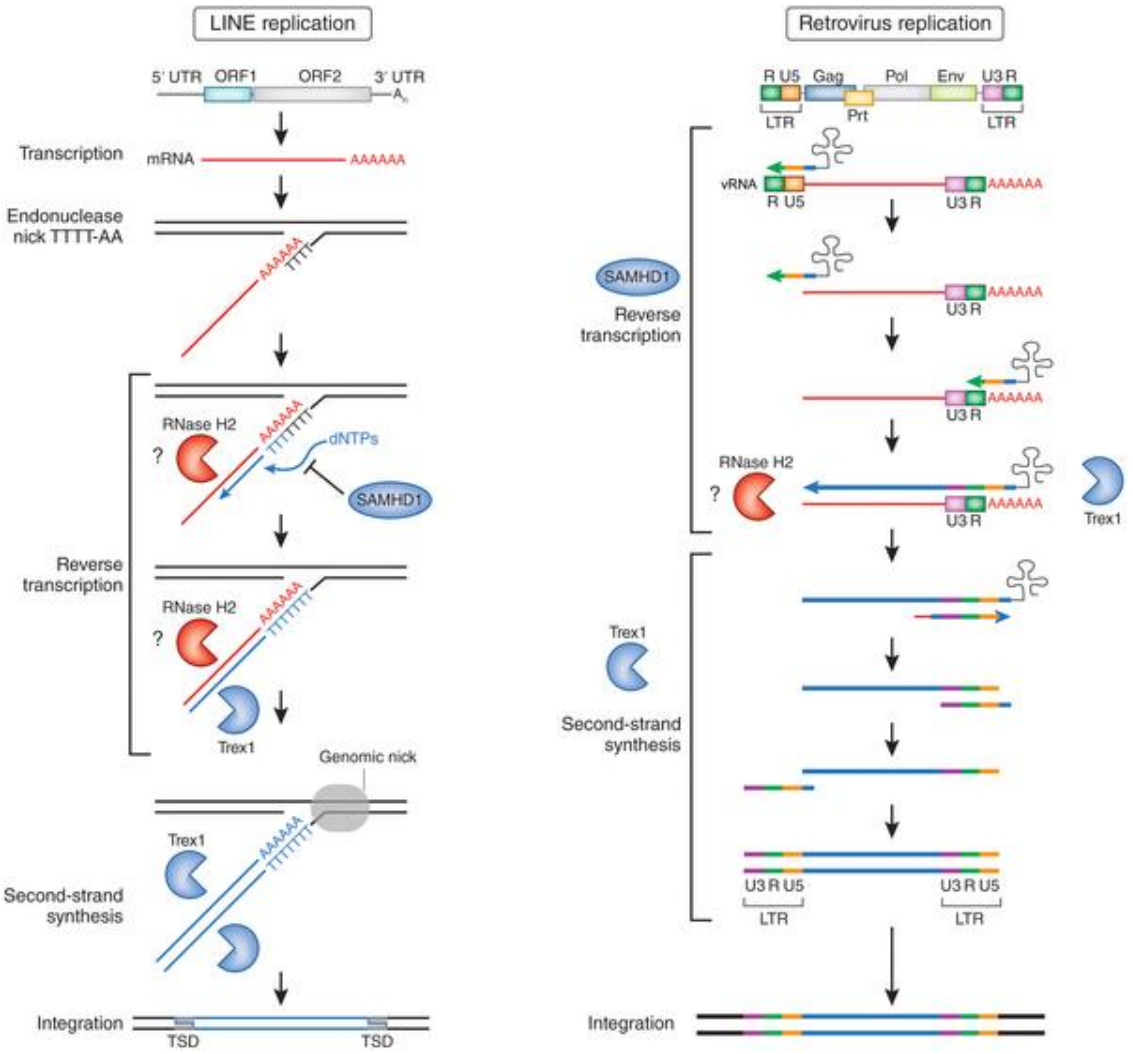


Figure 1.7 Retroelement replication and enzymatic pathways modulating this process

Various enzymes act to restrict the steps of LINE and retrovirus replication and retrotransposition by affecting products of reverse transcription: SAMHD1 inhibits reverse transcription through the degradation of cellular dNTPs; Trex1 metabolises the DNA products of reverse transcription; RNase H2 is hypothesised to degrade the RNA of RNA-DNA hybrids. These latter processes can block the retrotransposition of endogenous retroelements. U3, R and U5 denote regions of the ERV LTRs; TSD, target site duplication. Figure from Volkman *et al.*, 2014 (121)

1.5.2 Retrotransposons in autoimmunity

The understanding of how retrotransposons interact with the genetic environment has been relatively recent. Through their ability to reinsert themselves at various points in the genome, thereby facilitating genetic innovation, their role in historical human genomic evolution is appreciated (215). However these same mechanisms, can also have relevance

in human disease. These have been most extensively examined in malignancy where retroelement activity has been shown to be increased (226). This activity could potentially generate insertion mutations which disrupt normal gene function thereby predisposing to malignant processes (226).

More recently however retrotransposons have been implicated in the development of autoimmunity, for example a mouse model with a similar phenotype to SLE had amelioration of disease when treated with anti-retroviral therapy (227) and there are suggestions that HERV-K113 may be a genetic risk factor for some types of autoimmune diseases (228). Some have suggested that these ERVs could create so-called super antigens which could trigger autoimmune responses via molecular mimicry (229). However to date the link between retroelements and autoimmunity has mainly focused on viral nucleic acids present during the retrotransposition process triggering intracellular DNA/RNA sensing pathways and culminating in the production of type 1 interferons (121). Thus most research into this process has been done in diseases, such as SLE and pSS, where type 1 interferons play a defined pathological role (121, 230). For example an increase in LINE-1 transcripts was detected in the kidneys of patients with lupus nephritis and in the salivary glands of pSS patients. These LINE-1 RNA transcripts were subsequently implicated in the generation of type 1 interferons and activation of innate immune pathways and there was a suggestion that pDCs were associated with the increased interferon-α transcripts (231). The autoantibody Ro60, frequently found in pSS and SLE, can bind Alu elements and Alu transcripts were up-regulated in SLE whole blood samples relative to controls. Furthermore anti-Ro60-positive SLE immune complexes contained Alu RNAs and these could also trigger the production of type 1 interferons from PBMCs (232). Activation of downstream RNA/DNA sensing pathways is likely to be important in these processes as gain of function mutations in the pathways related to the sensing of viral RNA/DNA can result in marked interferon signalling and an autoimmune phenotype similar to SLE (121, 233-235). Indeed increased pDC TLR and RLR expression, potentially due to increased intracellular ligation, has been noted in IGS+ PSS patients when compared with IGS- patients (236).

Although retroelements have not been examined as extensively in RA they are nonetheless still likely to be relevant. There is evidence that LINE-1 transcripts and multiple ERV transcripts are increased in RA synovium when compared with OA or reactive arthritis (237-239). In keeping with this there is also down regulation of Trex1 in RA synovial fibroblasts. This means there will be a decrease in DNA degradation and

thus reduced inhibition of retroelement replication/activity (240). There is also evidence of ERV activity in peripheral blood of RA patients, particularly HERV-K (HML-2). The viral load of HERV-K is increased in RA plasma and synovial fluid when compared with healthy controls and OA patients and positively correlated with RA disease activity. There is also evidence of HERV-K *gag* and e*nv* proteins in RA and intriguingly these shared the same peptide sequences with rheumatoid factor epitopes on IgG1Fc (241-243). Finally there is HERV-K LTR repeat in the "shared epitope" RA risk allele, HLA-DQB1. This increases both the relative risk and the positive predictive value that an individual will develop RA compared with the shared epitope alone (244).

The current understanding of how these viruses have been integrated and sustained within the genome suggests that they may have been subject to some form of positive evolutionary selection pressure. Having increased retroelement activity may keep the immune system in a primed state which would allow a rapid and effective response against viral triggers. However the trade-off for this would be increased type 1 interferon signalling, which, in a susceptible individual, could trigger autoimmunity (121). Viral infection itself, with herpes viruses for example, has been shown to increase endogenous retroviral activity (242, 245). There is a current theory that infection may, in part, trigger the onset of autoimmune disease, such as RA (246). Thus how an individual responds to an infection and the subsequent degradation of retroelement products may play a role in the development of RA. These processes however have never been examined in early RA nor in the context of the RA IGS.

1.6 Hypotheses

The hypotheses underpinning my PhD relate to type 1 interferons in glucocorticoid and DMARD naive early RA.

Given the putative increased role of type 1 interferons at the onset of autoimmune disease I hypothesised that a similar effect may be occurring in early RA.

Furthermore, as pDCs are the main type 1 interferon producing cell, I hypothesised that pDCs produce the type 1 interferons in early RA that subsequently generate the IGS, and propose that pDCs are triggered by increased retroelement activity.

To address these hypotheses my PhD was divided into the following areas of study:

1) The IGS and early drug naïve RA

2) Peripheral blood pDCs and early drug naïve RA

3) Peripheral blood lymphocyte transcriptomics in IGS+/IGS- early drug naïve RA

4) Retroelement activity and the IGS in early drug naïve RA

Chapter 2: Materials and methods

2.1 Patient recruitment

2.1.1 Ethics

Ethical approval was provided by North East - Newcastle & North Tyneside 2 Research Ethics Committee for the project entitled *Prognostic and therapeutic biomarkers in an observational inception cohort: the Northeast Early Arthritis Cohort;* REC reference 12/NE/0251. In addition ethics was provided by South West 3 REC for the project entitled *Newcastle Autoimmune Inflammatory Rheumatic Disease Research Biobank;* REC reference 10/H0106/30.

2.1.2 Newcastle Early Arthritis Clinic

The Freeman Hospital Musculoskeletal Unit Early Arthritis Clinic is a NHS service for patients that have arthralgia with concurrent inflammatory features, as determined by the referring clinician. At visit 1 (week 1) patients were reviewed by the nurse specialist and have their disease activity assessed, undergo ultrasound examination of their hands and feet as well as have routine inflammatory and rheumatology blood tests performed. Disease activity is measured by the DAS-28 which is a composite score including tender joint count (TJC), swollen joint count (SJC), patient report visual analogue scale (VAS) and inflammatory markers CRP or ESR. At visit 2 (week 2) the patient is reviewed by a consultant rheumatologist and a diagnosis made on the basis of clinical history, examination and the above investigations.

Over the period of recruitment to this project (Dec 2013-July 2016) individuals which fulfilled the 2010 ACR/EULAR classification criteria for Rheumatoid arthritis (see Appendix A 1.0) were, where possible, recruited. All these patients were naïve to disease modifying anti-rheumatic drugs (DMARDs) and biological therapies. Any patient that had received any oral, intramuscular or intra-articular glucocorticoid steroids in the last 3 months were excluded. We also recruited patients that were positive for anti-CCP antibody (ACPA) and had arthralgia but had no evidence of inflammatory disease on ultrasound. These patients were termed to be in a "pre-RA" state.

Some of these early RA patients were followed up at 1, 3, 6 and 12 months and had peripheral blood and clinical parameters (including DAS-28) reassessed.

For all early RA patients where possible the drug history, including glucocorticoid steroid use was obtained at 3 and 6 months post diagnosis. Furthermore clinical response

to initial therapy at 3 months and 6 months was quantified by EULAR response criteria as defined in table 2.1.

Present DAS-28 score	**Improvement in DAS-28 score**		
	>1.2	**>0.6 or ≤1.2**	**≤0.6**
≤3.2	*Good response*	*Moderate response*	*No response*
>3.2 to ≤5.1	*Moderate response*	*Moderate response*	*No response*
> 5.1	*Moderate response*	*No response*	*No response*

Table 2.1 EULAR response criteria, using the DAS-28

2.1.3 Established RA and SLE patients

Patients who had a RA or SLE disease duration of >12 months and were established on anti-rheumatic treatments were also recruited. These were recruited from rheumatology clinics/ day unit services at the Musculoskeletal Unit, Freeman Hospital Newcastle upon Tyne. Where feasible patients who were not on oral glucocorticoid steroids were chosen, however this was not always possible. For all patients however an attempt was made to record the date of last glucocorticoid administration or dose of current treatment.

2.1.4 Healthy controls

Individuals with no personal history of autoimmunity or other medical conditions were used as healthy controls. They were recruited mainly from staff and students at Newcastle University.

2.2 RNA Analysis Techniques

2.2.1 RNA extraction

Whole blood RNA

Peripheral blood was collected in to Tempus blood RNA tubes (ThermoFisher Scientific) and stored at -80 °C. The tubes were then thawed and RNA extracted using the Tempus Spin RNA Isolation Kit (ThermoFisher Scientific, MA, USA) according to manufacturer's instructions. An additional step was included to exclude any

contaminating genomic DNA. This was carried out as per manufacturer's suggestion, where 100µl of Absolute RNA wash solution containing an optimised buffer and DNase enzyme mix (Applied Biosystems, CA, USA) was applied to the column and left for 15 minutes. This was then washed away (Wash buffer 2) and the remainder of the protocol was carried out as stipulated.

To determine the concentration and quality of RNA the NanoDrop ND-1000 spectrophotometer was used (NanoDrop Technologies, Wilmington, USA). The RNA was then frozen down at -80 °C.

Cell subset RNA

To isolate RNA from the flow cytometry sorted cell subsets (see flow cytometry sorting section 2.6.4) cells were first spun at 300g for 7 minutes to form a pellet. This was then re-suspended in 350µl of RLT Plus Lysis Buffer from Qiagen RNeasy Plus Micro kit, (Qiagen, Germany) to which β-mercaptoethanol (Sigma-Aldrich, MO, USA) had been added at ratio 1:100. This lysate was then added to a QIAshredder column (Qiagen, Germany) and spun at 1700g for 2 minutes. The eluate was then frozen at -80 °C pending the following steps.

The Qiagen RNeasy Plus Micro Kit (Qiagen, Germany) was used to extract the RNA as per manufacturer's instructions apart from one single modification. To optimise RNA yield the eluting solution was left on the column for 10 minutes and then spun as directed (1700g for 2 minutes). This eluate was then re-added to the column and the above step repeated. The concentration of RNA nucleic acids in this final eluate was again determined using the NanoDrop (as above) and the RNA was frozen at -80 °C.

Of note in this kit the lysate was first passed through a genomic DNA (gDNA) Eliminator spin column to remove any contaminating gDNA. To ensure adequate performance of this gDNA Eliminator spin column, a test run was performed where cell subset lysates were either passed or not passed through this column prior to all other steps being identical. Downstream purity with regards to gDNA removal was then checked (see PCR and Gel Electrophoresis sections).

2.2.2 Concentrating RNA samples

To standardise the cellular subset RNA content added to the NanoString nCounter Human Immunology V2 Panel (see section 2.7), upon thawing of the RNA 2µl of RNaseOUT recombinant ribonuclease inhibitor (40U/µl, Invitrogen) was added to the prevent

degradation and together this was centrifuged using the DNA 120 SpeedVac system (Thermo Scientific, Epson, UK) until completely dry. The pellets were then re-suspended in RNase Free water to final concentration of RNA of 10ng/µl. This was then re-frozen at -80°C prior to use.

2.2.3 Reverse transcription

Two separate reverse transcription techniques/protocols were used depending on the downstream application.

Whole blood interferon gene signature (IGS)

For analysis of the whole blood interferon gene signature (IGS) the following protocol was used. The total amount of RNA was normalised within each batch by adding an appropriate amount of RNA (depending on the concentration ng/µl) and RNase/DNase Free water (Sigma-Aldrich) to make a final volume of 10µl in a 0.2ml PCR strip. This was done in duplicate for each sample. For each reverse transcription negative controls were included; 10ul RNase/DNase Free water and for the sample where the RNA was relatively the most abundant 2 additional wells were created to which the DNA polymerase was later excluded. To all wells 1ul of random hexamers (1ug/ul, Integrated DNA Technologies, Inc, IA, USA) was added and the mixture was pulse spun to >800g and covered with a StarSeal adhesvive lid (StarLab, Germany). In a Takara PCR Thermal Cycler Dice the strip was heated to 70°C for 10 minutes and then chilled at 4°C for a further 10 minutes. A mastermix was generated of which 9ul added to each well. This mix included 0.01M DTT, 0.25mM dNTP, 1 unit SuperScript II and 4µl of 5X First Strand Buffer (all Invitrogen) with remaining volumes made up with RNase/DNase free water. An additional mix was made up where SuperScript II was excluded and the missing volume made up with RNase/DNase Free water. This separate mix was added to the reverse transcriptase negative control wells. The strip was then heated for 1 hour at 42°C. The resultant cDNA was then diluted down to 1:2.5 and 1:100 with RNase/DNase Free water and stored at -20°C.

Super-clean cDNA generation for retrotransposon analysis

For analysis of the whole blood retrotransposon activity the following separate reverse transcription protocol was used to ensure complete elimination of any contaminating genomic DNA. For future reference this was termed the “super-clean” protocol. 4µl of RNA (independent of starting ng/µl concentration), 1U of TurboDNase and 0.5µl of turbo

Dnase buffer was added (both Ambion, TX, USA) to a 0.2ml PCR tube. Negative controls were included; 4µl UltraPure™ DEPC-treated Water (ThermoFisher Scientific, UK) and a duplicate of the most abundant RNA sample from which future reverse transcriptase enzyme steps would be excluded. This mix was incubated in a Takara PCR Thermal Cycler Dice for 30 minutes at 37°C before 0.9µl of 100mM EDTA was added to inactivate the DNase and the tubes were incubated at 75°C for a further 10 minutes. Next 2.5ul of a master mix containing 4mM dNTPs (Invitrogen) and gene specific primers at final concentration of 2uM for each**.** Remaining volume was made up with UltraPure™ DEPC-treated water, which in this case was 0.5ul. These gene specific primers included those that specifically reverse transcribed LINE1 elements and the housekeeper gene TATA–binding protein (TBP). For our final analysis we only used LINE-1 and TBP sequences. Their nucleic acid sequences are shown in the following table 2.2.

Gene specific Primer	Sequence
LINE-1 elements	ACATGTGCACATTGTGCAGGTTAG
TATA –binding protein (TBP)	GGTGCAGTTGTGAGAG

Table 2.2 Sequences of gene specific primers used in reverse transcription

This mix was then incubated at 65°C for 5 minutes and then placed on ice for a further 2 minutes prior to a master mix being added to each tube which contained 4µl of 5X strand buffer (Invitrogen), 9mM MgCl (Life Technologies), 0.02M DTT and 40U RNase out (40U/µl) all from Invitrogen. This was then incubated at 25°C for 1 minute. SuperScript enzyme was diluted 1:3 dilution of in UltraPure™ DEPC-treated water and 1µl of this dilution was added to each tube, apart from the negative control tube which excluded any reverse transcription enzyme steps. For this tube 1µl of in UltraPure™ DEPC-treated water was added instead. The following PCR programme was subsequently run**:** 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 10minutes. RNaseH (5000U/µl, New England Biolabs, MA, USA) was diluted 2:3 with UltraPure™ DEPC-treated water and 1µl of this dilution added to each tube before the final incubation at 37°C for 20 minutes. The resultant cDNA was diluted 1:2.5 with UltraPure™ DEPC-treated water and frozen at -20°C.

2.2.4 Polymerase Chain Reaction

We used polymerase chain reaction (PCR) as part of the steps to verify that there was no gDNA contamination in either the RNA or cDNA that would be used to examine retrotransposon activity.

To verify the purity and lack of gDNA contamination in cDNA generated from the "super-clean" protocol we used *HBP1* gene specific primers that cross an exon intron boundary and can concurrently amplify both gDNA and cDNA. gDNA contaminated cDNA would therefore produce different fragment sizes (570bp and 152bp) whereas cDNA would only produce one band (152bp). Primer sequences can be found in the table 2.3. In brief 0.5µl of c-DNA was added to a 13µl PCR master mix containing 0.5µM forward and reverse primers (10mM, Sigma Aldrich, UK), PCR buffer (50mM KCL, 10mM Tris-HCL pH 8.3), 2mM MgCl2, 0.2mM dNTPs, 0.08 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, ABi, Life Technologies, UK) and UltraPure™ DEPC-treated water**.** Positive controls of genomic DNA (Gift from Professor John Loughlin) and a negative control of UltraPure™ DEPC-treated water were also amplified as above**.**

In an Applied Biosystems® Veriti® 96-Well PCR Thermal Cycler the following thermocycling conditions were used**:** a denaturation stage for 2 minutes at 94°C, followed by 32 cycles of 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds and a final step of 72°C for 5 minutes.

HBPI Primer sequence for c-DNA PCR	**Sequence**
Forward Primer	TCGAAGAGTGAACCAGCCTT
Reverse Primer	GAAGGCCAGGAATTGCACCATCC

Table 2.3 HBP1 primer sequence used for PCR of "superclean" cDNA to validate gDNA removal

With RNA generated from the individual cell subsets (Qiagen RNeasy Plus protocol) the following method was applied to check for adequate gDNA removal. Again *HPB1* primers were used (see table 2.4) but this time they were in the last exon of the 3'UTR region and so expressed only in gDNA. In brief 2ul of RNA was added to 8ul of a master mix containing 4ul 5X Phire reaction buffer, 0.4ul Phire Hot start DNA II polymerase (both Thermofisher scientific), 2mM dNTPs (Invitrogen) and 1.25mM forward and reverse HPBI primers. Positive controls of gDNA (Gift of Professor David Young, UK) at known concentrations (50ng, 5ng, 0.05ng and 0.005ng) as well as a negative control of water were also amplified as above.

HBPI Primer sequence for RNA PCR	Sequence
Forward Primer	TGCATATACATTGACTCTTGATGG
Reverse Primer	GTTGCCCCTAAATTTTGCA

Table 2.4 HBP1 primer sequence used on RNA to validate gDNA removal

In an Applied Biosystems® Veriti® 96-Well PCR Thermal Cycler the following thermocycling conditions were used: a denaturation stage for 30 seconds at 98°C, followed by 35 cycles of 98°C for 30 seconds, annealing at 58°C for 5 seconds, and extension at 72°C for 45 seconds, 72°C for 45 seconds and a single final step of 72°C for 1 minute.

For both of these PCR products were further analysed immediately afterwards by using gel electrophoresis (see below in 2.2.5).

2.2.5 Gel electrophoresis

The PCR products were electrophoresed through a 1% weight/volume agarose gel containing ethidium bromide (10 mg/ml; Sigma) at a concentration of 1 µl/100 ml agarose and UV visualised using a G:BOX gel doc system (Syngene, Cambridge, UK). A 2-Log DNA ladder (New England Biolabs, MA, USA) was used for the whole blood c-DNA super-clean protocol products where Contaminating DNA will produce 2 bands (570bp and 152bp), while uncontaminated cDNA will have just one product (152bp). A GeneRuler^{TM} 100bp and 1Kb DNA ladders (Thermo Fisher Scientific) was used to assess the band sizes for the cell subset specific RNA PCR products, where a detectable band at 1011bp indicated gDNA contamination.

2.2.6 Quantitative Real-Time PCR

For quantitative real time PCR (qRT-PCR) calculation of expression of interferon response genes (IRGs) 15µl of master mix containing 0.2 µl of 10uM universal ProbeLibrary probe (Roche), 0.4uµl of 10mM forward and reverse primers (SIGMA Aldrich), 4ul RNase/DNase Free water and 10 µL 2 X TaqMan® gene expression master mix (Life Technologies) was added to 5 µL of 1:2.5 diluted cDNA in a MicroAmp Fast Optical plate (Applied Biosystems). Probe and primer sequences for IRGs are shown in table 2.5 Regarding the housekeeper 18S expression 5ul of a separate master mix containing 0.1µl of 10uM 18S Probe, 0.1µl of 10mM forward and reverse primers,

(SIGMA Aldrich), and 4.7µl of 2 X TaqMan® gene expression master mix was added to 5µl of 1:100 diluted c-DNA. Probe and primer sequences for 18S shown in table 2.6.

mRNA expression was determined using a TaqMan 7900HT fast-real time PCR system (Life Technologies) with the thermal cycler set to 50°C x 2 minutes, 95°C x 10 minutes, then (95°C x 15 seconds, 60°C x 1 minute) x 40.

Gene	Primer		Roche Universal Probe Library Number
	Forward	Reverse	
MxA	GGAGAACATGGTGTGATAATCCT	CACCGTGACACTGGGATTC	83
ISG15	GCGAACTCATCTTTGCCAGTA	CCAGCATCTTCACCGTCAG	23
OAS1	CATCCGCCTAGTCAAGCACT	CAGGAGCTCCAGGGCATAC	87
IFI6	CGGGCTGAAGATTGCTTCT	AAAGCGATACCGCCTTCTG	25
IFI44L	TGACACTATGGGGCTAGATGG	GAATGCTCAGGTGTAATTGGTTT	15

Table 2.5 IRG forward and reverse primer sequences and corresponding probe number from Roche universal probe library

	18s
Forward primer	CGAATGGCTCATTAAATCAGTTATGG
Reverse primers	TATTAGCTCTAGAATTACCACAGTTATCC
Probe	FAM-TCCTTTGGTCGCTCGCTCCTCTCCC-TAMRA

Table 2.6 Housekeeper gene 18S primer and probe sequences used in IGS calculations

For qRT-PCR quantification of whole blood L1-5'UTR and L1-ORF2 expression 4µl of 1:2.5 diluted "super-clean" c-DNA was added to a master mix containing 5µl Fast SYBR Green Master Mix (Thermo Fisher Scientific), 0.4µl of 10mM forward and reverse primers (for L1-5'UTR, L1-ORF2 or housekeeper TBP) and 0.2µl RNase/DNase Free water in a MicroAmp Fast Optical plates (Applied Biosystems). Each sample was amplified in duplicate. Primer sequences for L1-5'UTR and L1-ORF2 and TBP are shown in table 2.7.

Gene or L1 element	Primer	
	Forward	Reverse
L1-5'UTR	ACAGCTTTGAAGAGAGCAGTGGTT	AGTCTGCCCGTTCTCAGATCT
L1-ORF2	TGCGGAGAAATAGGAACACTTTT	TGAGGAATCGCCACACTGACT
TBP	GAACATCATGGATCAGAACAACAG	ATAGGGATTCCGGGAGTCATG

Table 2.7 Primer sequences used for qRT-PCR quantification of LINE-1 (L1) elements including house keeper TATA-binding protein (TBP)

However since SYBR green does not use a probe as well as primers, there is a risk that there may be amplification of additional products due to mis-annealed primers or primer dimers. Melt analysis curves were therefore run after each assay where a single peak implies a single amplified product. An example of melt curves is shown in figure 2.1 where a single peak is show for L1-5'UTR, L1-ORF2 and TBP confirming a single amplified product and accuracy of the qRT-PCR amplification results.

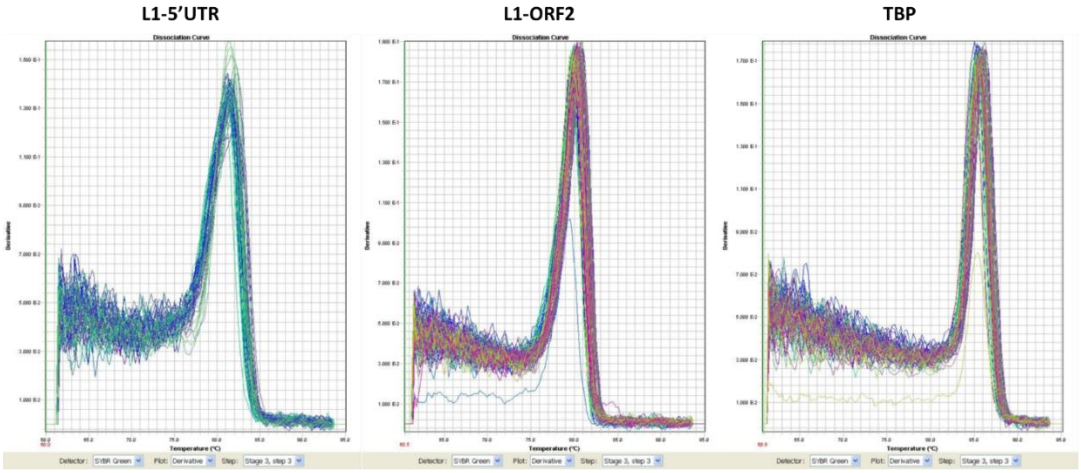


Figure 2.1 An example of melt/dissociation curves for each SYBR green amplified primer pair

Melt curves were performed for all the SYBR green qRT-PCR assays to ensure there was only a single product being detected/amplified. Representative curves are shown for each of the products where there is only a single, uniform curve observed suggesting only a single product was generated.

As a positive control HEK293T cDNA (see section 2.3.2), which contains LINE1 elements, was generated from a single "super-clean" reverse transcription, which had the same number of freeze-thaws cycles as sample cDNA and was run in duplicate on each MicroAmp Fast Optical plates (Applied Biosystems).

mRNA expression was determined using a 7900HT fast-real time PCR system (Life Technologies) with the thermal cycler set to 50°C x 2 minutes, 95°C x 10 minutes, then (95°C x 15 seconds, 60°C x 1 minute) X 40.

Of note when using the SYBR Green dye due its potential to bind to double stranded DNA dissociation/melt curves of RT-qPCR products were carried out to ensure there were no primer dimers or splice variants affecting the assay. In addition since SYBR green was used with non-library validated primer sequences we checked primer binding efficiency by performing the above SYBR green RT-qPCR on serial cDNA dilutions at 1:25, 1:250, 1:2500 and 1:25000. The target for primer binding efficacy was 100% +/- 5%.

2.2.7 Analysis of Quantitative Real-Time PCR

Results were analysed using SDS 2.4 software (Life Technologies). For IRG *MXA, IFI44-L, IFI6, OAS1* and *IGS15* expression levels were normalised to the level of housekeeper 18S using the calculation $2^{-\Delta CT}$, where ΔCT represents CT (target gene) – CT(18S).

For L1-5'UTR and L1-ORF2 there was sub-optimal primer efficiency and a mathematical model, called the Pfaffl correction, for relative quantification in real-time RT–PCR was used to take this variability into account (247). This is a mathematical model for data analysis to calculate the relative expression ratio on the basis of the amplification efficiency. A titration of cDNA undergoing qRT-PCR is performed to determine amplification efficiency (E) for both target genes (L1-5'UTR and L1-ORF2) and housekeeper genes (TBP). The relative expression (ratio) of L1-5'UTR and L1-ORF2 in the samples of interest is then calculated using the formula below.

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$

To provide an additional internal control after Pfaffl correction L1-5'UTR and L1-ORF2 expression was expressed as a relative ratio compared with HEK293T expression.

2.3 Calculation of the IGS

2.3.1 Interferon response genes (IRGs)

There are over 2000 interferon response genes (108). There is a spectrum in the published literature of the number of IRGs chosen to calculate an IGS with 3 being the lowest number shown to generate meaningful results (186). I examined 5 IRGs and this number was chosen as it increased our chance to show meaningful results as well as for practical and technical reasons when performing qRT-PCR.

I choose 5 IRGs; *MxA, IFI6, IFI44L, OAS1* and *ISG15.* These had all been previously shown to be increased in RA and SLE whole blood expression studies (158, 184, 200, 203, 206, 248-251). I attempted to choose those genes that had the highest differential expression. However some of the top expressed IRGs reported in the literature could also be induced by type 2 interferons. These genes were excluded when chosing IRGs for my score in an attempt to preferentially examine a type 1 interferon response. Once these genes had been identified I cross referenced them with those that had been used in published literature in the generation of an IGS in RA or ACPA+ arthralgia. The 5 final IRGs chosen as are listed in table along with their function and evidence of dysregulation and use. As I was going to be comparing SLE patients with the early RA patients I ensured that I chose IRGs that had demonstrated IGS related abnormalities in both RA and SLE (figure 2.8).

2.3.2 Calculating the IGS and defining a positive/negative IGS

The relative expression of the 5 IRGs was calculated by qRT-PCR as described in section 2.2.6 and 2.2.7. The mean expression of $2^{-\Delta CT}$ values for all 5 IRGs was termed the interferon gene signature (IGS) or "IGS score." Patients were defined as exhibiting a positive IGS if their IGS was ≥ 2 standard deviations above the mean healthy control IGS. It was termed negative if it fell below this cut-off. This is an acknowledged method and has been previously published (248, 252).

Interferon Response Gene (IRG)	Function	Evidence of derangement in RA, SLE or ACPA+ arthralgia	Degree of dysregulation in RA or ACPA+ arthralgia
MxA **(MX Dynamin Like GTPase)**	Encodes a guanosine triphosphate (GTP)-metabolizing protein that participates in the cellular antiviral response	RA (182-187, 200, 203, 204, 248, 253) SLE (250)	RA fibroblast: in top 5 IRG DEG (184). Whole blood: In top 15 DEG IRG in RA (185). In top 15 IRG in RA (187) In top 59 DEG IRGs in RA (204). 3x increased expression RA (183) Used to calculate IGS in RA/ACPA+ arthralgia (182, 186, 200, 203, 248, 253)
IFI6 **(Interferon Alpha Inducible Protein 6)**	Protein may play a critical role in the regulation of apoptosis	RA (182, 183, 187, 203), SLE (250, 251)	Whole blood: In top 15 IRG in RA (187) . 3x increased expression RA (183). Used to calculate IGS in RA/ACPA+ arthralgia (182, 203)
OAS1 **(2'-5'-Oligoadenylate Synthetase 1)**	Synthesizes 2',5'-oligoadenylates (2-5As). This activates latent RNase L, resulting in viral RNA degradation and the inhibition of viral replication	RA (182, 185-187, 204, 206, 249) SLE (158, 250, 251)	Whole blood: Top 15 DEG IRG in RA (185). In top 59 DEG IRGs in RA (204) In top 15 IRG in RA (187) . 3x increased expression RA (183) Used to calculate IGS in RA/ACPA+ arthralgia (186, 187, 206, 249)
ISG15 **(ISG15 Ubiquitin-Like Modifier)**	Multiple functions: chemotactic activity towards neutrophils, direction of ligated target proteins to intermediate filaments, cell-to-cell signalling and antiviral activity during viral infections	RA (184-187, 200, 204), SLE (158)	RA fibroblasts: in top 5 IRG DEG (184). Whole blood: In top 15 DEG IRG in RA (185). In top 59 DEG IRGs in RA (204) In top 15 IRG in RA (187) . 3x increased expression RA (183) Used to calculate IGS in RA/ACPA+ arthralgia (186, 187, 200)
IFI44L **(Interferon Induced Protein 44 Like)**	Protein exhibits a low antiviral activity against hepatitis C virus	RA (182, 183, 185, 187, 200, 204, 248, 249, 253) SLE (251)	Whole blood: In top 15 DEG IRG in RA (185). In top 59 DEG IRGs in RA (204) In top 15 IRG in RA (187) . 3x increased expression RA (183) Used to calculate IGS in RA/ACPA+ arthralgia (182, 200, 248, 249, 253)

Table 2.8 IRGs used to calculate the IGS

The 5 interferon response genes (IRGs) that were used to calculate the IGS are shown here. All genes have been shown to be dysregulated in RA and SLE. DEG: differentially expressed gene.

2.4 Cellular analysis techniques

2.4.1 Cell isolation

Peripheral blood mononuclear cells (PBMCs)

Blood was collected into a BD EDTA Vacutainer® tubes and then diluted 1:2 with Hanks Balanced Salt Solution (HBSS, ThermoFisher Scientific, UK) containing 2mM EDTA (Sigma Aldrich, UK). Density centrifugation was performed by layering 20ml of this diluted blood over 15ml of Lymphoprep (Axis-Shield Diagnostics Ltd, UK) which was centrifuged for 30mins at 895g (room temperature). The resulting interface contained the PBMC layer (see figure 1) and this was pooled (up to 2 tubes) and HBSS with 1% foetal calf serum (FCS, Sigma Aldrich, UK) was added until total volume of 50ml. This was centrifuged at 600g for 7 mins (4°C) and pellets from up to 2 tubes were again pooled topped up to 50ml with HBSS with 1% FCS and centrifuged at 250g for 7 mins (4°C) to remove any platelets. Any remaining tubes were pooled and cells re-suspended in 50ml HBSS 1% FCS and then cells in this PBMC suspension were counted.

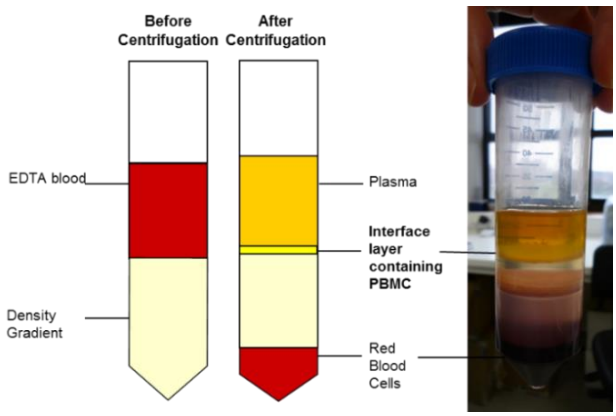


Figure 2.2 Pictorial representation of the interface containing the PBMC layer generated by density centrifugation

Plasmacytoid Dendritic Cells

Plasmacytoid dendritic cells (pDCs) were isolated from the PBMC population using the positive selection CD304 (BDCA-4/Neuropilin-1) MicroBead Kit (Miltenyi Biotech, Germany). The rationale for this method is shown in Figure 2.3. It was performed as per manufacturer's instructions, however to improve purity the isolated pDC suspension underwent an additional purification by passing the eluate through a secondary LS magnetic column. The purity checks to identify pDC yield were performed by quantifying co-expression of CD123 (PerCP/Cy5.5, clone 6H6 eBioscience, UK) and CD303 (APC,

clone AC144, Miltenyi Biotech, Germany) by using flow cytometry (see section 2.6). Purity >90% was deemed acceptable for downstream applications. All magnetic cell separation used LS columns from (Miltenyi Biotec, UK) that were pre-cooled at 4°C for at least 20 minutes prior to use.

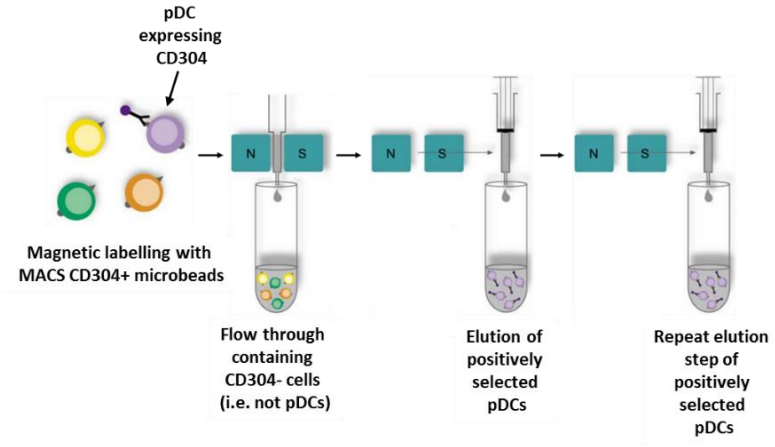


Figure 2.3 Pictorial demonstration of the positive selection magnetic microbeads method used to isolate CD304$^+$ cells (pDCs).

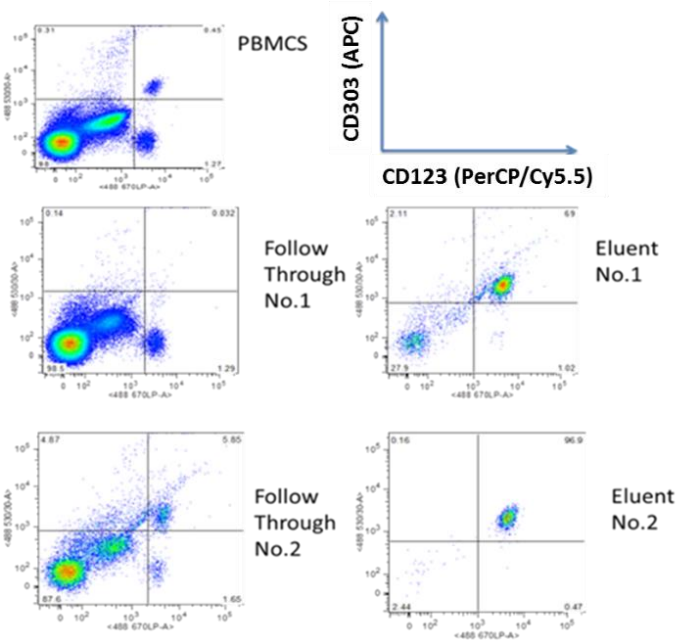


Figure 2.4 pDC purity was optimised following use of 2 eluting columns

CD304+ magnetic beads allowed positive selection of plasmacytoid dendritic cells to isolate from PBMCs. The use of these beads was optimised as shown above where the purity (as determined by CD123 (APC) and CD303 (PerCP/Cy5.5 co-expression) was best when the magnetic bead-cell mixture was passed twice through magnetic columns. A representative purity shown here is 97% purity.

$CD4^+$ T-cells

$CD4^+$ T-cells were isolated from whole blood using a human CD4+ T-cell enrichment cocktail (RosetteSep; StemCell Technologies). In brief this cocktail was added to whole blood at a ratio of 20:1, mixed well and incubated for 20 min at room temperature. The sample was diluted 1:2 with PBS (Lonza) with 2% FCS and enriched cells were separated by density gradient centrifugation (895 g, 30 min, room temperature) on Lymphoprep (Axis-Shield Diagnostics). $CD4^+$ T cells were recovered from the resulting interface and washed in PBS + 2% FCS (600g, 7 min, room temperature) and counted using a Burker counting chamber. The $CD4^+$ T-cells were further enriched for $CD45RA^+/RO^-$ naïve T-cells using a CD45RA positive selection magnetic selection kit (Stem Cell Technologies, UK) as per manufacturer's instructions. Purity was assessed by examining concurrent CD45RA and CD4 expression by flow cytometry (see section 2.6). Purity >95% was considered suitable for downstream applications. These were then frozen down (see section 2.3.6) and stored at -80ºC until required.

2.3.2 Cell Lines

The cell line HEK -293T (ATCC® CRL-3216™) was used (Gift from Professors David Young and Tim Cawston). The HEK-293T cell line, originally referred as 293tsA1609neo, is a highly transfectable derivative of human embryonic kidney 293 cells, and contains the SV40 T-antigen. It is semi adherent and due to its oncogenic potential and high expression of LINE-1 elements was used as a positive control for LINE-1 experiments.

2.3.3 Cell Culture

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were cultured in RPMI medium (Sigma-Aldrich, St Louis, USA) containing 10% (v/v) serum replacement (Invitrogen) and supplemented with, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, St Louis, USA). PBMCs were cultured in flat bottomed 96 well plates at a final density of $5x10^6$ cells/ml. This is a higher concentration than normally used for PBMC cultures. However during preliminary optimisation I found due to the low number of circulating pDCs at least 2 million PBMCs were needed to be cultured overnight to allow for enough pDCs to perform reliable phenotyping. Less than this resulted in erratic and unreliable results due to low cell number. Normal culture volumes (i.e. $1x10^6$/ml)

would have meant using large volume of reagents, such as the TLR agonists (see below), and this was not possible within the scope of this project's finances. Others have successfully used increased numbers of PBMCs in culture, for example between $2x10^6$/ml (149) and $1x10^7$/ml (254). I found that $5x10^6$ cells/ml gave reliably sufficient data without high death (as determined by DAPI) and allowed conservative use of reagents. To these cultures TLR 9 agonists CPG-B (ODN 2006) (InvivoGen, CA, USA) was added at a final concentration of 5µg/ml and cultures left in an incubator at 37°C (5% CO_2) for 18-22 hours. For some experiments the pDC depleted PBMCs (see 2.4.1) were also cultured in parallel with 5µg/ml TLR 9 CPG-B. Supernatants from these cultures were frozen at -20°C prior to further analysis whereas cells were immediately analysed by flow cytometry (see 2.7).

Plasmacytoid dendritic cells (pDCs)

pDCs were cultured in RPMI (Sigma-Aldrich, St Louis, USA) containing 10% (v/v) serum replacement (Invitrogen, UK) and supplemented with 10mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all Sigma-Aldrich, St Louis, USA) in a 96 well flat bottomed plate at a density of 200,000 cells/ml. TLR 9 agonists CPG-B (ODN 2006) (InvivoGen, CA, USA) was initially titrated (concentrations ranging between 0.05µg/ml to 10ug/ml) and cultured with the pDCs for 18-22 hours. For final experiments an optimal concentration of 5µg/µl for CPG-B was chosen. Supernatants from these optimised cultures were frozen at -20°C prior to further analysis whereas cells were analysed immediately by flow cytometry (see section 2.7).

Cell line cultures

HEK-293T cells were cultured in Dulbecco's Modified Eagle Medium with high glucose (Life Technologies, UK) supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and 10% FCS.

Upon thawing of a cryovial of frozen HEK 293-T cells they were washed once with warmed (37°C) culture medium and centrifuged at 400g for 7 minutes. The resultant pellet was then re-suspended in 24ml warmed medium and split into 2 T75 culture flasks. These were then incubated at 37°C with 5% CO_2 overnight and on day 2 the medium was entirely removed and replaced to ensure any residual DMSO was completely removed. Once the flasks were 80-90% confluent as they were semi-adherent trypsin (Sigma-Aldrich, St Louis, USA) was used to detach the cells and then they were either split or used for their desired application.

2.3.4 Mixed Lymphocyte Reactions (MLRs)

PDCs from both healthy donors and established RA patients were cultured overnight in either RF10 alone (as described above), or with RF10 and 5ug/ml CpG- B (ODN 2006, InvivoGen, CA, USA). PDCs were harvested the next day and washed twice in HANKS + 1% FCS to remove excess stimulus. Tryptan blue was used to determine viable cell number.

Naïve $CD4^+$ T-cells were thawed (as section 2.3.6) and washed twice in room temperature RF10. They were then labelled with 2.5µg/ml CFSE (Biolegend, USA) by adding 5µg/ml of CFSE in PBS (LONZA, Switzerland) to equal volume of T-cells in PBS and incubating this in the dark at room temperature for 15 minutes. The reaction was stopped by adding ice cold RF10 and the cells were washed twice in RF10 before being used in the MLR. Viable pDCs were co-cultured with CFSE labelled allogenic naive $CD4^+$ T cells in round-bottomed 96-well with a final ratio of 1:10 pDCs to T-cells and cultured for 6 days. At this point culture supernatant was collected and frozen at -20°C prior to further analysis and T-cell surface marker expression was phenotyped by flow cytometry (section 2.6).

2.3.5 Cell freezing and thawing

Cell freezing

Cells were pelleted (400g, 7min 4°C) and re-suspended in 37 °C warmed FCS (Sigma-Aldrich) with a maximum concentration of 20 $x10^6$ cells per 500ul. Warmed 37°C FCS with 20% dimethyl sulfoxide **(**DMSO, Sigma Aldrich) was added drop wise in equal volume, with the final concentration of DMSO at 10%. This suspension was added to cryovials (1ml per vial) and slowly frozen to -80°C in a freezing box that facilitated a fall of temperature of 1°C/minute.

Cell thawing

Frozen cryovials from heathy and patient donors were thawed as follows. Cryovials were warmed to 37 °C for 10 minutes in a standard waterbath. Contents of 2-3 cyrovials were each added to a universal container and warmed (37°C) thawing medium HBSS (Sigma-Aldrich) with 10% FCS was added was added dropwise to a final volume of 25mls or until the cryovial suspension volume had been diluted by a factor of 10. Suspensions were spun at room temperature (400g for 7 mins) and the pellet was resuspended in warmed thawing medium and the above spin repeated. After this any duplicate universals were pooled, resuspended in thawing medium and the cells were counted ready to use for downstream applications.

For PBMCs used for flow sorting there was a slight modification in the thawing protocol. The thawing medium was RPMI-1640 with 10% FCS (both Sigma-Aldrich, St Louis, USA). For the first resuspension of cells 0.6mg/ml DNAse I (grade II from bovine pancreas, Sigma-Aldrich, St Louis, USA) was added and the cells left for 5 mins at room temperature. After this the above protocol was applied.

2.3.7 Cytospins

Cells of interest were pelleted and re-suspended in 50ul FCS (Sigma-Aldrich, St Louis, USA) which resulted in a 1:1 mix of cell medium and FCS. Slides were labelled with a pencil and placed in to the cytospin cartridges along with the filter facing the centre of the cytospin. Cells were aliquoted in to each cytospin well and spun at 500rpm for 10 minutes. The slides were dried overnight. The next day they were placed in pre-prepared vials containing the following; ice cold ethanol (20 seconds, to fix the slide); May-Grunwald (red dye, 10 seconds); Giemsa (blue dye, 45 seconds). Slides were left for at least one hour to dry before being imaged.

2.4 Serum isolation

Peripheral blood was collected into a BD Vacutainer® Tube (BD Biosciences, CA, USA) containing clot activator and gel for serum separation (red top). This was then centrifuged at room temperature at 1800g for 12 minutes. The serum was divided into 500ul aliquots and frozen at -80°C until further use.

2.5 Cytokine measurement

2.5.1 ELISAs

Interferon-α was measured from cell culture supernatants using Human interferon-α Platinum ELISA (eBioscience, CA, USA) as per manufacturer's instructions. Circulating BAFF in the serum of patients and healthy controls was measured using the Human BAFF/BLyS/TNFSF13B Quantikine ELISA kit (RnD Systems, Bio-Techne Ltd, UK) again according to manufacturer's instructions.

Performance testing Human BAFF/BLyS/TNFSF13B Quantikine ELISA was carried out to ensure that there was no interference from heterophillic autoantibodies in the patient samples which could compromise accuracy of these assays. To do this samples with and without known heterophillic antibodies were used as well as spiked concentrations of known respective cytokine. To some of these samples Heteroblock at 32µg/ml (Omega Biologicals Inc, MT USA) was added and the concentration of detected cytokine levels

and spike recovery recorded. There did not appear to be any interference from heterophillic antibodies, with normal spike recovery in all cases. Therefore Heteroblock was not used in further patient serum assays.

2.5.2 MSD multiplexing technology

Interferon-α was also measured in the serum and in some culture supernatants by MSD technology using the Human interferon-α2α Ultra-Sensitive Kit technology (Meso Scale Discovery, Maryland, USA). Other inflammatory cytokines including IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α were also measured by MSD technology using the V-PLEX Proinflammatory Panel 1 (human) Kit.

The same performance testing as explained above (2.5.1) was performed on the Human interferon-α2α Ultra-Sensitive Kit technology (Meso Scale Discovery, Maryland, USA) with no evidence of interference from heterophillic antibodies. Previous testing in the laboratory had confirmed no evidence of heterophillic antibody interference in the V-PLEX MSD kits.

2.6 Flow cytometry

2.6.1 Cell surface protein expression

PBMCs (either fresh of following overnight culture) or isolated cell subsets were washed once in FACS buffer (phosphate-buffered saline (PBS) containing 3% FBS, 0.1% sodium azide and 2 mM EDTA) by centrifuging at 400g for 3-5 minutes. Cell pellets were resuspended in either FACS buffer (MLR phenotyping/purity checks) or BD Horizon Brilliant Stain Buffer (BD Biosciences, CA, USA) (DC subset phenotyping) containing selected fluorophore labelled surface marker antibodies (see Tables 2.9 – 2.13 for fluorophore labelled antibodies used for each experiment set) and 4µg/ml IgG (Gift from Prof Sophie Hambleton, UK) at a final volume of 50ul. This was incubated at 4ºC in the dark for 30 mins and if being used, CyStain® was added for the last final 10 minutes of incubation. Cells were then washed twice in FACS buffer (400g for 3 minutes) and resuspended in a final volume of 200µl FACS buffer and data were acquired. PDC phenotyping panels were acquired on a BD LSR Fortessa™ whereas MLR associated pDC and T cell phenotyping was acquired on a FACSCanto II (both Becton Dickinson, NJ, USA).

The T cell staining panel underwent intracellular protein expression steps (see below 2.6.2) which involved a fixation step but other samples were fixed with 1% paraformaldehyde and kept for up to 7 days if immediate acquisition was not possible.

Surface marker	Fluorophore	Clone	Dilution Factor	Source
Lineage: CD3	FITC	HIT3a	1:25	Biolegend CA USA
Lineage: CD19	FITC	HIB19	1:10	Biolegend CA USA
Lineage: CD20	FITC	2H7	1:10	Biolegend CA USA
Lineage: CD203c	FITC	NP4D6	1:10	Biolegend CA USA
CD11c	PerCP-Cy5.5	Bu15	1:10	Biolegend CA USA
ILT7	PE	17G10.2	1:25	Biolegend CA USA
CD14	BV510	M5E2	1:25	Biolegend CA USA
CD123	BV650	6H6	1:25	Biolegend CA USA
CD141	BV711	1A4	1:25	Biolegend CA USA
HLA-DR	AF700	L243	1:10	Biolegend CA USA
CD1c	APC-Cy7	L161	1:10	Biolegend CA USA
CCR7	APC	150503	1:25	RnD Systems, Abigndon, UK
Alive/Dead	*CyStain® (DAPI)*	*n/a*	*1:5*	*Partec, Japan*

Table 2.9A Fluorophore labelled antibodies used for DC subset phenotyping – Tube 1

Surface marker	Fluorophore	Clone	Dilution factor	Source
Lineage: CD3	FITC	HIT3a	1:25	Biolegend CA USA
Lineage: CD19	FITC	HIB19	1:10	Biolegend CA USA
Lineage: CD20	FITC	2H7	1:10	Biolegend CA USA
Lineage: CD203c	FITC	NP4D6	1:10	Biolegend CA USA
CD11c	PerCP-Cy5.5	Bu15	1:10	Biolegend CA USA
CD40	PE	C40-1457	1:25	BD Biosciences, CA, USA
CD14	BV510	M5E2	1:25	Biolegend CA USA
CD123	BV650	6H6	1:25	Biolegend CA USA
CD141	BV711	1A4	1:25	Biolegend CA USA
HLA-DR	AF700	L243	1:10	Biolegend CA USA
CD1c	APC-Cy7	L161	1:10	Biolegend CA USA
CD86	APC	FUN-1	1:25	BD Biosciences, CA, USA
Alive/Dead	*CyStain® (DAPI)*	*n/a*	*1:5*	*Partec, Japan*

Table 2.9B Fluorophore labelled antibodies used for DC subset phenotyping – Tube 2

Antibody	Fluorophore	Clone	Dilution Factor	Source
CD303	APC	201A	1:25	Miltenyi Biotech, Germany
CD123	Percp-Cy5.5	6H6	1:25	eBioscience, CA USA

Table 2.10 Fluorophore labelled antibodies used for MLR pDC purity check

Antibody	Fluorophore	Clone	Dilution Factor	Source
CD4	APC	UCHT1	1.50	Biolegend CA USA
CD45RA	FITC	H1100	1.25	BD Biosciences, CA, USA

Table 2.11 Fluorophore labelled antibodies used for MLR Naïve CD4 T cell purity check

Antibody	Fluorophore	Clone	Dilution Factor	Source
CD123	Percp-Cy5.5	6H6	1:25	eBioscience, CA USA
HLA-DR	APC-H7	L243	1:50	BD Biosciences, CA, USA
PDL1	BV421	29E.2A3	1.10	Biolegend CA USA
ICOSL	APC	MIH12	1.10	Miltenyi Biotech, Germany
ILT7	PE	17G10.2	1:25	Biolegend CA USA
Lineage: CD3	FITC	HIT3a	1:25	Biolegend CA USA
Lineage: CD19	FITC	HIB19	1:10	Biolegend CA USA
Lineage: CD20	FITC	2H7	1:10	Biolegend CA USA
Lineage: CD203c	FITC	NP4D6	1:10	Biolegend CA USA

Table 2.12 Fluorophore labelled antibodies used for pDC phenotyping from overnight PBMC cultures

Antibody	Fluorophore	Clone	Dilution Factor	Source
CD3	BV510	UCHT1	1.50	Biolegend CA USA
CD4	APC-Cy7	RPA-T4	1.50	Biolegend CA USA
CD25	PE-Cy7	BC96	1.25	Biolegend CA USA
FoxP3	Alexaflour 647	206D	1.10	Biolegend CA USA
CFSE	On FITC channel	-	-	Biolegend, CA USA

Table 2.13 Fluorophore labelled antibodies used for MLRs $CD4^+$ T cell phenotyping

2.6.2 Intracellular protein expression

To assess intracellular expression of FoxP3 T cells were first harvested and surface stained with respective antibody mix (as outlined in 2.6.1 above). They were then fixed and permeabilised using staining buffer set (eBioscience, CA USA) as per manufacturer's instructions. To reduce background staining, the cells were blocked with 2% mouse serum (Sigma-Aldrich) for 15 minutes before addition of anti-human FoxP3 AlexaFlour 647 antibody. Cells were incubated at 4°C for 30 mins, washed and re-suspended in FACS buffer and were then ready to acquire.

2.6.3 Flow cytometry analysis

Dead cell exclusion

All data was analysed using FlowJo Version 8.7.1 (Treestar inc). Where possible dead cells were excluded by CyStain® (Partec, Japan) where positive uptake indicated cell death. Cellular debris and were excluded by their position on the forward and side scatter area and doublets were excluded on the basis of their side scatter height vs side scatter area.

DC subsets

The measurement of peripheral blood pDCs was identified according to their surface marker expression (panel used in table 2.9). The combination of markers used to identify the pDCs is shown in table 2.14 and the gating strategy is shown in figure 2.5.

CD203c was used within the lineage cocktail to exclude any basophils. These are also $CD123^{+}$ and fall within the lymphocyte gate. CD203c is expressed by all basophils and is increased upon activation (255).

Of note CD303 was avoided as this can modulate the intracellular production of type 1 interferons via (113). As this involves the modification of intracellular signalling pathways I wished to avoid any potential confounding effect of other phenotypes that could result from this and so avoided the use of CD303 when performing phenotyping (or flow cytometry cell sorting – section 2.6.4) of pDCs.

	Markers used to identify pDCs
Plasmacytoid dendritic cells (pDCs)	Lin – (CD19, CD20, CD3, CD203c), HLA-DR^{+}, $CD14^{-}$, $CD1c^{-}$, $CD141^{\text{negative or dim}}$, $CD11c^{-}$, $CD123^{+}$

Table 2.14 Surface marker expression used to identify pDCs

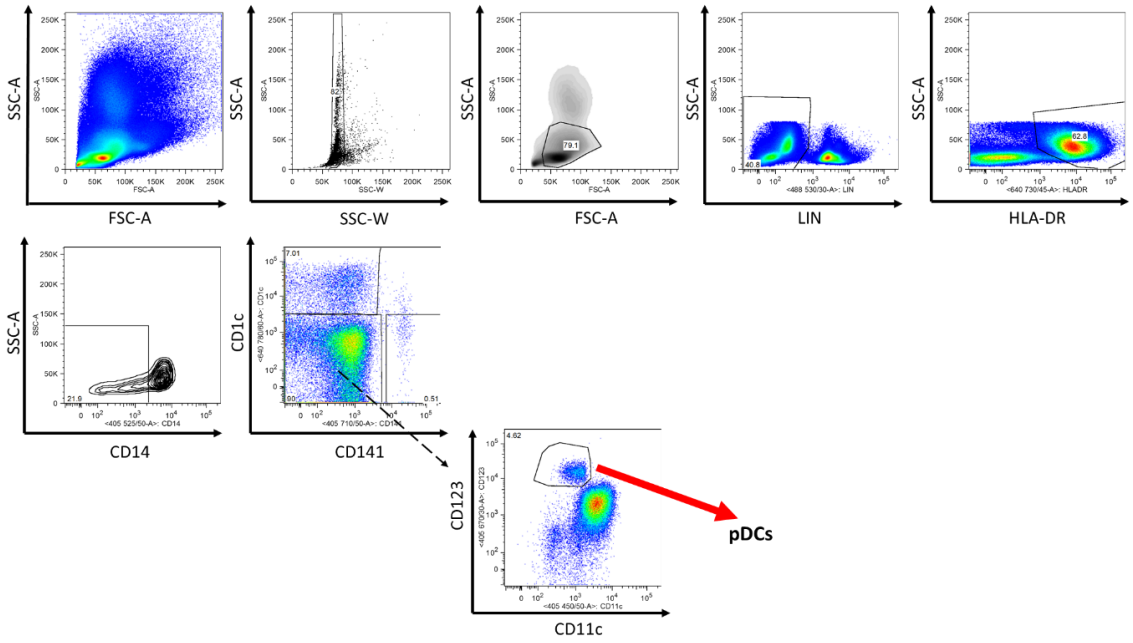


Figure 2.5 Gating strategy

This is the gating strategy used to identify the pDC subsets from isolated PBMCs. PDCs were defined as: Lin- HLA-DR+ CD14- CD141-/dim, CD1c-, CD11c - CD123+. Lin included lineage markers to exclude CD3 (T-cells), CD19, CD20 (all B-cells) and CD203c (Basophils).

T cell proliferation

Naïve CD4 T cell proliferation was measured using CFSE dilution. Percentage having undergone CFSE dilution was used as a marker of percentage undergone proliferation. This is demonstrated in figure 2.6

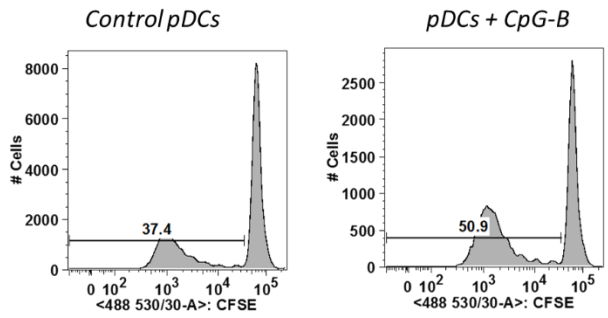


Figure 2.6 CFSE dilution and resultant percentage proliferated T cells in the MLR

This figure shows the percentage of naive CD4 T cells having undergone a dilution of CFSE signal following a 6 day pDC and CD4 T cell MLR. This percentage is then used as marker or percentage of cells having undergone proliferation over this period.

2.6.4 Flow cytometry cell sorting

PBMCs were pelleted at 400g for 7 minutes (4 °C) and re-suspended in cold FACS buffer at a final concentration of 20 x 10^6 cells/ml. To each FACS tube 500µl of this cell suspension was added and topped up with an additional 500µl of FACS buffer. This was spun at 400g for 5 minutes at 4°C and the pellet re-suspended in the fluorophore labelled antibody mixture (antibodies used listed in table 2.15). 2µl of human IgG (4µg/mg) was again added and the final volume of the staining mixture was made up to 50µl by adding BD Horizon Brilliant Stain Buffer (BD Biosciences, CA USA).

The cells and staining mixture were incubated for 30 minutes in the dark at 4 °C as above. After washes all tubes were pooled to a final volume of 1ml per 50 x 10^6 of stained PBMCs. CyStain® (DAPI) (ratio of 1µl to 50µl of cell suspension) was added 15 minutes before acquiring. For some samples the suspension was strained through a 70um cell strainer (EASYstrainer, Greiner Bio-One, GmbH, Germany) and washed with another 500ul of FACS buffer. This was performed immediately prior to sorting due to small cell clumps.

The BD FACSARIA II (Becton Dickinson, NJ, USA) was used to sort the 6 immune cell subsets. The gating strategy is shown in figure 2.7 and the phenotype used to identify each subset shown in table 2.16.

Surface marker	Fluorophore	Clone	Dilution Factor	Company
CD56	FITC	HCD56	1:25	Biolegend CA USA
CD11c	PerCP-Cy5.5	Bu15	1:10	Biolegend CA USA
CD8	PE	HIT8a	1:25	BD Biosciences, CA, USA
CD4	PE-CY7	RPA-T4	1:25	Biolegend CA USA
CD14	BV510	M5E2	1:25	Biolegend CA USA
CD123	BV650	6H6	1:10	Biolegend CA USA
CD3	BV786	UCHT1	1:25	BD Biosciences, CA, USA
HLA-DR	AF700	L243	1:10	Biolegend CA USA
CD1c	APC-Cy7	L161	1:10	Biolegend CA USA
CD19	APC	HIB19	1:10	BD Biosciences, CA, USA
CD20	APC	2H7	1:10	Biolegend CA USA
Alive/Dead	*CyStain® (DAPI)*	*n/a*	*1:50*	*Partec, Japan*

Table 2.15 Fluorophore labelled antibodies used for immune cell flow cytometry cell sorting

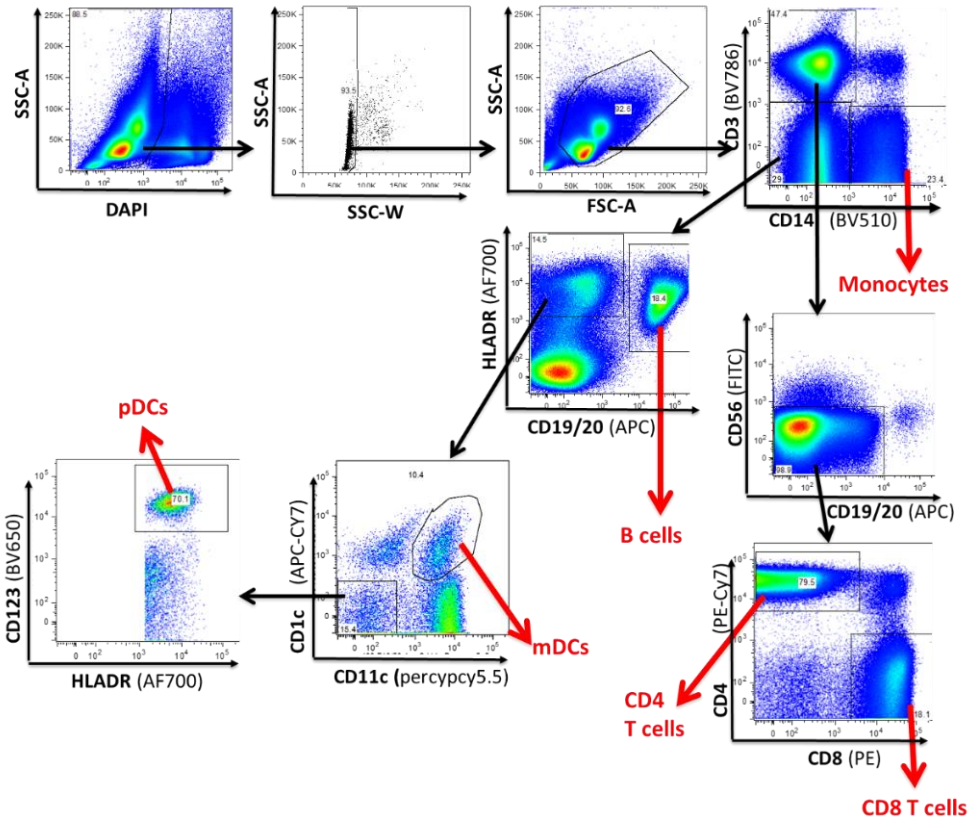


Figure 2.7 Gating strategy used to cell sort 6 different immune cell subsets

Flow cytometry was used to sort 6 distinct cell subsets. The gating strategy is shown**.** Subsets sorted included plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs^{+}), B cells, CD8^{+} T cells, CD4^{+} T cells and CD14^{+} monocytes.

	Markers used to identify immune cell populations
Plasmacytoid dendritic cells (pDC)	CD19^{-}, CD20^{-}, CD3^{-}, CD14^{-}, HLA-DR^{+}, CD1c^{-}, CD11c^{-}, CD123^{+}
Myeloid dendritic cells (mDC)	CD19^{-}, CD20^{-}, CD3^{-}, CD14^{-}, HLA-DR^{+}, CD1c^{+}, CD11c^{+}
B cells	CD19^{+}, CD20^{+}, CD14^{-}, CD3^{-}
CD8+ T cells	CD19^{-}, CD20^{-}, CD14^{-}, CD3^{+}, CD4^{-}, CD8^{+}, CD56^{-}
CD4+ T-cells	CD19^{-}, CD20^{-}, CD14^{-}, CD3^{+}, CD4^{+}, CD8^{-}, CD56^{-}
CD14+ monocytes	CD3^{-}, CD14^{+}

Table 2.16 Cell subsets sorted by follow cytometry and surface marker protein expression used to identify those populations.

2.7 NanoString Technologies

2.7.1 Methodology and genes examined

The NanoString nCounter Human immunology V2 Panel (NanoString Technologies Inc, WA, USA) was used to examine immune cell specific transcriptome. For a full list of genes included please see Appendix A 2.0. An additional panel of 20 genes was custom generated to specifically identify retrotransposon activity and DNA/RNA intracellular sensing pathways (shown in table 2.17, sequences included in Appendix A 3.0).

Standard probes	Customised probes
ISG15	AluYa5
DDX58 (RIG-1)	AluYb5
IDO2	LTR 5' end
STING	LTR 3' end
IRF9	L1-5'UTR
cGAS	L1-ORF2
MAVS	
CCR9	
IFI6	
IFI44L	
OAS1	
TREX1	
RNaseH2	
SAMHD1	

Table 2.17 Additional probes/genes included in the customised panel plus NanoString nCounter chip

A total volume of 50ng RNA was used for each of the cell subsets and loaded onto the chip and the protocol was followed according to standard nCounter instructions. In brief each target gene of interest is detected using a pair of reporter and capture probes carrying 35- to 50-base target-specific sequences. At the 5' end each probe also carries a unique colour code allowing the molecular barcoding of the genes of interest (Figure 2.8 A). The target mRNAs and reporter-capture probes are hybridized in a solution phase, excess probes are removed, and the probe/target complexes aligned and immobilised in the nCounter cartridge (Figure 2.8.B) This is then placed in a digital analyser for image acquisition and data processing (Figure 2.8.C). Each CodeSet includes a number of housekeeping genes to correct for RNA input amount and/or quality differences.

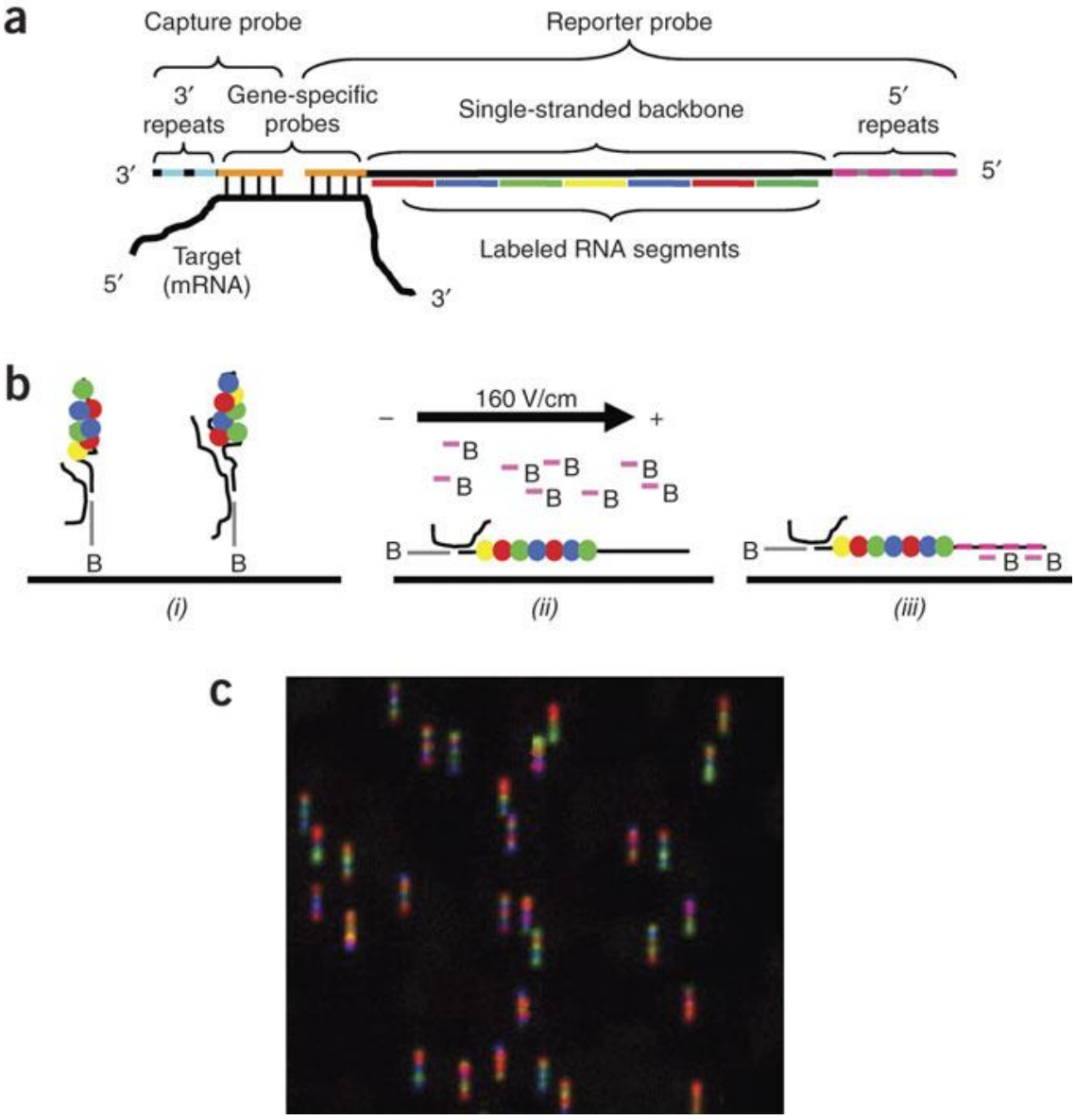


Figure 2.8 NanoString nCounter technology

A: Capture probe and reporter probe hybridize to a complementary target mRNA in solution via the gene-specific sequences notably the 5' end of each probe carries a unique colour code allowing the molecular barcoding of the genes of interest. **B**: Schematic representation of binding, electrophoresis, and immobilization. **C** False-color image of immobilized reporter probes. Figure from Geiss et al., *Nature Technology* 2008 (256)

2.7.2 Computational modelling

The analysis of this data was greatly assisted by the help of Andrew Skelton from Newcastle University Bioinformatics Support Unit (BSU), who performed the following steps to allow interpretation of the findings. R *Core team (2016)* (257) was used in association with the Bioconductor repository (258) for this analysis

Pre-processing

Pre-processing steps were used to transform the data into a shape where it can be easily manipulated for the experimental questions. These steps included initial quality control (QC) inspection, and normalisation.

NanoString data is provided in raw and normalised counts. Working with the raw counts adheres to the underlying assumptions of DESeq2 (Negative Binomial Distribution) (259). Normalisation is typical in high-throughput experiments to make samples comparable to one another. When working with DESeq2, the recommended protocol is a library size scaling factor normalisation which was performed in this case.

Using the whole dataset of observations for each of the 6 immune cell subsets principle components analysis (PCA) was performed. PCA was used as a data dimensionality reduction technique to get an overall view of the dataset, and identify samples that showed similar variation.

Generalised Linear models

Generalised Linear models (GLM) implemented using the DESeq2 package allow for a flexible framework in interrogating integer count data. Due to the different experimental questions in this project, there were 3 different model fits. The different model fits grouped together known experimental conditions, to simplify coefficient estimation. These included:

Term1 – '*CellType_DiseaseType'*; where for each cell type, healthy samples were compared against RA.

Term2 – '*CellType_DiseaseType_IGSClass*'; where for each cell type, positive IGS early RA patients were compared against negative IGS in early RA patients.

Term3 – '*CellType_IGSClass*'; where each cell type, positive IGS Patients (RA Only), are compared against negative IGS healthy controls.

Differential gene expression was calculated using the Wald test. Differences between

these cohorts were considered to be statistically significant when the adjusted (taking into account false discovery rates [FDR]) p value was < 0.05 and Fold changes were > 1.5.

Ingenuity Pathway Analysis (IPA)

The differentially expressed HGNC gene symbols from each list that was generated by the above GLMs were uploaded to the Ingenuity® Pathway Analysis (IPA®) software.

IPA® uses a knowledgebase derived from the scientific literature to relate genes or proteins based on their interactions and functions. Based on the uploaded dataset, the program algorithmically generates biological networks, defines canonical pathways and functions as well as any potential unifying upstream modulators.

A p value is generated during this process where significance is <0.05. An activation Z score (or Z score) is also generated by applying the differential genes expressed and the strength of their known associations with biological networks. The Z score infers activation state, for example if a pathway is upregulated there is a positive score and this related to an increase in associated biological functions whereas for a negative score the converse is true. Values > ±2 denote a strong signal. However for some biological networks there is evidence that a gene set is implicated in that network but not enough published literature to clearly predict whether this causes a net increase or decrease of that biological function. In these cases a Z score is not generated, although a p value is.

2.8 Statistical methods

Statistical analysis included ordinal and nominal logistic regression, univariate multiple regression analysis, 1-way ANOVAs, Wilcoxon signed rank, Kruskal-Wallis, Mann-Whitney U and Chi^2 tests. These tests were performed using GraphPad Prism (ver. 5.0, San Diego USA) IBM SPSS statistics (ver. 22.0 Armonk, NY: IBM Corp) and JMP Statistical Visualization Software (ver. 11, SAS Inc, NC). Significance was defined when $p<0.05$ however for some analyses where multiple parameters were simultaneously assessed, Bonferroni corrections were applied. As mentioned above, R Core team (2016) was used in association with the Bioconductor repository (258) for the NanoString transcriptomics analysis.

Chapter 3: The interferon gene signature in early rheumatoid arthritis

3.1 Introduction

Type 1 interferons, such as the interferon-α family, are cytokines conventionally produced upon the immune system's interaction with viruses and bacteria. Many interferon response genes (IRGs) control pathogen infection by targeting various pathways and functions required for pathogen proliferation. However type 1 interferons can also affect host cells, particularly immune cells, thereby amplifying the production of defensive cytokines and antibodies (260). For example type 1 interferons promote the differentiation of monocytes into an inflammatory phenotype dendritic cell (127, 129, 261) and can promote the class switch of B cells into antibody and sometimes autoantibody producers (145).

While these effects are effective and appropriate when the body is facing infection, type 1 interferons, and the subsequent upregulation of IRGs termed an interferon gene signature (IGS), have also been implicated in autoimmunity (262). This has mostly been looked at with regards to systemic lupus erythematous (SLE) where type 1 interferons and the IGS have been shown to predict disease flares and associate with certain aggressive presentations, such as lupus nephritis (156, 157). Furthermore some early phase 2 trials targeting the type 1 interferon pathway have been met with encouraging results (161, 263).

In established rheumatoid arthritis (RA) a positive IGS is present in about 20% of patients however it does not associate with disease activity and there is very little clearly known regarding its role in RA pathogenesis (185, 264). It has been shown to correlate with a poor response to certain treatments, particularly rituximab (186, 200, 202) but conversely predicts a better outcome to others, such as tocilizumab and TNF-α inhibitors (203, 206). At the same time, in established RA certain ongoing therapies, namely glucocorticoids, can modulate the IGS (23) which could mask associations between type 1 interferons and disease activity or pathogenesis.

I have therefore examined the IGS in DMARD-naive early RA patients and looked at both its prevalence and effect on disease outcome, as well as associations with disease parameters and clinical phenotype. I also looked at its expression in a longitudinal cohort of early RA patients at repeated points over a year to examine how its expression may change with both time and the initiation of conventional DMARD therapy.

3.2 Chapter Aims

In this chapter I wished to answer the following questions:

1. What is the prevalence of a positive IGS in drug naïve early RA?
2. Does the IGS change with time and therapy in early RA?
3. What association does the IGS have with inflammatory cytokines in early RA?
4. Does the IGS associate with ANA and RA associated autoantibodies?
5. Do serum derived factors trigger type 1 interferon production?
6. Is there any association between the IGS, disease activity in early RA or on clinical outcomes?

3.3 Results

3.3.1 IGS calculation and cohort demographical data

Using qRT-PCR 5 interferon response genes (IRGs) were quantified in the whole blood. These IRGs included *MxA, IFI44L, OAS1, ISG15* and *IFI6.* The rationale for each IRG chosen is explained in Chapter 2 methods (table 2.8). In brief IRGs previously shown to be highly differentially expressed in RA and SLE cohorts when compared to healthy controls were chosen (158, 184, 200, 203, 206, 248-251). Care was taken to choose IRGs that were preferentially activated by type 1 interferons to minimise any potentially confounding contribution from type 2 interferons.

In keeping with previously published methods (248, 252) the IGS was calculated by averaging of the normalised expression of the 5 IRGs. This generated a score which was termed the IGS, sometimes termed the IGS score. A "positive" or "negative" IGS in the patient cohorts was determined using a healthy control population; any patient sample IGS that was greater than 2 standard deviations above the healthy control mean was termed positive and values that fell below this cut off was termed negative, or normal. This was again a published method to stratify patient cohorts (248, 252).

The whole blood IGS was calculated in four separate cohorts; early drug naive RA, established RA and established SLE, as well as healthy controls. Principal component analysis (PCA) was used to assess whether a particular IRG appeared to be more highly differentially expressed. Ultimately all 5 IRGs were upregulated in a similar manner (data not shown).

There were 50 patients in the early RA cohort and their demographic and clinical data are shown in table 3.1. All patients met the 2010 ACR/EULAR RA classification criteria.

There were 23 individuals in each of the three other cohorts; established RA, SLE and healthy controls (table 3.2). Some early RA data are also included in this table 3.2 for statistical comparison.

As expected, the disease groups contained significantly more women than men and they were also significantly older. However between the established RA and early RA groups there was no significant difference between percentage seropositive or disease activity. The criteria for established disease was disease duration >12 months and for both SLE and established RA the median disease duration was 10 years.

A subgroup of the early RA cohort was followed up at intervals over a year (1, 3, 6 and 12 months). The specific demographics of this cohort as well their initial DMARD and glucocorticoid drug history is shown in table 3.3.

In view of the significant differences between the groups with regards to age and sex, correlations between the IGS score and these parameters were performed. There was no significant association between age and the IGS nor was there any association between sex and the IGS for either the entire cohort, early RA cohort or when examining pooled disease or healthy control populations separately (data not shown).

Early Rheumatoid Arthritis patients	Number = 50
Age (years, median and range)	57.5 (30-91)
Male : Female ratio	2:3
DAS-28 (median and range)	4.36 (1.33 – 7.63)
CRP (mg/L, median and range)	7.5 (0-114)
ESR (mm/hr, median and range)	22.5 (2-68)
Tender joint count (median and range)	4 (0-25)
Swollen joint count (median and range)	2 (1-24)
Patient VAS (median and range)	57 (8-100)
Duration of symptoms (weeks, median and range)	12 (3-52)
Early morning stiffness (minutes, median and range)	52.5 (0-380)
Anti-CCP positive (n and percentage)	34 (68%)
Rheumatoid factor positive (n and percentage)	32 (64%)
Seropositive - either anti- CCP+ or RF+ (n and percentage)	n=36 (72%)
Patients starting MTX (n and percentage)	41 (82%)
Patients starting HCQ (n and percentage)	18 (36%)
Patients starting SSZ (n and percentage)	12 (24%)
Patients starting LFU (n and percentage)	2 (4%)
Patients given depot glucocorticoid at diagnosis (n and percentage)	41 (82%)

Table 3.1 Early RA cohort demographics

The demographical and disease activity data for the early glucocorticoid and DMARD naive early RA cohort that had their whole interferon gene signature (IGS) quantified is shown. All met the 2010 ACR/EULAR criteria for a new diagnosis of RA. There were 50 patients in total all of who were started on a form of DMARD therapy at diagnosis. MTX, methotrexate, HCQ, hydroxychloroquine; SSZ sulfasalazine; LFU, leflunomide. RF rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody.

	Early RA patients	Est RA patients	Est SLE Patients	Healthy Controls	Difference between groups (p value)
Number	50	25	23	23	-
Age (years, median and range)	57.5 (30-91)	68 (28-81)	55 (33-69)	36.5 (25-62)	<0.0001*
Male : Female ratio	2:3	2:3	1:8	1:1	<0.0001*
Disease duration, (years median and range)	0[#]	10 (1 – 22)	10 (1 – 35)	-	-
DAS-28	4.36 (1.33 – 7.63)	5.45 (3.11 - 8.43)	-	-	0.059[+]
Seropositive RA (either RF or anti CCP+) (n and percentage)	36 (72%)	20 (87%)	-	-	0.235[+]
Patients on oral immunomodulatory therapy at time of recruitment (n and percentage)	0	21 (91%)	20 (87%)	0	-
Patients receiving glucocorticoids at time of recruitment or in the preceding 1 month (n and percentage)	0	3 (13%)	6 (26%)	0	-

Table 3.2 All cohorts demographics

The demographical and disease activity data for the disease cohorts (early RA, n=50, established RA [est RA] n=25, SLE [Est SLE] n=23) and healthy controls (n=23) examined are shown here. * one way ANOVA [+]Mann-Whitney U tests. [#] Disease duration is "0" as recruited at diagnosis.

Longitudinal early RA IGS cohort	Total n=15
Age (years, median and range)	64 [35-84]
Male : Female ratio	3:2
DAS-28-ESR (median and range)	4.28 [2.61-5.73]
CRP (mg/L, median and range)	7 [4-84]
ESR (mm/hr, median and range)	18 [2-48]
Tender joint count (median and range)	4 [0-16]
Swollen joint count (median and range)	2 [1-12]
Patient VAS (median and range)	49 [8-79]
Duration of symptoms (weeks, median and range)	8 [3-52]
Early morning stiffness (minutes, median and range)	32.5 [0-380]
Anti-CCP positive (n and percentage)	11 (73%)
Rheumatoid factor positive (n and percentage)	12 (80%)
Seropositive - either anti- CCP+ or RF+ (n and percentage)	12 (80%)
Patients starting MTX (n and percentage)	11 (73%)
Patients starting HCQ (n and percentage)	4 (27%)
Patients starting SSZ (n and percentage)	0
Patients starting LFU (n and percentage)	0
Patients given depot glucocorticoid at diagnosis (n and percentage)	13 (87%)

Table 3.3 Longitudinal IGS cohort demographics and drug history

A subgroup of early RA patients (n=15) were followed up at 1, 3, 6 and 12 months after their baseline IGS sample. At these time points clinical parameters and IGS were re-examined. The demographic data are shown here as well as their initial DMARD and glucocorticoid therapies.

3.3.2 IGS prevalence in disease cohorts

The positive/negative status of the early RA, established RA and SLE cohorts was examined. Twice as many patients were IGS+ in early RA compared with established RA (42% vs 20%) (figure 3.1). This compared with SLE where 56% were IGS^{+}. However when comparing early and established RA this difference was not significant.

I then examined the IGS score itself between the groups (figure 3.2) and found that the IGS score was significantly higher in early RA compared to established RA. It was also significantly higher in early RA compared with healthy controls but this difference was not observed when comparing established RA and healthy controls. Furthermore there was no significant difference between early RA and SLE patients although there was a slight trend towards increased IGS scores in SLE patients. There was however a significant difference between established RA patients and SLE patients.

These data all suggest an increase in the expression of IRGs and the IGS in early drug naive RA compared with established RA.

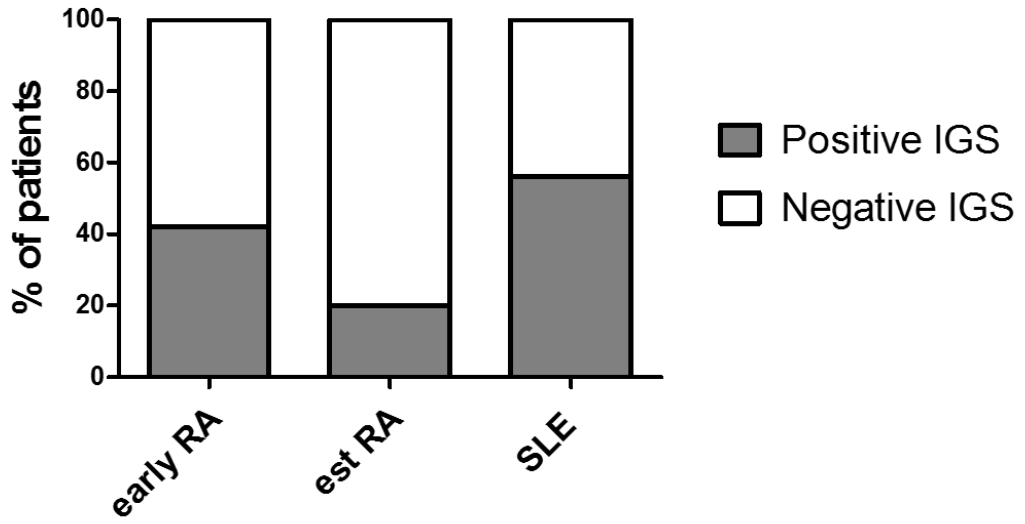


Figure 3.1 Increased prevalence of a positive IGS in early RA compared with established RA

Early RA patients (n=50), established RA (n=25) and SLE patients (n=23) had their whole IGS status (+/-) calculated. The percentage of patients who were IGS+ was: 42% early RA patients, 20% established RA and 56% SLE patients. These differences did not meet significance Chi^2 test.

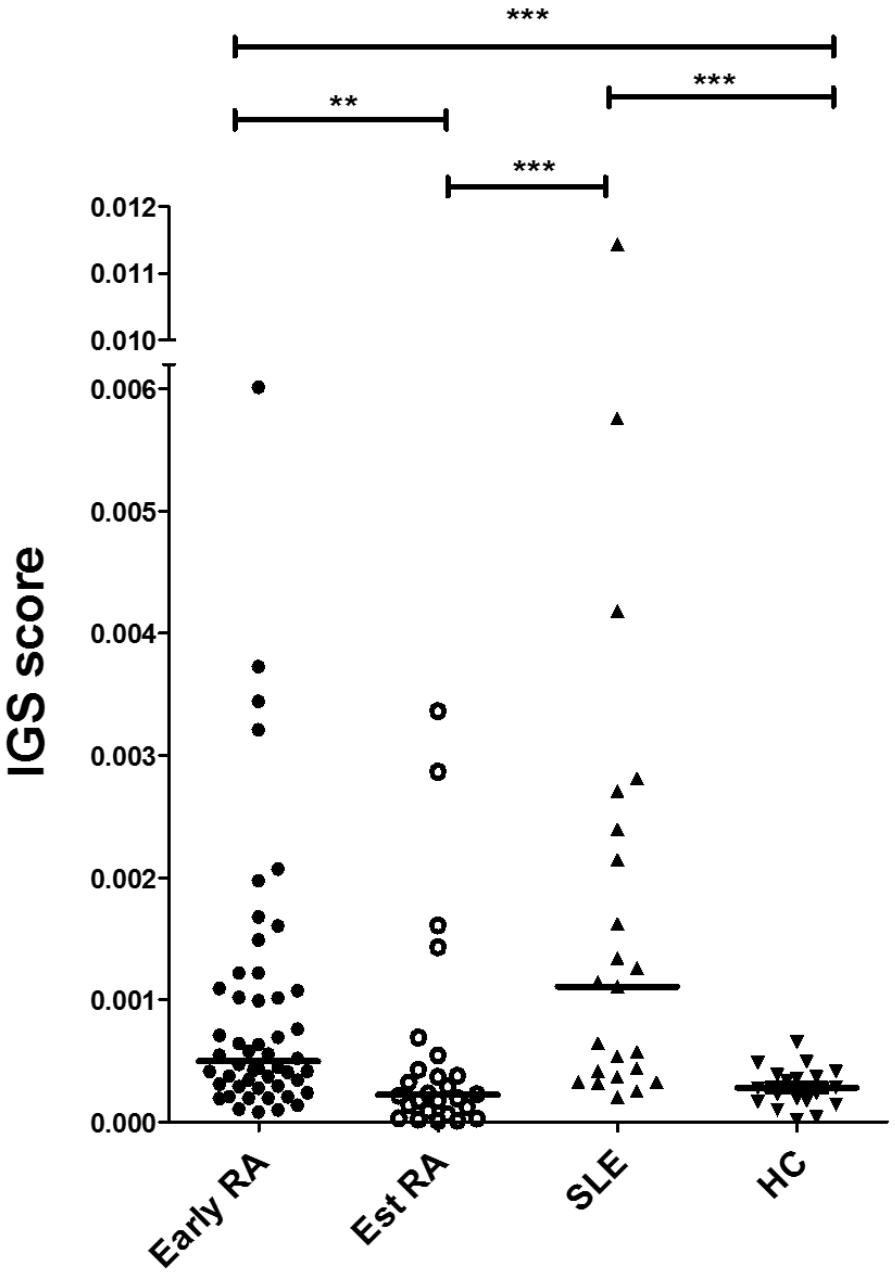


Figure 3.2 Significantly increased IGS score in early RA compared with established RA

The whole blood IGS (IGS score) was calculated across disease cohorts: early RA (n=50), established RA (Est RA, n=25), established SLE patients (SLE, n=23) and healthy controls (HC, n=23). Horizontal bars depict median values. Mann-Whitney U tests. *p<0.05, ** p<0.01, *** p<0.001.

3.3.3 Longitudinal expression of the IGS

Given the IGS increase in early RA, I wished to examine if it fell with time/treatment, which would explain the lower IGS score in established RA. Following diagnosis and initiation of therapy the IGS was repeatedly measured in 15 early RA patients over 12 months (baseline, 1, 3, 6, 12 months). Compared with baseline I found a significant fall in the IGS at 1 month, 3 months, 6 months and 12 months (figure 3.3.A)

I next looked at the individual IRGs to see which ones were most affected (figure 3.3.B). For *MxA* there was a non-significant fall at 1 month. However at 3, 6 and 12 months there was a significant fall in relative *MxA* expression. With *IFI6* and *IFI44L* there was a significant reduction at all time points. For *ISG15* the largest fall in expression was at 1 and 3 months but this was not sustained at 6 and 12 months. *OAS1* showed no significant change in expression over the 12 months, although at 1 month there was a trend towards reduced expression.

It is known that glucocorticoids affect the expression of IRGs (23) and I therefore excluded 4 patients that had received glucocorticoids beyond baseline. The IGS score was re-examined and again showed a significant and sustained fall over 12 months that could not therefore be explained by glucocorticoid use (figure 3.4.A). I similarly looked at individual IRGs excluding patients that had received additional glucocorticoids, and found a similar pattern as previously described (figure 3.4.B).

Treatment for this cohort included a single baseline intramuscular glucocorticoid depot (n=10) and methotrexate monotherapy (n=8), hydroxychloroquine monotherapy (n=1) or methotrexate with hydroxychloroquine (n=2).

Given the change in IGS with time, I examined the IGS and individual IRG expression with relation to early RA patient reported symptom duration. There were no associations seen (data not shown). However when examining symptom duration there was a recorded upper limit of 52 weeks due to information collection methods which may have been skewing the data via a ‘ceiling’ effect. I therefore excluded 8 patients with a disease duration of 52 weeks but there was still no significant association between symptom duration and the IGS or any individual IRGs (data not shown).

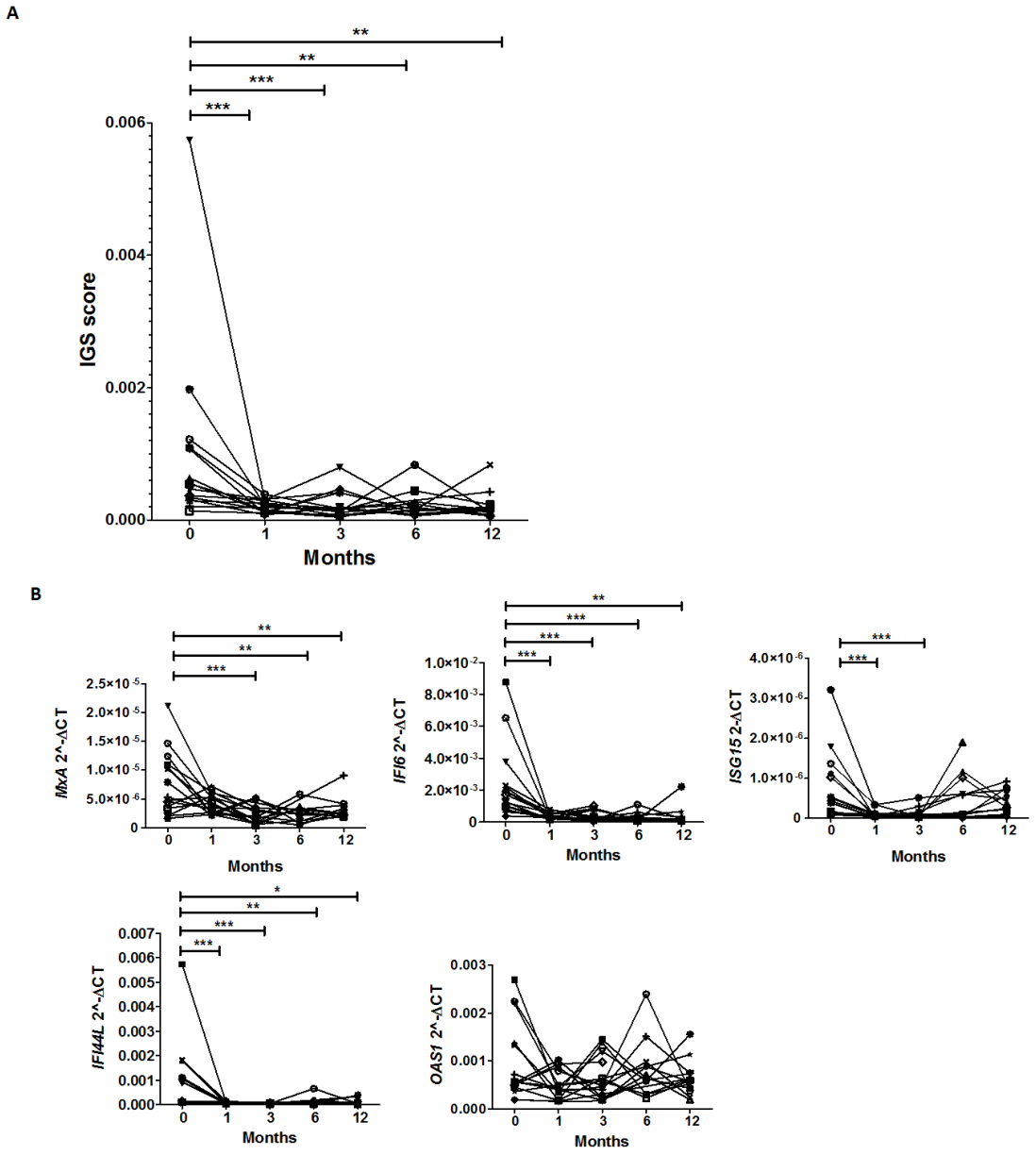


Figure 3.3 Significant and persistent fall in the IGS after initiation of treatment

Early RA patients (n=15) had their whole blood IGS repeated at 1, 3, 6 and 12 months after diagnosis (0 months). Each line represents a different patient. **A:** Whole blood IGS expression over 12 months. **B:** Individual interferon response gene (IRG) expression over 12 months. IRGs included *MxA, IFI6, IFI44L ISG15* and *OAS1*. Wilcoxon Signed Rank Tests. *p<0.05, ** p<0.01, *** p<0.001.

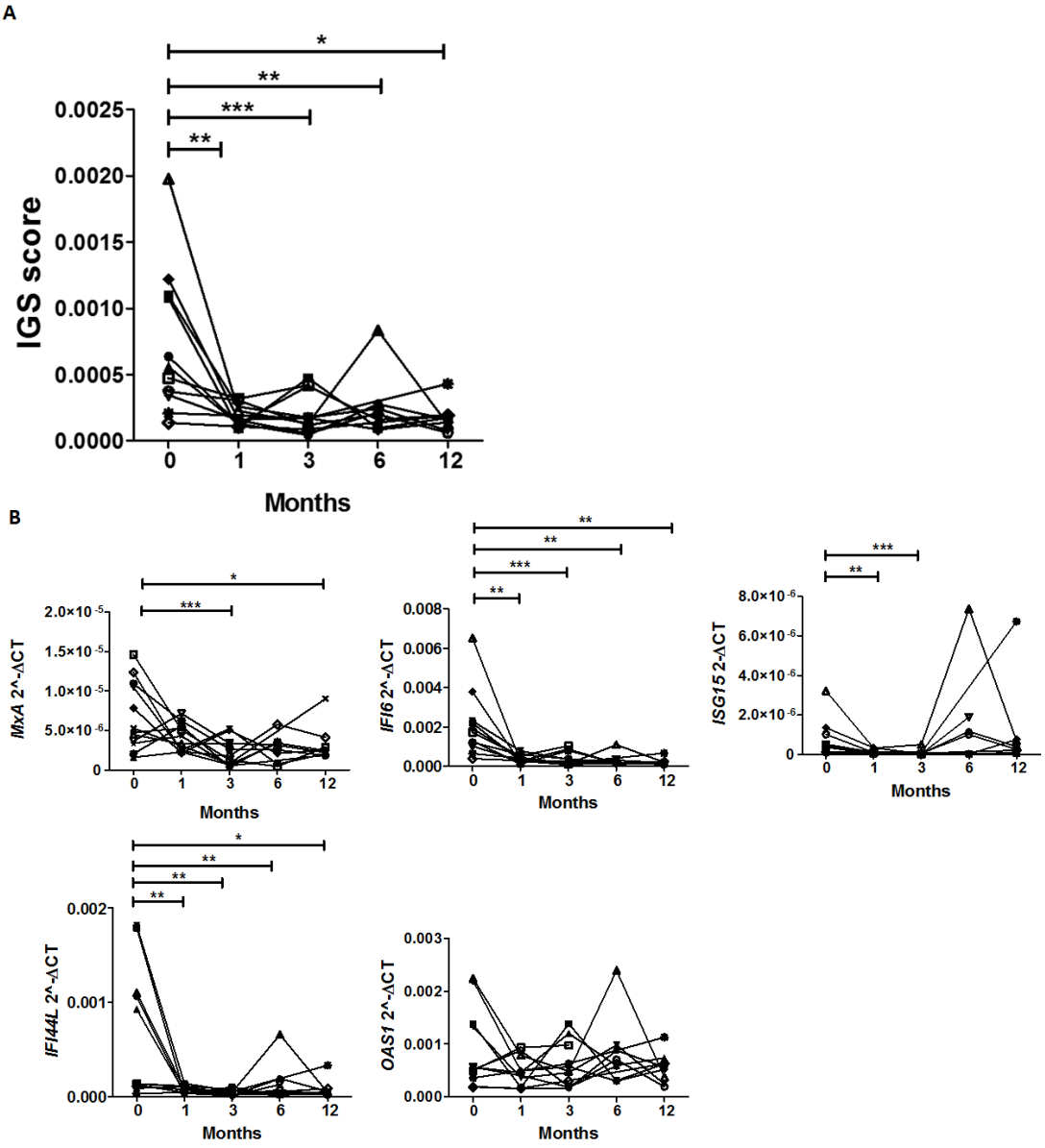


Figure 3.4 Significant and persistent fall in the IGS after initiation of treatment - excluding patients that had received additional glucocorticoids

Early RA patients that had not received additional glucocorticoids beyond baseline (0 months) (n=11) had their whole blood IGS repeated at 1, 3, 6 and 12 months. Each line represents a different patient. **A:** Whole blood IGS expression over 12 months. **B:** Individual interferon response gene (IRG) expression over 12 months. IRGs included *MxA, IFI6, IFI44L ISG15* and *OAS1*. Wilcoxon Signed Rank Tests. *p<0.05, ** p<0.01, *** p<0.001.

3.3.4 The IGS and serum cytokines

To correlate serum interferon-α with the IGS an ultra-sensitive interferon-α chemoluminescent assay was performed using early RA and SLE patients' serum samples. However, in all cases interferon-α was below the detection threshold (0.58pg/ml) (data not shown).

To see if the IGS was associated with a general inflammatory response in early RA circulating cytokines were examined for any association with the IGS. These were analysed using an MSD mutli-plex immunoassay and included interferon-γ (IFN-γ), IL-6, IL-12 p70, TNF-α, IL8, IL-1β, IL-2, IL-13, IL-4, IL-10 and BAFF was also examined by standard ELISA. There was no significant association between the IGS and any of the cytokines measured (data not shown). Despite this I was interested to investigate if these circulating cytokine levels followed the same pattern as the IGS following initiation of therapy. I therefore examined their expression at 1, 3, 6 and 12 months post diagnosis. There was no change in TNF-α, IL-1β, IL-4, IL-13 or IL-12 at any time point. IFN-γ expression significantly increased at 12 months only. IL2, IL6 and IL-10 levels fell at 1 month. IL-2 increased again subsequently to levels similar to baseline. IL-6 remained supressed at 3 months but also returned to baseline levels by 6 months, whereas IL-10 remained reduced at 3 and 6 months. IL-8 was the only cytokine to remain reduced over the entire 12 months (Figure 3.5).

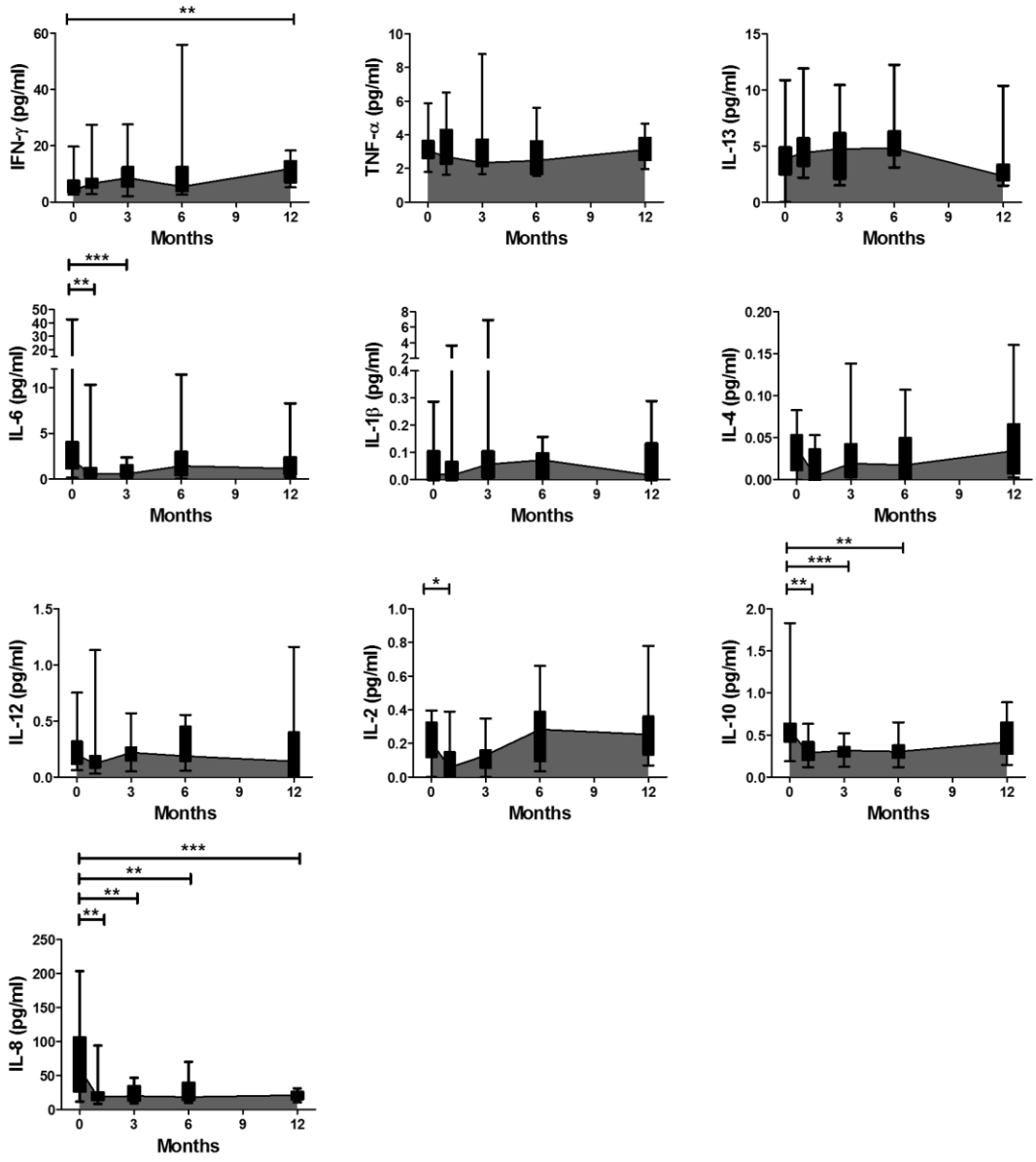


Figure 3.5 Circulating cytokines during the first 12 months of RA

Early RA patients (n=15) had baseline circulating serum cytokines measured using MSD mutli-plex immunoassay. Cytokines included IL-12 p70, TNF-α, IL-1β, IL-13, IL2, IL6, IL-10, interferon-γ (IFN-γ), IL-8 and IL-4. Measurements were repeated at 1, 3, 6 and 12 months after initiation of immunomodulatory therapies. Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Wilcoxon Signed Rank Tests. *p<0.05, ** p<0.01, *** p<0.001.

3.3.5 The IGS and autoantibodies

Type 1 interferons have been shown to affect B cell function (145), thus the IGS was examined in the early drug naive RA cohort with respect to autoantibody status, in particular rheumatoid factor (RF), anti-cyclic citrullinated peptide titres (anti-CCP) and anti-nuclear antibody (ANA).

Early RA patients were first separated by serostatus as defined by their anti-CCP titres or RF titres which were either "positive" or "negative" according to clinical laboratory standards. Patients were further split into double negatives (i.e. anti-CCP-, RF-), double positives (i.e. anti-CCP+, RF+), single positives (i.e. anti-CCP+ or RF+) or single negatives (i.e. either anti-CCP- or RF-). There was no significant difference in median IGS score between the groups, (figure 3.6). Furthermore most early RA patients were ANA negative (43/50, 86%), and there was no significant difference in the IGS+ and IGS- cohorts in terms of ANA status (data not shown).

The laboratory report for titres had an upper limit of detection and was reported as >340 U for anti-CCP titres and >131 IU/ml for RF titres. This may have had a skewing (ceiling) effect on any potential correlations and so serum samples were re-analysed by the clinical laboratories to provide accurate titres on samples whose values were previously above the detection threshold. Re-examination of the data using these more accurate titres now demonstrated a different relationship between the IGS and RF versus anti-CCP (figure 3.7). Median RF titre was significantly higher for IGS+ than IGS- patients and there was a significant positive association between the IGS (as a continuous variable) and the RF titre (figure 3.7.A). This was in contrast to anti-CCP+ titres in whom these significant associations were not seen (figure 3.7.B).

Given the different relationships noted between the IGS and RF and anti-CCP titres, I examined whether any other immune parameters associated differentially with autoantibodies, particularly those cytokines known to influence B cell function. There was a significant positive association between RF titres and BAFF levels, a positive trend with IL-6 and a significant inverse association with IL-13 (figure 3.8.A). In contrast there was no association or trend between anti-CCP titres and BAFF or IL-13 levels. There was a significant positive association with IL-6 levels but this was driven by a single outlier with very high anti-CCP titres and IL-6 levels (figure 3.8.B). When this individual was removed, significance was lost (figure 3.8.C).

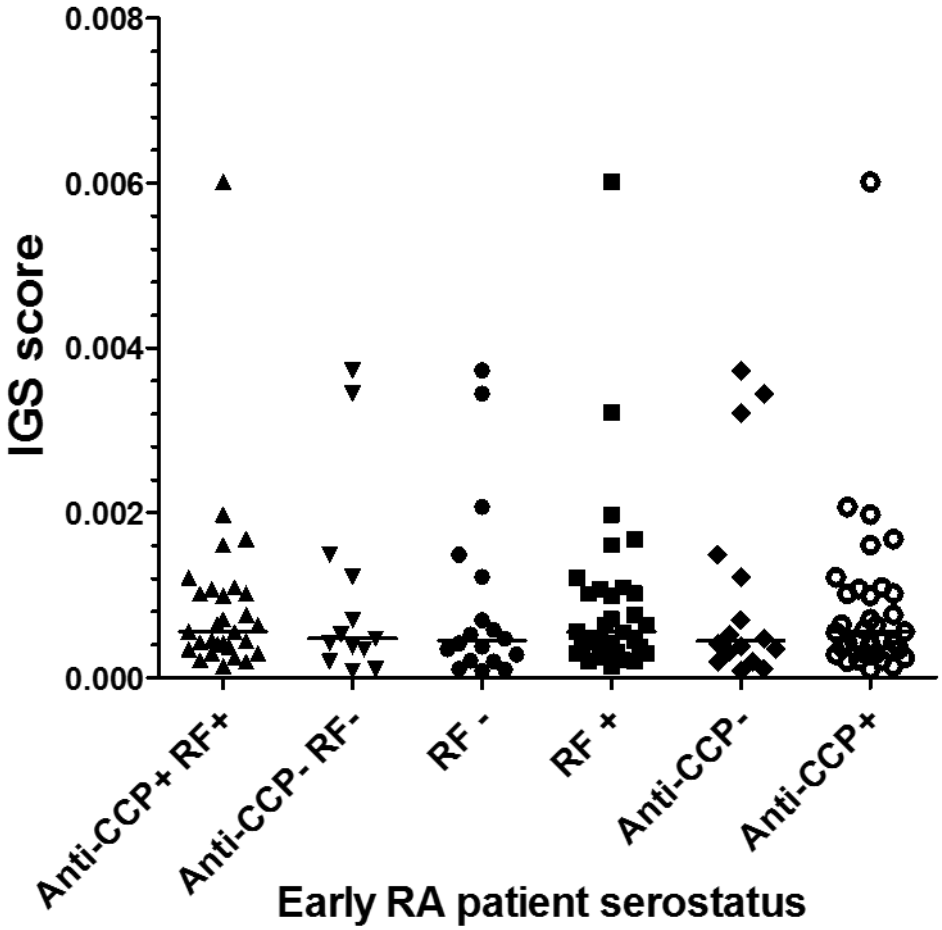


Figure 3.6 The IGS in relation to RF and anti-CCP status

Early RA patients (n=50) had their whole blood IGS calculated and patients grouped by their serostatus for (positive or negative) RF and anti-CCP autoantibodies as defined by conventional laboratory parameters. Cohorts included double negatives (i.e. anti-CCP-, RF-, n=13), double positives (i.e. anti-CCP+, RF+, n=29), single positives (i.e. anti-CCP+ n=34 or RF+ n=32) or single negatives (i.e. anti-CCP- n=16 or RF- n=18). Horizontal bars depict median values. Kruskal-Wallis test

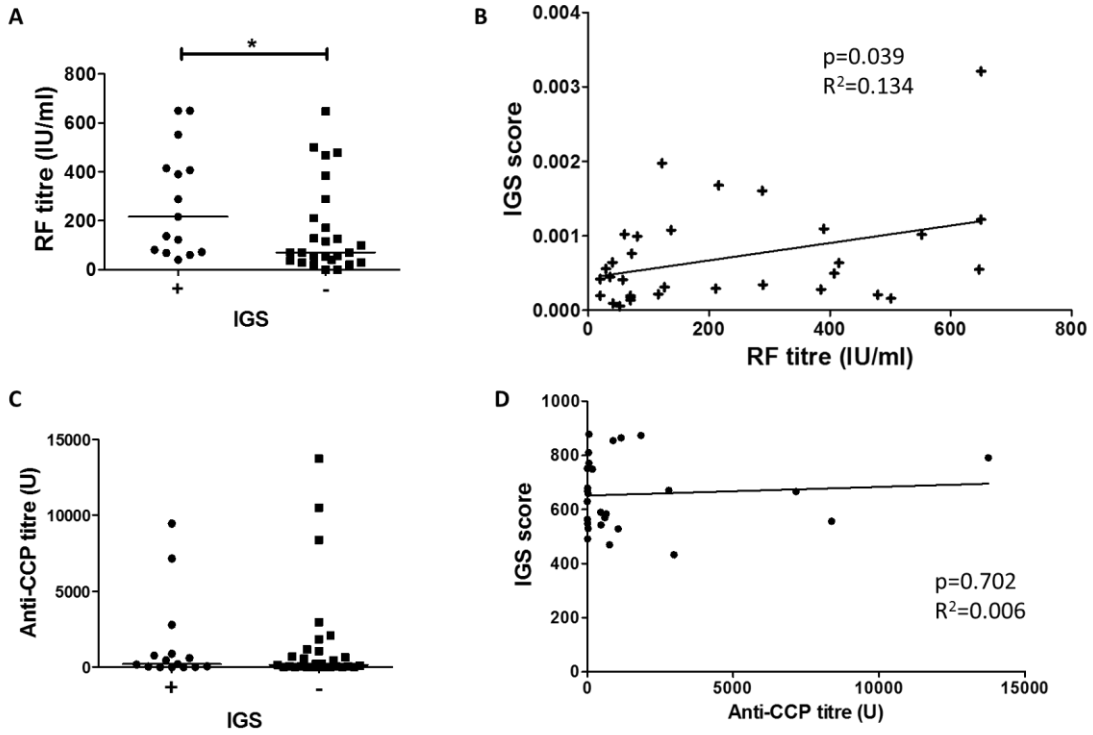


Figure 3.7 Autoantibody titres and the IGS score

Early RA patients had their RF, anti-CCP titres and whole blood IGS quantified. Associations were sought between the IGS and autoantibody titres. **A:** RF+ early RA patients (n=41) were split into IGS+ (n=15) and IGS- (n=26); horizontal lines depict median RF titres **B:** Plot shows linear regression between RF titres in RF+ early RA patients and the IGS (as a continuous variable) **C:** Anti-CCP+ early RA patients (n=42) were split into IGS+ (n=13) and IGS- (n=29); median anti-CCP titre depicted **D:** Plot shows linear regression between anti-CCP titres in anti-CCP+ early RA patients and the IGS (as a continuous variable). Mann-Whitney U tests and linear regression. * $p<0.05$

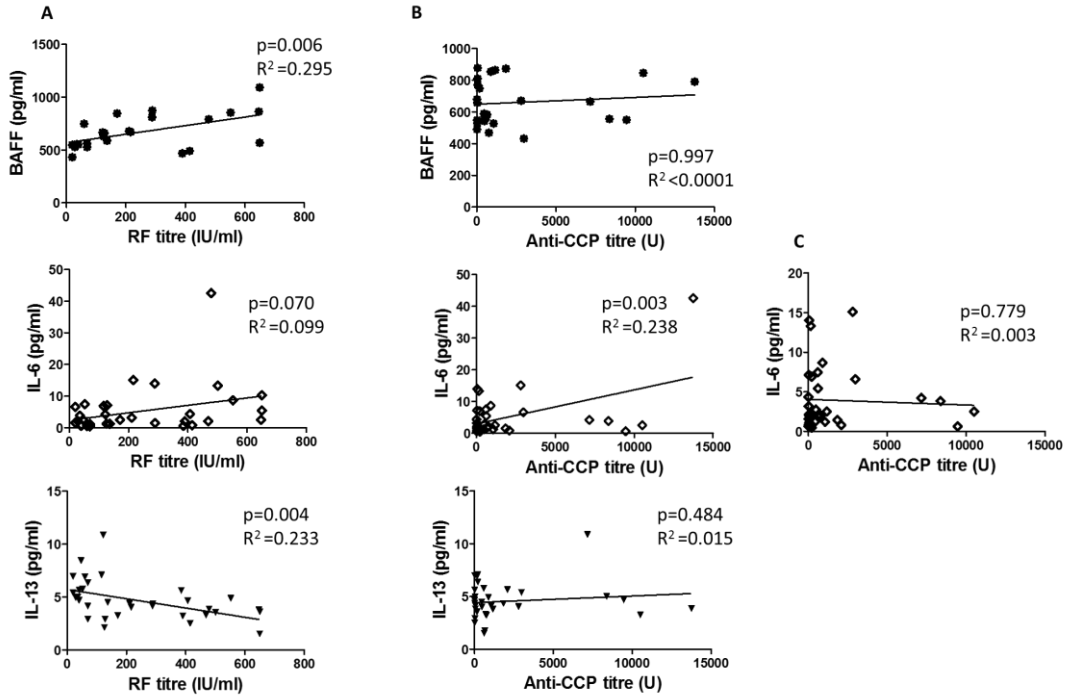


Figure 3.8 Associations between RF and anti-CCP titres and circulating cytokines

Early RA patients had circulating IL-13, IL-6 and BAFF quantified by MSD mutli-plex immunoassay and ELISA. Associations were sought with autoantibody titres **A:** Plots show linear regression between RF titres in RF+ early RA patients (n=22) and serum BAFF, IL-6 and IL-13. **B:** Plots show linear regression between anti-CCP+ titres in anti-CCP+ early RA patients (n=25) and serum BAFF, IL-6 and IL-13. **C:** Linear regression between anti-CCP+ titres and serum IL-6 with single outlier removed. Linear regression for all.

3.3.6 Do factors in serum stimulate interferon-α production?

I designed experiments to determine if serum-derived factors were driving the production of interferon-α. PBMCs from a healthy donor were cultured overnight with autologous serum as well as serum from early RA patients and healthy controls. Cultures were also repeated with pDC-depleted PBMCs as well as with pDCs alone. Interferon-α was measured in the culture supernatants.

All results were close to the assay detection threshold (0.58pg/ml) which is shown as a dotted red line on all graphs. Values below this threshold are therefore technically unreliable but are included for comparison only. Despite these caveats there appeared to be increased interferon-α production from PBMCs cultured in RA serum when compared with healthy control serum (Figure 3.9.A). There was no detectable interferon-α in the serum samples themselves (data not shown). When the early RA cohort was divided into IGS+ and IGS- patients there was no clear difference between the interferon-α production induced from the serum of either cohort (figure 3.9.B). As pDCs are the main interferon-α cell subset healthy control PBMCs with the pDCs-depleted (>60% depleted) were cultured under the same conditions in parallel. There was a significant reduction in interferon-α production in the pDC-depleted PBMCs thus highlighting that pDCs were primarily responsible or the interferon-α detected (figure 3.9.C). This was significant with IGS- RA patient serum (figure 3.9.D) but not IGS+ serum. When isolated healthy pDCs were cultured with patient serum, only IGS+ patient serum stimulated interferon-α production (figure 3.9.E).

Since I had shown associations between RF and the IGS I sought potential associations between autoantibody titres in serum and interferon-α production in PBMC cultures. However there was no significant association with either RF or anti-CCP+ titres (data not shown). Although notably, some measurements were again below the assay detection threshold.

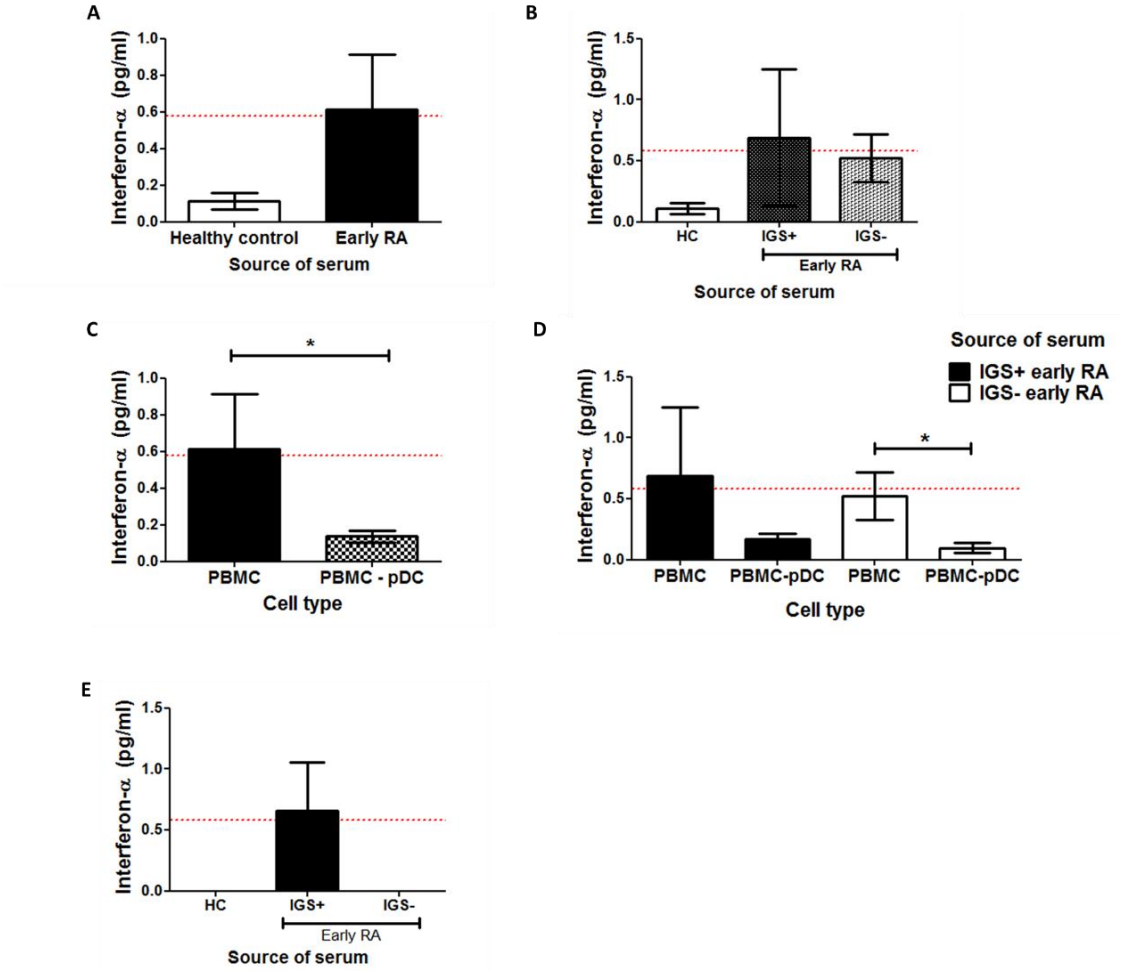


Figure 3.9 Interferon-α production following culture of healthy donor PBMCs (+/- pDCs) with early RA and healthy control serum

PBMCs from a healthy donor were cultured overnight with serum from either healthy controls (including autologous serum) or early RA patients. Interferon-α was measured in the supernatant by ELISA. **A:** PBMC Interferon-α production following culture with serum from early RA patients (n=19) and healthy controls (n=8). **B:** Healthy control PBMC Interferon-α production following culture with serum from IGS+ (n=10), IGS- (n=9) early RA patients and healthy controls (n=8) **C:** Interferon-α production from healthy control PBMCs with or without pDC depletion when cultured with early RA serum (n=10) **D:** Interferon-α production from PBMCs with or without pDC depletion when cultured with IGS+ (n=5) and IGS- (n=5) early RA serum. **E:** Interferon-α production from purified healthy control pDCs cultured with serum from IGS+, IGS- early RA patients and healthy controls (n=2 each) The red line on each graph demonstrates the lower detection threshold of the assay (0.58pg/ml). Mean and SEM depicted. Mann-Whitney U tests * $p<0.05$

3.3.7 The IGS and disease activity in early RA

Early RA patients had their DAS-28 calculated alongside their IGS. Using multiple regression, and incorporating age and sex into the model, baseline DAS-28 positively associated with baseline IGS score (figure 3.10.A). A positive trend was seen between the baseline IGS and the DAS-28 at 3 months (figure 3.10.B) and a significant association between the baseline IGS and the 6 month DAS-28 (figure 3.10.C).

Given the DAS-28 was a composite score of TJC, SJC, ESR and VAS I questioned if any of these parameters were more highly associated with the IGS. Using multiple regression analysis I found that TJC and SJC associated most strongly with the IGS. These findings were robust to the inclusion of age and sex (figure 3.11).

A principal components analysis (PCA) was performed to identify if certain IRGs within the IGS were driving this association more than others. Ultimately all 5 IRGs in combination were important for the above observations with equally weighted contributions (data not shown).

For comparison I performed a similar analysis in the established RA cohort. There was no significant association between DAS-28 (or any of its components) and the IGS score in established RA (data not shown).

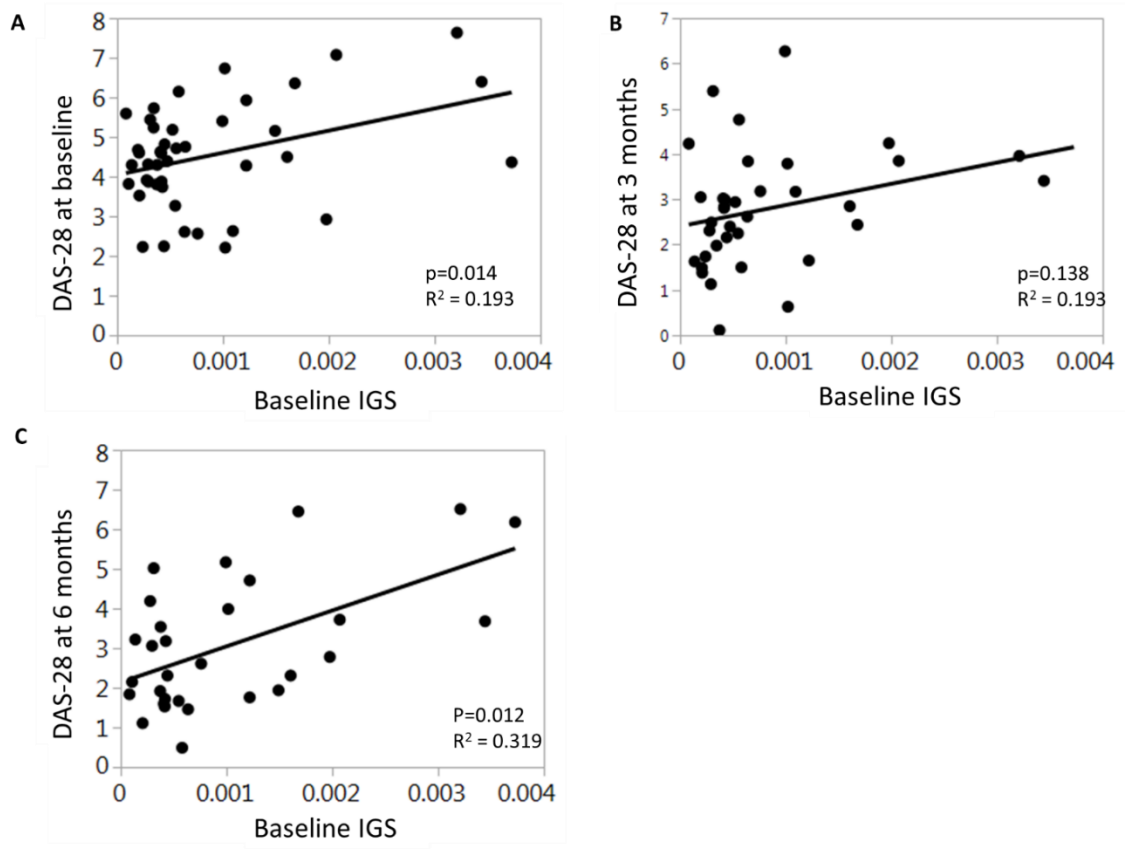


Figure 3.10 The baseline IGS associates with contemporaneous disease activity as well as with 6 month disease activity

Early RA patients had their whole blood IGS quantified at the time of diagnosis (baseline). They also had disease activity measured (DAS-28) at baseline (n=50), 3 months (n=37) and 6 months (n=32) after diagnosis. Plots demonstrate age and sex matched multiple regression for: **A:** Baseline IGS and baseline DAS-28 **B:** Baseline IGS and 3 month DAS-28 **C:** Baseline IGS and 6 month DAS-28.

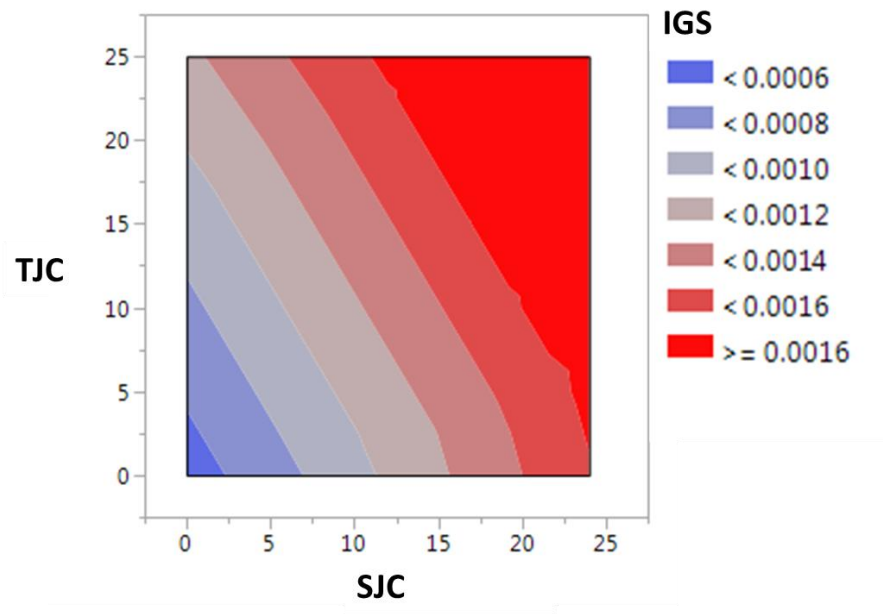


Figure 3.11 Associations with DAS-28 is predominantly driven by the combination of SJC and TJC

Early RA patients (n=50) underwent a multiple regression analysis to determine which DAS-28 components underpinned the associations between baseline DAS-28 and whole blood IGS (shown in figure 3.10). DAS-28 components included swollen joint count (SJC); tender joint count (TJC); patient VAS; ESR and age and gender was also included. Swollen joint count (SJC) (p=0.017) and tender joint count (TJC) (p=0.043) were the key drivers of the association. This is demonstrated in the above schematic where an increase in SJC or TJC corresponds with an increase in IGS as depicted by changing colour (blue, low IGS; red high IGS).

3.3.8 The IGS predicts a poorer response to initial therapies

In light of the relationship between baseline IGS and 6 month DAS-28, I questioned whether the baseline IGS could be a predictive factor for parameters of clinical response and disease activity.

Patients who require additional glucocorticoids administrations in the first 6 months will have a worse disease activity/disease course than those who don't. I examined the number of glucocorticoid depots required to control disease activity at up to 6 months following diagnosis (excluding glucocorticoids administered at baseline). This was again compared with the predictive function of baseline DAS-28, ESR and CRP. Using ordinal logistic regression (with Bootstrap probabilities included to guard against the relatively small sample size and any abnormal skewing of data) I found that the baseline IGS score predicted glucocorticoid requirements in the initial 3 and 6 months post diagnosis (figure 3.12). The IGS was superior at predicting these outcomes as baseline DAS-28, CRP and ESR had no ability to predict the number of glucocorticoid administered.

Multiple linear regression was performed to assess the ability of the IGS to predict 6 month DAS-28. This was compared with the ability of baseline DAS-28, ESR and CRP to make the same prediction. It demonstrated the baseline IGS was highly significant at in predicting DAS-28 at 6 months, wheras all other baseline variables (CRP, ESR, DAS-28) were non-significant (data not shown).

In addition the EULAR response to initial therapy (good, moderate or poor) was calculated for patients at both 3 months and 6 months from baseline. There was a significant inverse association between baseline IGS score and the probability of a good EULAR response at both 3 and 6 months (figure 3.13). There was however no such association seen for baseline ESR, CRP or DAS-28. All of these above findings were robust to corrections for age and sex.

I used a multivariate general linear model to examine for a potential influence of specific DMARD therapies initiated during the first 6 months of treatment on these findings. There was a slight suggestion that hydroxychloroquine mitigated the effects of a raised IGS when used in conjunction with methotrexate. However, this was non-significant and was shown to be unstable when using likelihood ratio tests which questioned the validity of any finding (data not shown). This effect was presumably due to the relatively small number in the follow up cohort.

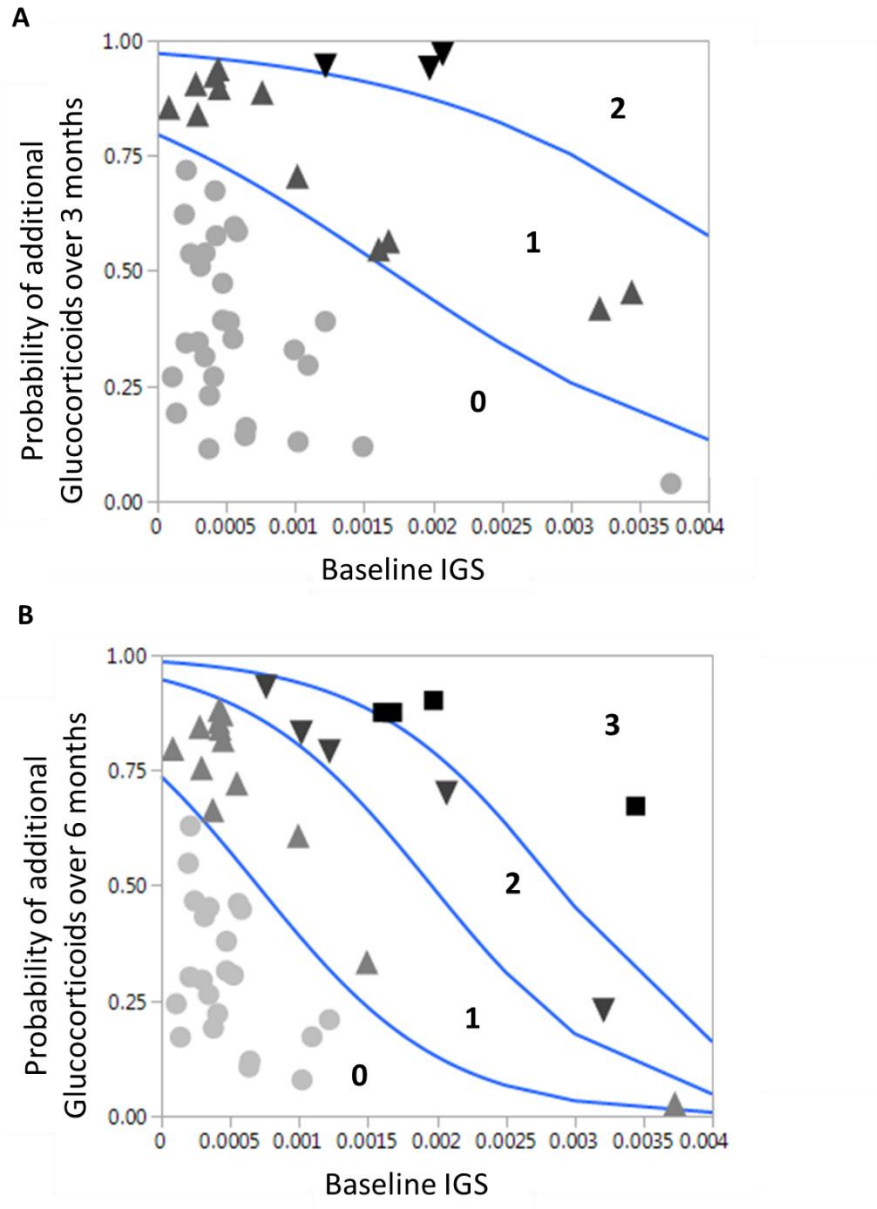


Figure 3.12 The baseline IGS predicts glucocorticoid requirements at 3 and 6 months

In early RA patients the baseline IGS was calculated and the number of additional glucocorticoid requirements (beyond any baseline administration) required in the first 3 and 6 months was recorded **A:** The baseline IGS significantly predicted the number of glucocorticoids required in the first 3 months after diagnosis ($p=0.023$, $n=37$) **B:** The baseline IGS significantly predicted the number of glucocorticoids required in the first 6 months after diagnosis ($p<0.0008$, $n=32$). Ordinal logistic regression, age and sex corrected with bootstrap probabilities included. Numbers 0-3 denote number of glucocorticoid administrations beyond baseline; thus a low IGS has a high probability of having 0 extra glucocorticoids, whereas a high IGS has a low probability of having 0 extra glucocorticoids.

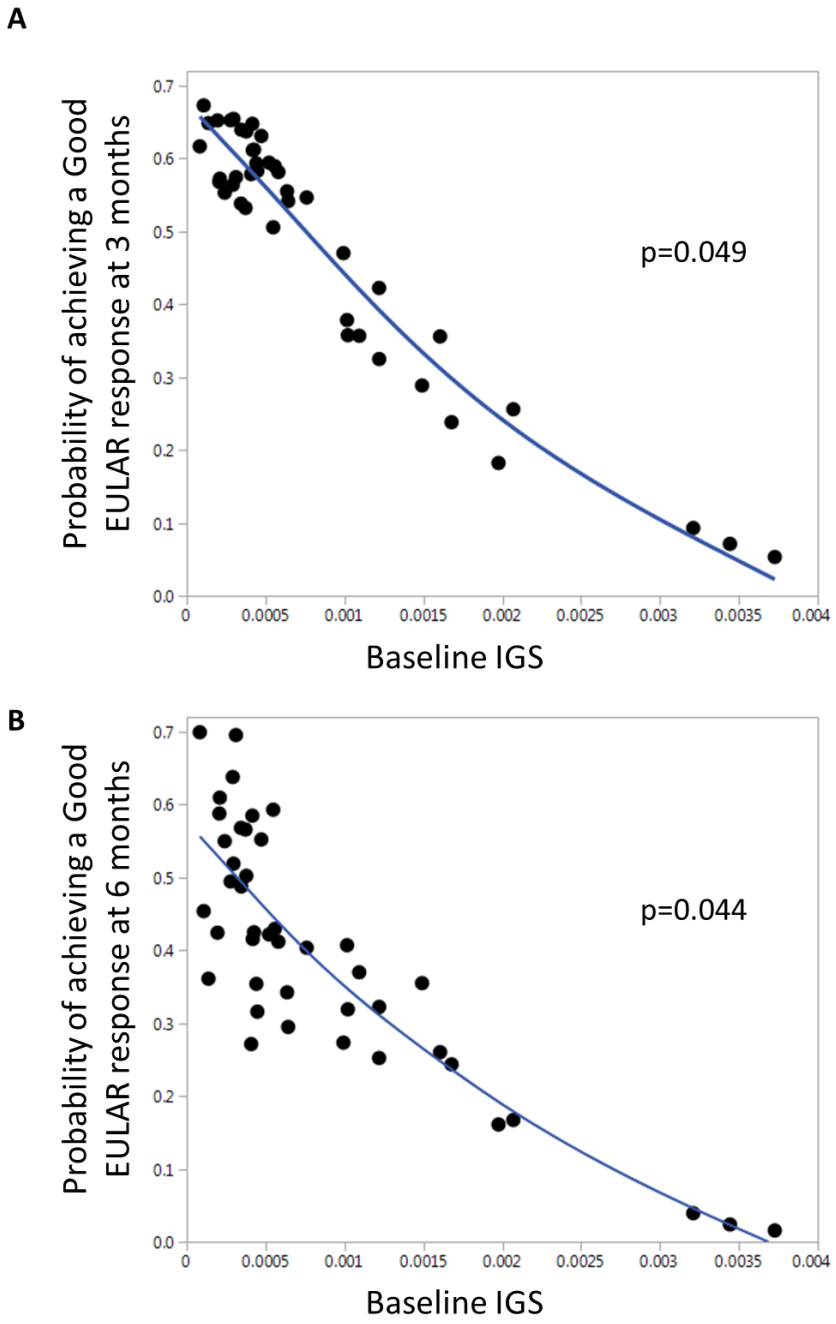


Figure 3.13 Inverse association between baseline IGS score and probability of achieving a good EULAR response at 3 and 6 months

In early RA patients the baseline IGS was calculated and a relationship sought between the probability of achieving a good EULAR response at **A:** 3 months (n=37) and **B:** 6 months (n=32). Nominal logistic regression age and sex corrected.

3.4 Discussion

In this chapter I sought a potential role for the IGS in early RA. I found the IGS was increased in early RA compared with established RA and fell during the 12 months after diagnosis. This fall was independent of any confounding glucocorticoid administration. In early RA the IGS demonstrated clinical relevance as it associated with RF titres as well as baseline and 6 month disease activity (DAS-28) scores. In addition the baseline IGS was able to predict at 6 months the DAS-28, the number of additional glucocorticoids required as well as predict the clinical response to initial therapies. In all cases a higher IGS resulted in a worse clinical outcome. Importantly the baseline IGS outperformed conventional parameters of disease activity such as baseline DAS-28, CRP and ESR when making these predictions.

The significance of the IGS in RA pathogenesis has been examined before however most previous studies have been performed in established RA cohorts, where findings may have been confounded by concurrent immunomodulatory therapies (23). In contrast I focussed on early, DMARD-naive RA patients. There are numerous IRGs that are upregulated in response to type 1 interferons (108). I examined 5 IRGs, which were either most differentially expressed or shown to be relevant in previous RA and SLE studies (158, 184, 200, 203, 206, 248-251). I demonstrated an increased prevalence of IGS-positive patients in early RA, which subsequently fell with treatment (figure 3.1-3.4). Reassuringly my data in established RA and SLE cohorts were consistent with previous reports (186, 262). This increased prevalence of type 1 interferons in early RA mirrors other human autoimmune conditions, including type 1 diabetes, primary Sjogrens syndrome (pSS) and systemic sclerosis, where early exposure to these cytokines reinforces aberrant signalling pathways leading to breach of tolerance (148, 265, 266). Indeed historically interferon-α therapy used for the treatment of viral hepatitis was shown to induce a symmetrical inflammatory arthritis which, in some cases, was seropositive and consistent with a *de novo* diagnosis of RA (267-269). More recent developments have shown that certain genetic variants that increase RA susceptibility, particularly in patients harbouring the shared epitope, are associated with the type 1 interferon pathway, such as IRF5 and STAT 4 (169, 173). In addition, upregulation of genes related to the type 1 signalling pathway predicted progression to RA in a seropositive arthralgia patient cohort (182, 183), and in established RA a higher IGS is seen in patients with a shorter disease history (191). In a mouse model, transfer of type 1 interferon producing dendritic cells was sufficient to propagate an inflammatory arthritis

which persisted even beyond the period where type 1 interferons were being produced (270). In other murine models intra-articular type 1 interferon injections induced an arthritis phenotype (177) and inhibition of interferon-α signalling pathways by a variety of means prevented the development of inflammatory arthritis in susceptible animal models (172, 177-179, 271). Finally in mouse models of arthritis secondary to infection, blocking type 1 but not type 2 interferons was sufficient to supress arthritis (272). Cumulatively this evidence, along with my data, suggest that type 1 interferons may be an important pathophysiological mechanism early in RA.

Consistent with an increased prevalence of a positive IGS in early RA I found a significant fall in the IGS at 3, 6 and 12 months after diagnosis and initiation of treatment (figure 3.3-3.4). At diagnosis (baseline) most patients received a depot intramuscular glucocorticoid injection and to reduce confounding (23) those who received additional glucocorticoids were excluded from longitudinal analysis. Glucocorticoid exposure is likely to have influenced the IGS at 1 month but, by 3, 6 and 12 months other factors must have contributed to the reduced IGS, such as reduced disease activity and direct DMARD effects. This supports the observation that patients with a longer duration of RA had a lower IGS (191) although longitudinal analysis of established SLE has showed no change in the IGS even during dynamic disease activity (273, 274). Apart from steroids and a single report following a lymphotoxin-beta receptor-immunoglobulin fusion protein therapy (23, 187), no other therapies have been shown to modulate the IGS in RA. It is therefore intriguing that I saw this in early RA following conventional DMARD therapy. Notably however there was no change in *OAS1* expression over 3 months (figure 3.3-3.4) but the PCA of the IGS showed all of the IRGs were similarly upregulated. It may be that OAS1 is relatively resistant to treatment-induced changes although this is currently only speculative. In light of these findings I also examined the IGS in the context of pre-diagnosis symptom duration but no association was seen. This was slightly unexpected, however symptom duration is a self-reported retrospective measure and therefore subject to recall bias. Perhaps more importantly, time itself may not be crucial to the fall of the IGS, but rather initiation of DMARDs and reduction in disease activity are the primary factors.

Multiple studies have shown no association between the IGS and disease activity in established RA (186, 187). This is despite established associations between the IGS and SLE disease activity (155) as well a single study reporting associations between serum interferon-α, extra-articular complications and disease activity (188). Nonetheless I

demonstrate for the first time an association between IGS and disease activity in early RA at both baseline and at 6 months (figure 3.10). When examining the composite parameters of the DAS-28 (ESR, TJC, SJC and patient VAS) I found that the association with IGS was mainly driven by TJC and SJC. Interestingly a recent Phase IIB trial of IFNAR1 blockade in SLE, demonstrated a reduction in symptoms of joint inflammation, such as TJC and SJC (162). This may support a role for type 1 interferons in driving direct synovial pathology.

My data also showed that in early RA the IGS had a predictive capacity; the baseline IGS predicted the DAS-28 at 6 months, EULAR response to initial therapies as well as glucocorticoid requirement over the 3-6 months after diagnosis and initiation of DMARDs (figure 3.12-3.13). Conventional models of inflammation, such as CRP, ESR and baseline DAS-28 were not able to make the same predictions, highlighting a distinct role for type 1 interferons in disease pathogenesis and treatment response. In SLE, simultaneous TLR7 and TLR9 signalling in pDCs causes relative glucocorticoid resistance, resistance to apoptosis as well as stimulating interferon-α release (275, 276). A similar mechanism may be occurring in RA also. A positive IGS predicts an inferior response to certain biological therapies in established RA (186, 200, 202), whereas this data show that the IGS also affects response to oral DMARDs and glucocorticoids in early disease. Some patients developing widespread polyarthritis following interferon-α therapy had substantial improvement following hydroxychloroquine (HCQ) therapy (269, 277). HCQ can bind nucleic acids directly, masking their TLR-binding epitope and consequent TLR signalling capacity (278). HCQ can also affect endosomal acidification thus modulating pDC TLR7 signalling and subsequent interferon-α production (155). This has been shown *in vitro* and also *ex vivo* when examining pDCs from HCQ treated SLE patients (279, 280). Finally patients with primary antiphospholipid syndrome have a reduced IGS if taking HCQ (281). In light of this an attempt was made to see whether the inclusion of HCQ in initial DMARD therapy modulated the effect of the IGS. Although there was a suggestion that HCQ abrogated the effect of IGS on disease outcome, my study had insufficient power to substantiate this observation. Therefore whether simultaneous TLR7/9 signalling in pDC relates to the poorer outcome seen in IGS positive RA patients, and whether HCQ may be particularly beneficial in IGS+ patients, remain to be elucidated. Overall however my data suggest a prognostic rather than therapy-specific influence of the IGS.

I did not detect circulating interferon-α in serum, which is consistent with published work in both established RA and SLE where, despite high IGS expression in cellular RNA, there is often no detectable cytokine in the serum (186, 187, 282). One potential explanation is that lymphocytes are being exposed to interferon-α at other sites, such as within lymph nodes. Supporting this suggestion is the observation that, upon activation, most interferon-α producing pDCs migrate to lymph nodes from the peripheral blood (283, 284). I also examined for associations with other inflammatory cytokines but none significantly associated with the IGS at baseline. It is interesting that the IGS did not associate with interferon-γ as in SLE there is a suggestion that type 2 interferons, such as interferon-γ, may be important in upregulating IRGs associated with a positive IGS (163). However, in my cohort not only was there no association between IGS and interferon-γ at baseline but 12 months later interferon-γ levels had significantly risen, whereas the IGS had fallen (figures 3.3-3.5). When I chose the 5 IRGs to generate my IGS I specifically excluded any IRGs that could also be cross stimulated by type 2 interferons. Some of these genes were very highly expressed in the RA microarrays used to identify my IRGs, and potentially could have been upregulated due to interferon-γ exposure secondary to the known RA Th1 response (66). Due to my chosen IRGs however this potential confounding association would have been minimised and thus any potential association with type 2 interferons lost. Others suggest the IGS detected in RA synovial fluid is driven by TNF-α (285), but again, I did not identify a relationship between TNF-α levels and the IGS. These data are reassuring in allowing us to draw conclusions that the IGS reported here is predominantly generated by exposure to type 1 interferons. However caveats regarding different cytokines level at localised sites, such as lymph nodes would still remain.

Treatment with interferon-α can induce de novo autoantibodies or increase titres of pre-existing autoantibodies. This is most marked for ANA where, depending on the patient group, between 18% and 72% exhibited increased titres following treatment with interferon-α (267, 268, 286-289). This pattern is similar to that seen in SLE, where the IGS associates with ANA and anti-RNA binding protein antibodies (282). Conversely, I did not see an association between IGS and ANA titres in early RA. I did, however, see an association between IGS and RF factor but not anti-CCP titre (figure 3.7). This is intriguing and, in patients who developed a polyarthritis following interferon-α therapy, 34% also developed RF autoantibodies but far fewer developed anti-CCP antibodies (180). Furthermore, *in vitro,* interferon-α can promote RA B-cells to produce RF (290).

The difference between the effect of the IGS and anti-CCP may in part be due to differences in mechanisms related to their generation. RF is a predominately a non-switched IgM against self-IgG and some have supposed that, contrary to anti-CCP generation, RF is generated following TLR signaling in a T cell independent manner (291). B cells express TLR 7 and 9 (292) so it is conceivable that triggers for pDC interferon-α production could also trigger RF generation. Conversely in SLE a novel mechanism has been proposed where the IGS is generated by opsonisation of dead cells by complement and IgM (293). Potentially IgM RF in RA may be contributing to this observation via a similar mechanism. Nonetheless my finding is contrary to what has been reported in established RA where IGS did not associate with RF, anti-CCP, or ds-DNA titres (294). However, in this published study the median disease duration was 98 months. Given the fall in IGS following initiation of DMARDs in early RA (figure 3.3-3.4) it may well be that any associations become blurred over time. An alternative explanation is that type 1 interferons are more important for inducing RF generation in early disease. Indeed the proportion of IgM anti-CCP antibodies was higher in early RA than in established RA (4) and this potentially may be due increased exposure to type 1 interferons observed in early RA.

There are a variety of ways that type 1 interferons can affect B cells and promote auto-reactivity and autoantibody production. BAFF/BLyS is under the control of IRF transcription factors (295) and interferon-α induces its expression in other cell subsets (145, 146). Autoreactive B cells require higher levels of BAFF/BLyS for survival and increased BAFF/BLyS following can rescue autoreactive cells that would otherwise have been deleted at the transitional phase (296, 297). It is interesting therefore that RF titres significantly associated with the IGS as well as with BAFF/BLyS (figure 3.8) although conversely there was no association between BAFF/BLyS and the IGS itself. However I only measured circulating levels of BAFF/BLyS; a membrane bound form also exists, which some propose to be a more potent stimulus for B-cells than soluble BAFF/BLyS (298). Indeed, RA fibroblast-like synoviocytes express membrane-bound BAFF/BLyS and are critical for the local activation of B-cells (299). Thus increased BAFF/BLyS signaling may be a mechanism whereby the IGS promotes RF generation. Type 1 interferons can also affect B-cells independently of BAFF/BLyS. They lower the BCR signaling threshold (139), increase TLR7 expression (140), promote B cell longevity by upregulating BCl2 (141) and also upregulate cell surface molecules such as CD69, CD86 and HLA-DR that affect B-cell survival and function (139). Finally type I interferon

exposure also affects the B-cell response to IL-6, which is involved both in B-cell survival and differentiation (142). There was a positive association between IL-6 and RF titres but not anti-CCP titres (figure 3.8). The association with the IGS in the RF+ cohort could therefore also be driven by the promotion of increased sensitivity to IL6 signaling. However with both these associations, although there is clear differential activation of anti-CCP and RF producing B cells, it is not clear why this occurs.

Although type 1 interferons affect B cells, these cells can in turn affect the production of type 1 interferons by pDCs. In SLE an evolving body of literature implicates serum derived factors in the production of type 1 interferons, namely autoantibody containing immune complexes. Autoantibody-containing IgG from SLE patients can directly bind to FcγRIIa (CD32) expressed on pDCs. These complexes are then internalised and subsequently trigger intracellular signalling pathways and initiate the production of interferon-α (147-151). To determine whether serum derived factors, such as immune complexes, acting on pDCs may have contributed to the IGS I cultured PBMCs (with or without pDC depletion) overnight with serum from IGS positive and IGS negative early arthritis patients as well as from healthy controls. Given the observation between RF titres and IGS I hypothesised that RF containing immune complexes may have been implicated in interferon-α production. There were clear caveats to my findings, the main being that most of the values were below or close to the assay detection threshold. Nonetheless there was a suggestion that early RA serum induced greater production of interferon-α from PBMCs and pDCs appeared to play a significant role (figure 3.9). However this effect was not related to serum RF or anti-CCP titres. Potentially this could be explained by other RA autoantibodies, such as anti-CarP antibodies (103), participating in pathological immune complexes. However purified immune complexes isolated from established RA serum did not induce type 1 interferon production from pDCs when a similar experiment was performed (150). There could be a difference between early versus established RA, but additional serum derived factors (not immune complexes) could have underpinned these observations. For example neutrophils can extrude mitochondrial DNA or the alarmin human neutrophil peptide 1 (HNP1) both of which can trigger type 1 interferon production (300, 301). Furthermore neutrophil cell death termed NETosis causes extrusion of intracellular contents, including nuclear material, in a lattice structure termed a NET. NETs are increased in RA serum and have pathological relevance in SLE where they directly activate pDCs and, via TLR9 ligation, cause type 1 interferon release (77, 302-304).

Finally I averaged the IRGs to calculate an IGS but some methods calculate an IGS value by comparing each individual gene to a healthy control population. A disadvantage of this method is that it does not generate a "positive/negative" cut off value but rather a continuous variable and thus limits the researcher being able to identify the prevalence of a "positive IGS" as I wished to do in this study. However an advantage of this method is that it does minimise skewing of the IGS by certain IRGs that may be more abundantly expressed. This would need to be taken into account when interpreting the findings, however the PCA of the IRGs in my IGS demonstrated no undue effect of one IRG over the other suggesting that this concern may be minimal.

However, as indicated, I used IRGs that had previously been shown to be upregulated in established RA and in ACPA+ arthralgia. A recently published abstract demonstrates IRG expression in incomplete early SLE and established complete SLE is different and suggest that IRG expression may be a dynamic process with an intrinsic variability in expression over time (305). It would have been preferable therefore to only use genes that had been shown to be abnormal in early RA. However the paucity in the literature examining the IGS in early RA is likely to have hampered my evidence based choice of genes. Nonetheless there is a single study looking at drug naïve early RA IRG expression in which *IFI44L*, *MxA* and *RSAD2* are used to generate the IGS (248). Two of these genes (*MxA* and *IFI44L*) do overlap with the genes chosen to calculate my IGS whereas *RSAD2* is also commonly activated by type 2 interferons and so was excluded from my choice. Theoretically as more is known about type 1 interferons in early RA, the gene list I used may need to be reviewed.

A further option to identify suitable IRGs would be to culture PBMCs from early RA patients with type 1 interferons and measure the upregulated IRGs and subsequently chose the most highly differentially expressed genes. One caveat to this method however choice of interferon-α for culture. There are 13 subtypes of interferon-α and while it is appreciated that interferon-β can have separate effects downstream of the IFNAR due to altered phosphorylation associated protein (306) or by binding uniquely to IFNAR1 independent of IFNAR2 and initiating downstream signalling pathways independent of JAK-STAT phosphorylation (307), the permeations of what each of the different interferon-α subtypes on cellular response or IRG upregulation is unknown. Furthermore it is unclear what interferon-α subtype is predominant in RA. Indeed different stimuli can promote the different release of interferon-α subtypes (308). Although the above

experiment would have provided a "positive control", given these caveats, I felt using IRGs known to be dysregulated *in vivo* in RA was preferable.

3.5 Conclusions

RA is highly heterogeneous disease and I hypothesize that, in a subgroup of patients with a susceptible genetic background, type 1 interferons contribute to initiation or reinforcement of early pathogenic pathways. These may involve the activation of B cells and the production of autoantibodies, such as RF.

A positive IGS in early RA has relevance to clinical progress as it predicts lack of response to initial treatment, which I have shown for the first time. RA therapeutic strategies are increasingly focused on early treatment and there may be merit in targeting pathways triggered by type 1 interferons in a subset of patients with a high IGS at disease onset. For example, anti-interferon-α monoclonal antibodies and IFNAR1 blockers are both currently being trialled in SLE (161, 263) and JAK1/3 inhibitors, recently licenced for the treatment of RA in some parts of the world, also inhibit type 1 interferon signalling (309).

In summary, I have demonstrated a high prevalence of the IGS in patients with early RA. This predicts increased disease activity as well as refractoriness to initial treatment. I suggest that a subset of early RA patients may therefore benefit from therapeutic strategies that target type 1 interferons and their downstream pathways.

3.6 Future work

One of the key steps in the discovery of any prediction model is the validation of the findings. It would also be useful to increase the number of patients examined independently of validation. A benefit of this would be the ability to examine the role of different DMARDs, particularly HCQ, in modulating the effect of the IGS. Furthermore since the IGS associated most strongly with the TJC and SJC correlating the IGS with musculoskeletal ultrasound findings is likely to be additionally informative.

I would also like to follow a larger cohort of early RA patients over 12 months to assess, more robustly, if there was any relationship between the fall in DAS-28 following treatment and if this was reciprocal to the changes in IGS at 1, 3, 6, and 12 months after diagnosis. It would be interesting to see if those with increased disease activity at 6 months also had a persistently higher IGS also. I was not able to assess this aspect of the dynamic interaction between the IGS and the DAS-28 at 6 months due to limitations in patient recruitment and the finite timing of the PhD. Nonetheless with increased patient number and matched DAS-28/IGS over the first 6 months certain modelling techniques could be used to identify if a relationship persisted between these agents once treatment was initiated.

I saw an interesting association with the IGS and RF but not anti-CCP. I only looked at the autoantibodies themselves and it would be ideal to examine immunoglobulin (IgA, IgM and IgG) levels within the serum to see if the IGS was associated with a global B cell activation or if it was primarily limited to RF or only IgM. In keeping with this it would also be interesting to follow up RF and anti-CCP titres and see if they changed with time parallel to the fall in the IGS.

I could also culture B cells from either IGS+ or IGS- early RA patients (as well as healthy controls) and stimulate them with interferon-α. This would examine the proliferative capacity of the B cells as well as the production of immunoglobulins, particularly IgM. Cell culture supernatant could be analysed specifically for RF and anti-CCP antibodies.

The results of experiments attempting to identify if serum derived factors were responsible for induction of type 1 interferons were very close to the assays' lower detection threshold. I would repeat these but over a longer time frame and also with a higher percentage of patient serum. In SLE immune complexes bind to FcγRIIa (CD32) on pDCs to initiate interferon-α release. To see if this mechanism was also involved in early RA I would also perform these cultures with a FcγRIIa blocking antibody. As both

NETs as well as immune complexes may be responsible for these observations, future work would include measuring NETs in the circulation of IGS+/IGS- early RA patients, as well as using purified immune complexes from RA serum in culture.

Finally I did not detect any interferon-α in the serum using MSD technology. Some have used a reporter assay (bioassay) to detect interferon-α in the serum and found that this is more sensitive than other conventional assays. This could be considered in the future to identify if there is increased interferon-α in the circulation. This would contribute to the understating of the location of the interferon-α producing cells, i.e. lymph node/joint or periphery.

Chapter 4: Plasmacytoid dendritic cells in early RA

4.1 Introduction

T cells and B cells are generally considered to be the pathological cell subtypes that are deranged in rheumatoid arthritis (RA), a theory supported by the therapeutic success of T and B cell targeted treatments (92). However, the role of other cell subsets in RA pathophysiology is becoming increasingly appreciated. Dendritic cells (DCs) are of growing interest, not least because they can modulate both the innate and adaptive immune system and therefore have direct effects on both T cells and B cells (32).

It is not only their ability to deviate T and B cell phenotype that makes DC of interest. DC also have tolerogenic capacities as shown by work in both cancer and autoimmunity (310, 311). Thus, DCs can induce cell death or anergy and can skew T cells towards a regulatory phenotype. This highlights the complexity of DC function and has further consolidated the important role of DCs in immune modulation and the need to understand their role in RA. Plasmacytoid dendritic cells (pDCs) are a relatively newly identified DC subset. The majority of work investigating their role in autoimmunity has been in SLE and focused on their prolific interferon-α production and the subsequent generation of an interferon gene signature (IGS) (157). They have not been studied in early RA and what role they play in RA pathogenesis remains unclear and to date contradictory (85, 90). In other environments pDCs have been reported to play a potentially tolerogenic role in part due to the expression of programmed death ligand 1 (PDL1) and inducible T-cell co-stimulator ligand (ICOSL) which promotes the generation of regulatory T cells, or Tregs (56-61). Some report reduced Tregs in RA, but this is not a universal finding (75, 96-99). Nonetheless the potential effect of modified pDC function on Treg generation in RA has not been examined.

Current evidence for the role of DCs in general in RA points towards a role in disease maintenance and progression, however most of this work has been performed in established RA and it remains unclear whether DCs initiate autoimmunity or facilitate breach of tolerance (81). Other cell types, such as $CD4^+$ T cells have been examined in more detail in early RA, with evidence that the function and phenotype of these cells in early disease may be distinct from what is seen in established RA (9, 248, 291, 312). Thus examining pDCs at the start of disease prior to initiation of disease modifying therapies is likely to provide additional useful information into their role in RA. I therefore studied pDCs in early RA with focus on the IGS, maturation/activation phenotype, as well as their ability to generate Tregs.

4.2 Aims

In this chapter I wished to address the following questions:

1. Is the percentage of circulating pDCs or their activation status deranged in early RA?
2. Does the IGS associate with pDC frequency or phenotype?
3. Is there an association between pDC subset frequency/phenotype and clinical parameters or pro-inflammatory cytokines?
4. What happens to pDC subsets with disease duration and treatment?
5. Do pDCs from RA patients differ from healthy controls functionally? In particularly relating to their ability to induce Tregs?

4.3 Results

4.3.1 Patient cohorts

Cohorts for this analysis included early DMARD and steroid naïve early RA, ACPA+ arthralgia patients (also DMARD and steroid naïve), and healthy controls who had no personal history of rheumatological disease nor any other significant past medical history. Finally established RA and SLE patients cohorts were included, and for these latter cohorts inclusion criteria included disease duration of greater than 12 months.

Patient and healthy control demographic data demonstrated a significant difference in age and sex of the different cohorts (table 4.1).

The frequency of pDCs in peripheral blood was identified by flow cytometry where the proportion of the pDC in the lymphocyte gate recorded. The population was first divided into healthy controls and disease patients which included all the disease groups. There was no association between age and the circulating frequency of pDC for either cohort nor was there any association with gender (figure 4.1).

Although age and sex did not affect pDC frequency, due to the highly significant differences in demographics between my cohorts, I corrected for age and sex when studying DC populations across the cohorts. However when looking at an individual cohort in isolation (e.g. early RA only) I did not correct for age and sex.

The specific demographic data for the longitudinal early RA cohort examined in this chapter (which is a subset of the larger early RA cohort), are shown in Chapter 3, table 3.4.

Patient Cohort	Early RA	Est RA	SLE	ACPA+ arthralgia	HC	Difference between cohorts
Number	44	11	23	11	30	-
Age (years) median [range]	57 [33-84]	62 [33-78]	53 [33-67]	43 [20-75]	37 [23-62]	p<0.0001
Male : Female ratio	3:4	1:10	1:11	1:2	3:2	p=0.0008

Table 4.1 Demographical data for cohorts used

Patient cohorts included those with early RA (n=44), established RA (Est RA, n=11), SLE (n=33), ACPA+ arthralgia (n=11) and healthy controls (HC, n=30). Their demographic data are displayed including median age (and range) and male:female ratio. Cohorts were compared by one-way ANOVA.

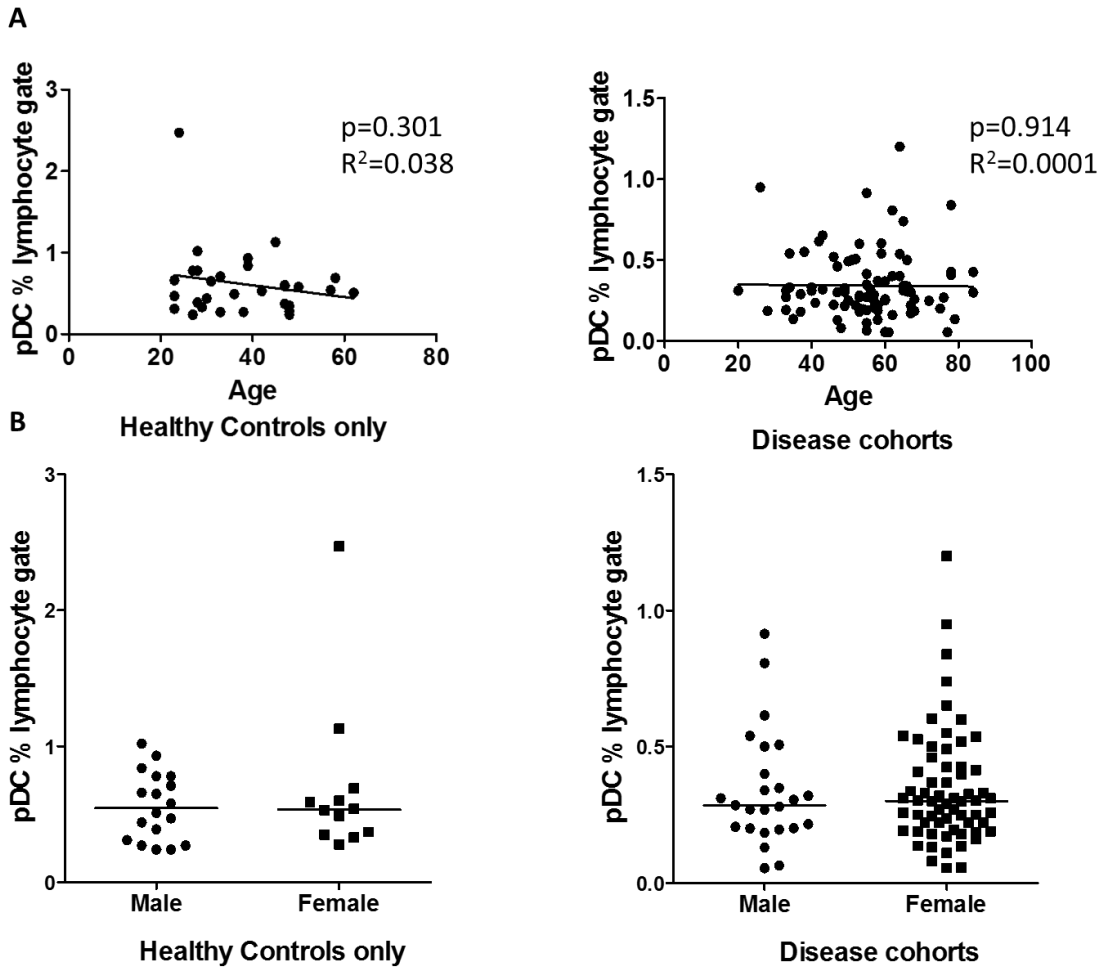


Figure 4.1 pDC frequency in the peripheral blood by age and gender

Subjects were divided into healthy controls (p=30) and disease categories (n=89). The latter included RA (n=44), established RA (n=11), SLE (n=33), ACPA+ arthralgia (n=11). PDCs were identified in the peripheral blood and the percentage of cells falling within the lymphocyte gate was recorded using flow cytometry. **A:** Plots show linear regression between age and pDC frequency for both healthy control and disease cohorts. **B:** pDC frequency by gender for healthy controls and disease cohorts. Horizontal lines depict median values. p>0.05 Mann-Whitney U tests.

4.3.2 pDC frequency by disease category and associations with the IGS

There was a significant reduction in the frequency of pDCs in early RA and SLE compared with healthy controls. Similar trends were seen with the other disease cohorts (ACPA+ arthralgia and established RA) but these did not reach significance (figure 4.2).

Given the role of pDCs in the production of type 1 interferons (43) associations were sought between pDC frequency and the interferon gene signature (IGS) in the early RA cohort. PDC frequency was comparable between IGS+ and IGS- early RA patients nor was there any association with the IGS (as a continuous variable) (figure 4.3).

Given that pDCs produce type 1 interferons upon activation, I also examined the pDC phenotype with respect to the IGS. However there was no significant association between any of the activation markers studied (CD40, CD86, CCR7, HLA-DR and ILT7) and the background IGS (data not shown).

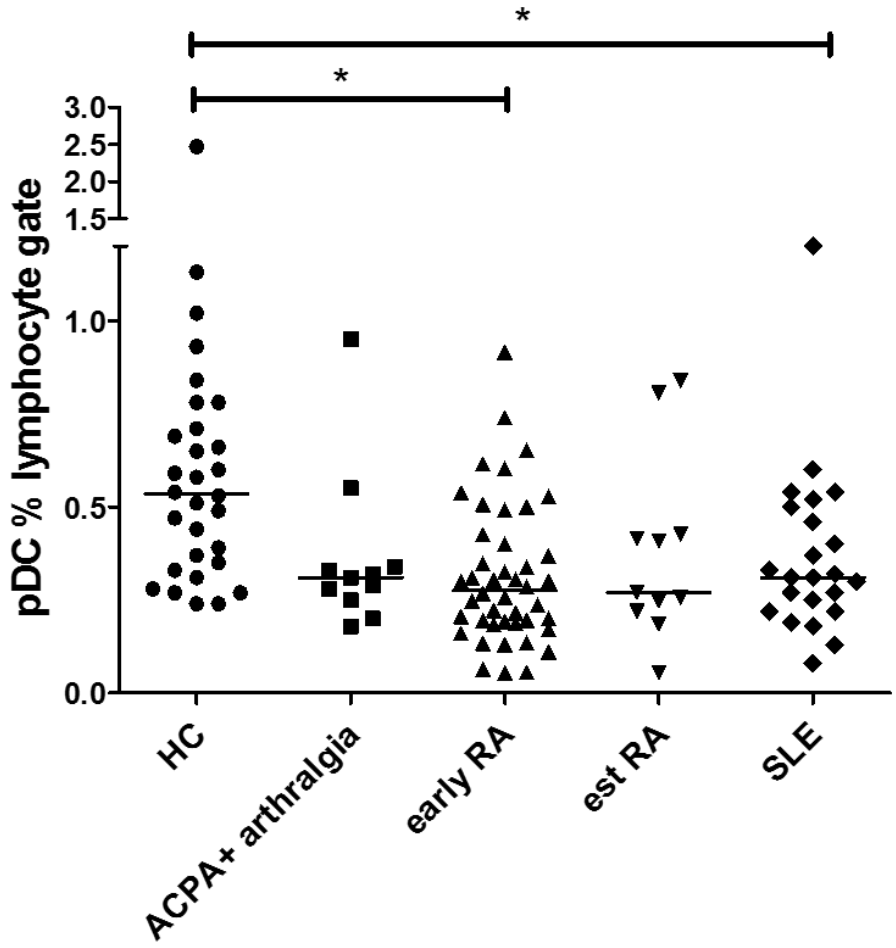


Figure 4.2 Proportion of pDC amongst peripheral blood lymphocytes in disease states compared with healthy controls

Peripheral blood pDCs, were identified by flow cytometry and recorded as a percentage of the circulating lymphocyte population. This value was compared across disease cohorts; early RA (n=44); established RA (est RA, n=11); anti-CCP+ (ACPA+) arthralgia (n=11); SLE (n=23) and healthy controls (n=30). Univariate generalised linear model (age and sex corrected). Horizontal lines depict median values. * $p<0.05$

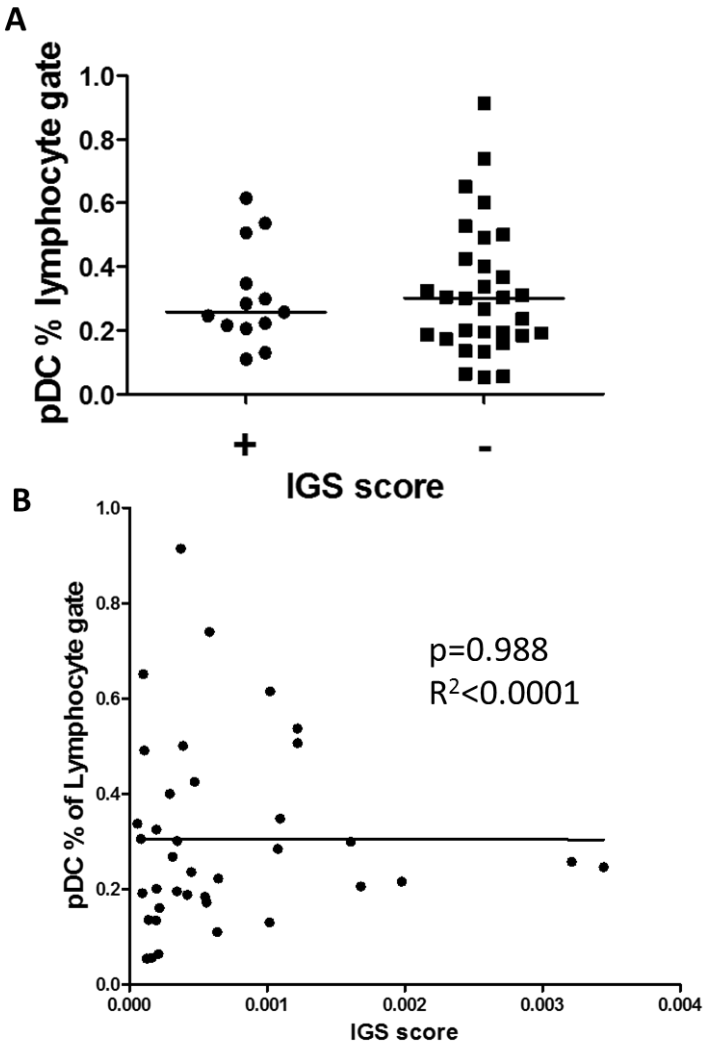


Figure 4.3 IGS and pDC frequency in early RA patients

Early RA patients (n=39) were examined for associations between pDC frequency within peripheral blood lymphocytes and the IGS **A:** IGS+ (n=13) and IGS- (n=26) early RA patients and pDC frequency. Horizontal lines depict median values. Mann-Whitney U test. **B:** Plot shows linear regression between pDC frequency and the IGS (continuous variable).

4.3.3 pDCs and early RA clinical phenotype

DCs can affect B cell function as well as T cell function (313). Given that B cell dysfunction in RA, commonly manifests in the production of autoantibodies, I questioned if serostatus had any effect on the percentage of circulating pDCs (figure 4.4).

There was a significant reduction in pDC frequency in seropositive early RA patients compared with healthy controls and a non-significant trend for seropositive patients to have a lower pDC frequency than seronegative patients (figure 4.5). In light of this RF and anti-CCP titres were compared with pDC frequency. However, there were no significant associations (data not shown).

As DC activation/maturation status can also affect interactions with other immune cells I examined pDC phenotype and serostatus. There was a trend towards increased CCR7 expression in early RA seropositive patients compared with healthy controls. Conversely seronegative patients had significantly less CCR7 when compared with healthy controls. Furthermore when directly comparing pDCs from early RA seropositive and seronegative patients there was significantly higher CCR7 expression in seropositive patients. No differences were seen in CD86, CD40, HLA-DR and ILT7 expression between the cohorts, nor was there any significant difference when examining ACPA+ arthralgia patients for all surface markers (figure 4.5).

An association was subsequently sought between CCR7 expression and pDC frequency where an inverse trend was seen (figure 4.6.A). This was significant when including only RF positive patients (figure 4.6.B) and there was a similar trend for anti-CCP positive patients, although not significant (figure 4.6.C). There was however a significant positive association between anti-CCP titres and pDC CCR7 expression; this was not reproduced with RF titres (figure 4.7). These associations were not detected with any other pDC activation markers (data not shown).

Finally in view of their potentially pathogenic role relationships between disease activity (DAS-28, VAS, TJC, SJC, ESR, CRP) and circulating pDC frequency and phenotype were sought; but no significant associations were seen (data not shown). There were also no significant relationships between serum cytokines (interferon-γ, IL-6, IL-12 p70, TNF-α, IL-1β, IL-2, IL-13, IL-4, IL-10 and BAFF) and pDC frequency or phenotype (data not shown).

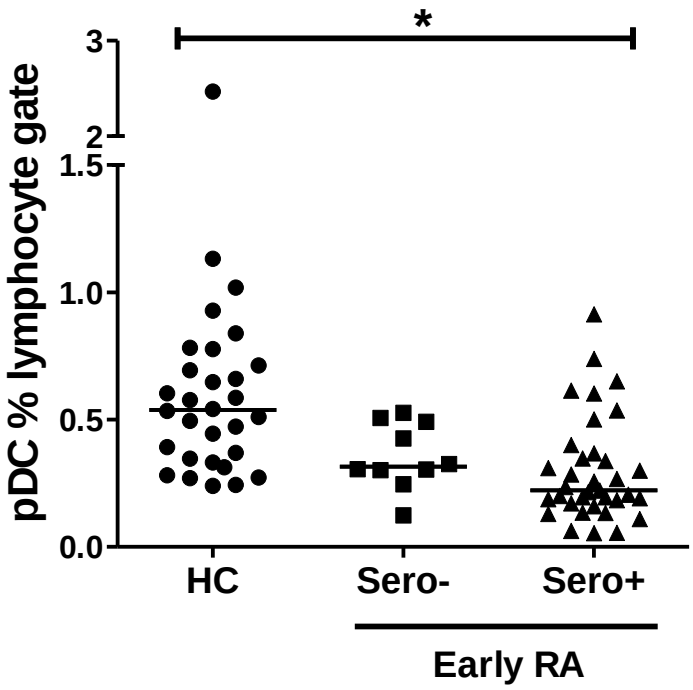


Figure 4.4 pDC frequency in relation to serostatus

Early RA patients (n=45) were divided into seropositive (n=35, either RF+ or anti-CCP+) and seronegative (n=10, both RF- and anti-CCP-) and pDC frequency (as determined by flow cytometry) compared with healthy controls (n=23). Horizontal lines depict median values. Univariate generalised linear model (age and sex corrected) between early RA and healthy controls and Mann-Whitney U test for comparisons within early RA. * $p<0.05$.

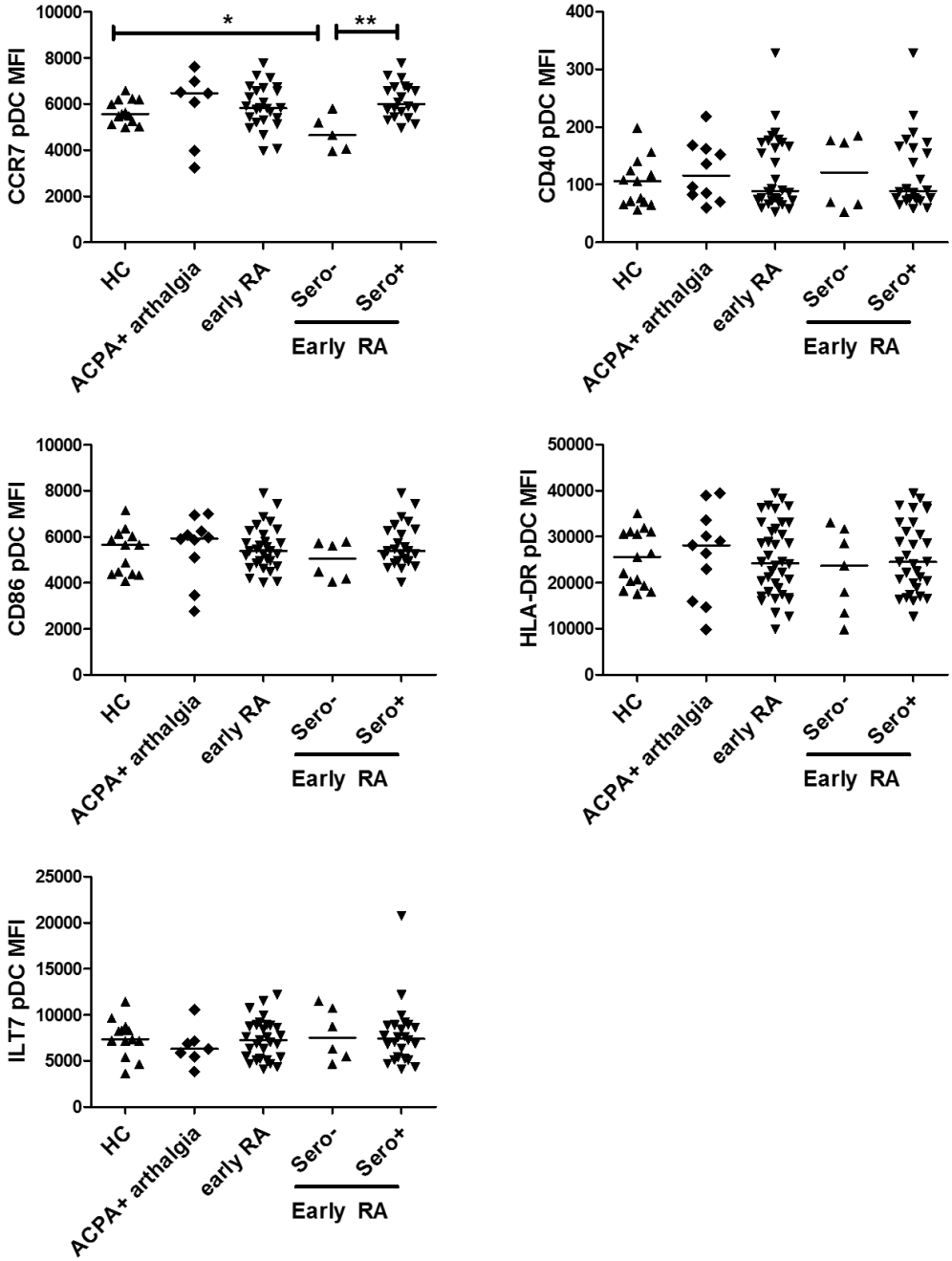


Figure 4.5 pDC surface phenotype in RA and pre-RA

PDC cell surface marker expression of CD86, CD40, ILT7 and HLA-DR was quantified by flow cytometry (median fluorescence intensity, MFI) in healthy controls (HC, n=13), anti-CCP+ arthralgia (ACPA+ arthralgia, n=7 for ILT7 and CCR7 expression, n=11 for remaining parameters) and early RA patients (n=30-32; further split into seronegative, n=5-7 and seropositive early RA, n=25). Horizontal lines depict median values. Univariate generalised linear model (age and sex corrected) between healthy controls and early RA and Mann-Whitney U test for comparisons within early RA with ACPA+ arthralgia *$p<0.05$, ** $p<0.01$.

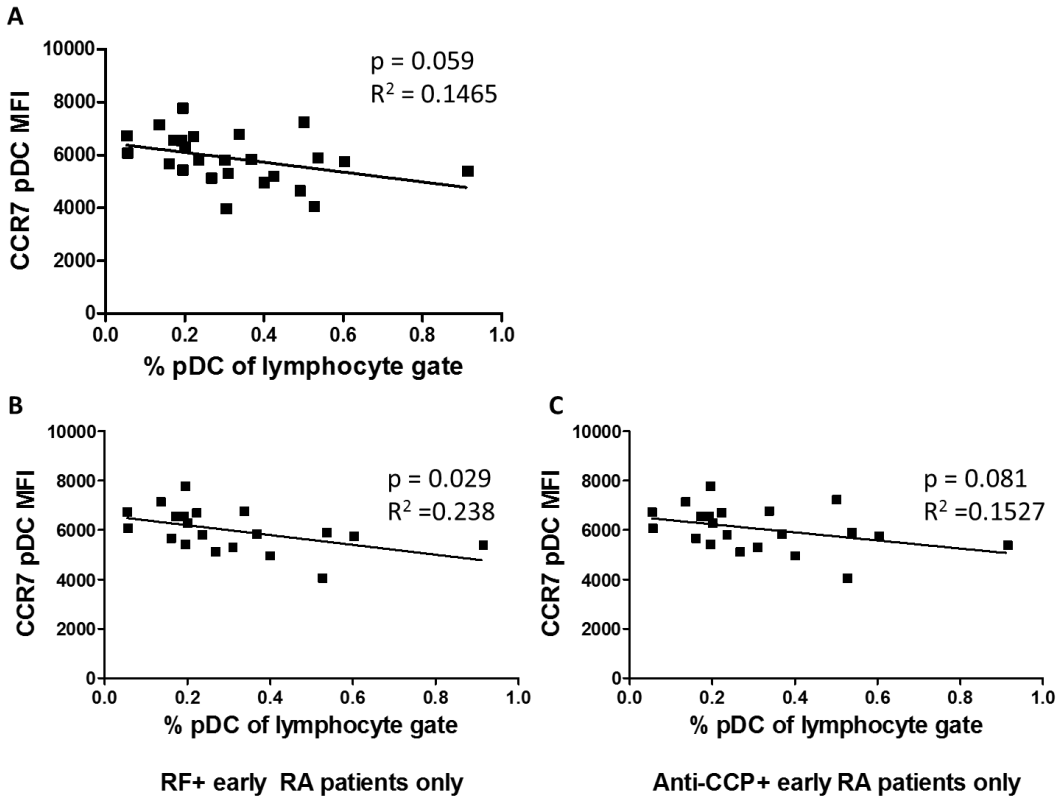


Figure 4.6 Relationship between percentage of circulating pDC and CCR7 expression in early RA

PDC CCR7 expression was quantified using flow cytometry derived median fluorescence intensity (MFI) and associations sought with pDC frequency. **A:** Plot depicts linear regression in all early seropositive RA patients (n=25) between pDC CCR7 expression and pDC frequency. **B:** Plot depicts linear regression in all RF+ early RA patients (n=25) between pDC CCR7 expression and pDC frequency. **C:** Plot depicts linear regression in anti-CCP+ early RA patients (n=21) between pDC CCR7 expression and pDC frequency.

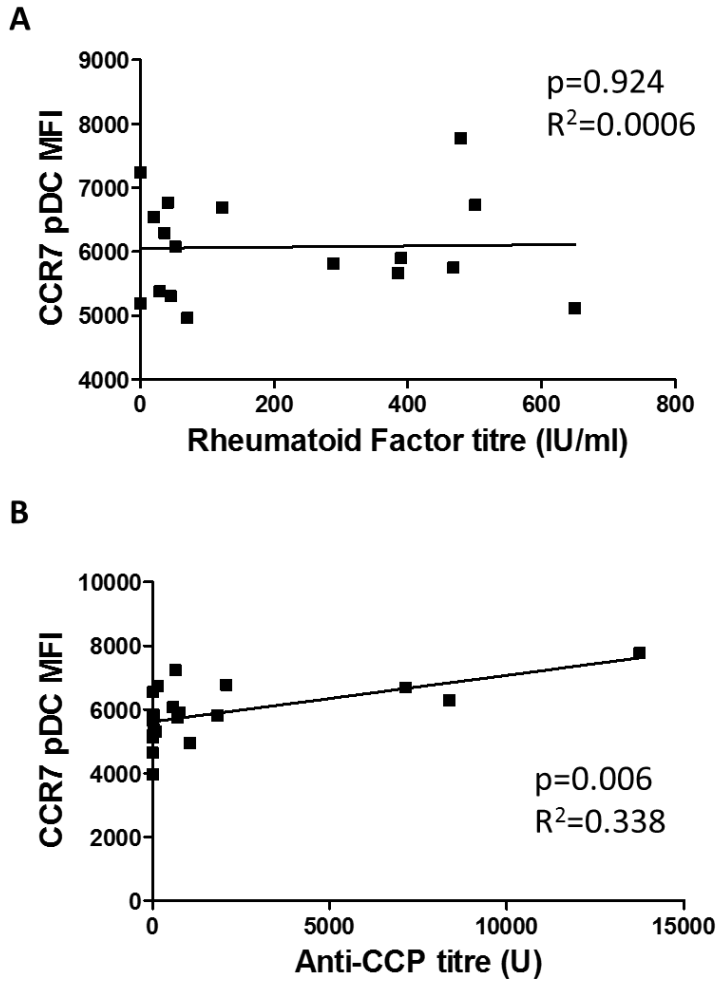


Figure 4.7 Relationship between RF and anti-CCP titre and pDC CCR7 expression
PDC CCR7 expression was quantified using flow cytometry derived median fluorescence intensity (MFI) in early RA patients and associations sought with autoantibody titres. **A:** Plot depicts linear regression between pDC CCR7 expression and RF titres. **B:** Plot depicts linear regression between pDC CCR7 expression and anti-CCP titres.

4.3.4 Longitudinal pDC frequency and phenotype

A cohort of 15 early RA patients were followed up at time points 1, 3, 6 and 12 months after diagnosis. Their demographic data are shown in chapter 3, table 3.4. At each time point (including baseline) pDC frequency and phenotype were studied.

There was no significant change in pDC frequency over the observation period (figure 4.8). However there was a significant fall in pDC CD86, HLADR and CD40 expression at 3 months 6 months and 12 months. There was also a difference in CD40 expression between 3 and 6 months, and 3 and 12 months; and between 1 and 6 months for HLADR expression (figure 4.9).

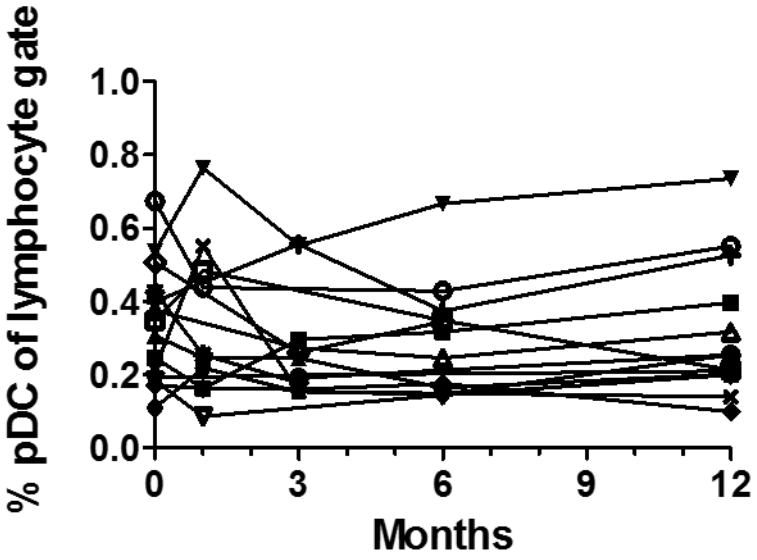


Figure 4.8 Longitudinal measurements of circulating pDC frequency in early RA

The percentage of pDC falling within the flow cytometry lymphocyte gate were enumerated longitudinally in an early RA cohort (n=15) at baseline and then 1, 3, 6 and 12 months after diagnosis. Each line represents an individual patient. Wilcoxon signed rank tests.

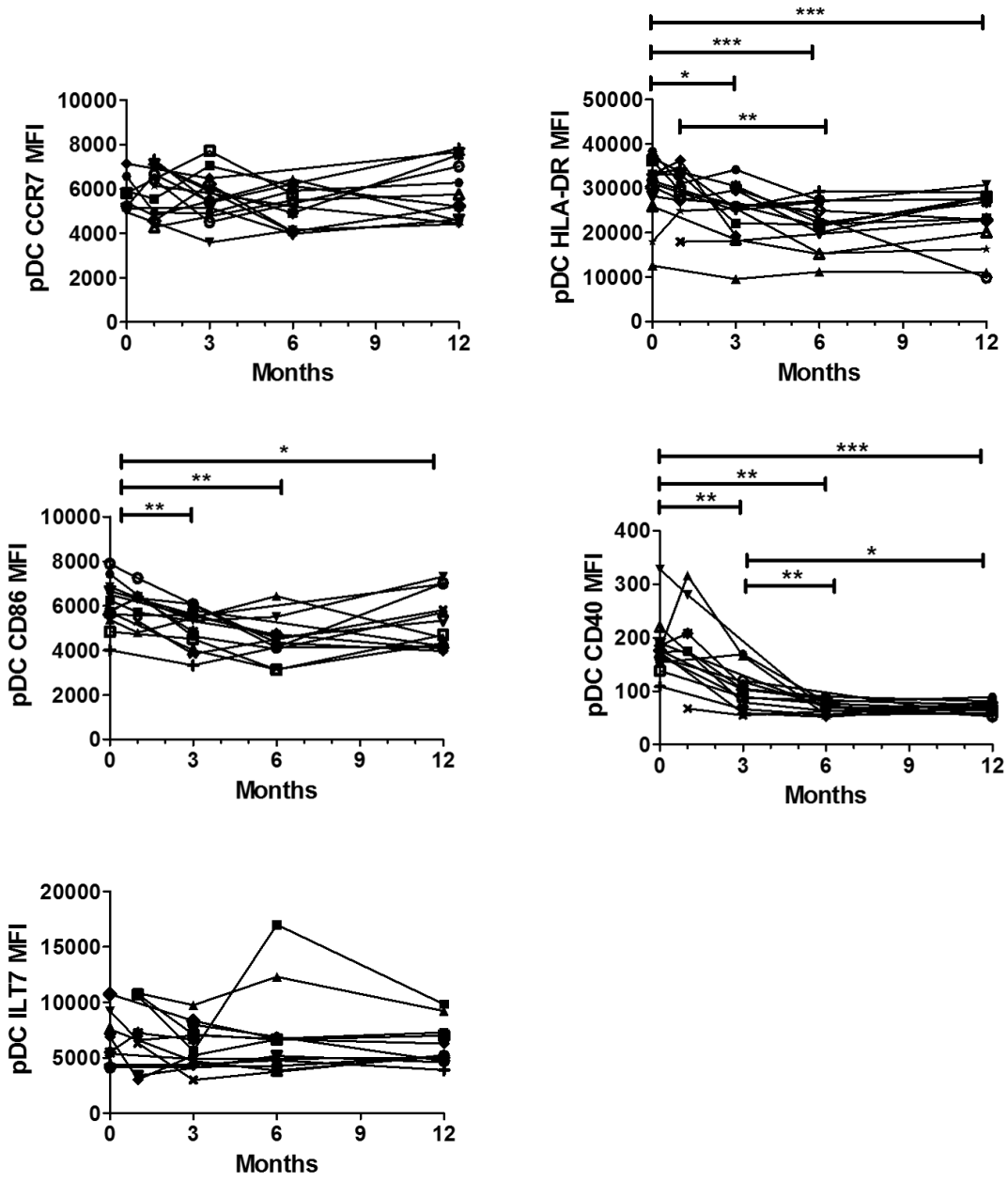


Figure 4.9 Longitudinal measurements of pDC activation markers in early RA

PDC cell surface marker expression (as determined by flow cytometry median florescence intensity, MFI) was quantified in an early RA cohort (n=15) at baseline and at 1, 3, 6 and 12 months after diagnosis. Each line represents an individual patient. Wilcoxon signed rank tests.*p<0.05, ** p<0.01, *** p<0.001.

4.3.5 pDC mixed lymphocyte reactions (MLRs)

Demographics of the established RA patients and healthy controls whose pDCs were used in the mixed lymphocyte reactions (MLRs) are shown (table 4.2). Notably the healthy controls were significantly younger than the patient population, although male:female distribution was comparable. Ideally early RA patients would have been used for this work but patient sample material was limited hence the use of established RA patients.

To quantify pDC response to maturation stimuli, PBMCs were stimulated with TLR-9 ligand CPG-B (5ug/ml) for 18-24 hours and compared with unstimulated controls. The fold change in pDC expression of HLA-DR, ICOS-L and PDL1 was then examined. Healthy controls and early RA patients upregulated pDC surface expression of ICOSL to a greater extent than established RA patients, although not to a significant degree (figure 4.10.A). This was inverse to what was seen with pDC PDL1 expression where healthy controls upregulated PDL1 significantly less than both early and established RA patients. Furthermore established RA patients upregulated PDL1 significantly more than early RA patients (figure 4.10.B). HLA-DR expression was comparable across groups and had a smaller range of fold-change induction than that seen for ICOSL and PDL1 (figure 4.10.C).

Mixed leukocyte reactions (MLRs) were performed between allogenic healthy control naive $CD4^+$ T cells and pDCs from either established RA patients or healthy controls. Optimal conditions for the MLRs were previously determined by the work of a Masters' student (Oliver Eltherington) within the laboratory and are not re-examined. These subsequent experiments were performed with his assistance.

MLR $CD4^+$ T cells were CFSE labelled and subsequent CFSE dilution (as determined by flow cytometry) was used to quantify the percentage of cells that proliferated. There was increased proliferation in MLRs from pDCs primed with CPG-B compared with control pDCs which was significant for RA but not healthy controls. However there was no difference between healthy controls and RA patient primed pDCs in the final level of proliferation achieved (figure 4.11.A). The co-expression of FOXP3 and high levels of CD25 has been described as one method of identifying Tregs (75). There was no difference in the proportion $CD3^+CD4^+$ $FOXP3^+CD25^+$cells following culture with pDCs from either healthy controls or RA patients (Figure 4.11.B).

Cytokine levels (interferon-γ, IL-13 and IL-10) were quantified in the supernatant of the pDC and naive T cell MLRs. Both the RA and healthy control cohorts demonstrated a

trend towards increased IL-13 from the matured CPG-B primed pDCs, however this was not significant and there was no difference between the cohorts (figure 4.12.A). Other cytokine (IL-10 and interferon-γ) levels were comparable across both groups for all conditions (figure 4.12.A) with no significance noted. I examined fold-changes in cytokine production between the unstimulated and CPG-B stimulated pDCs for both cohorts (figure 4.12.B). In the RA patients there was a slight trend for reduced fold-change production with the primed pDC MLRs, but again this was not significant.

Patient cohort	Rheumatoid Arthritis	Healthy Controls	Difference between groups (p value)
Number	5	6	-
Age, years median [range]	55 [53-68]	32 [29-32]	0.030
Male : Female ratio	2:3	1:5	0.480

Table 4.2 Demographics data for pDC origin used in mixed leukocyte reaction

Mixed leukocyte reactions (MLRs) were performed using pDCs from healthy donors (n=6) and patients with established RA (n=5). The demographic data for each groups is shown. Mann-Whitney U tests.

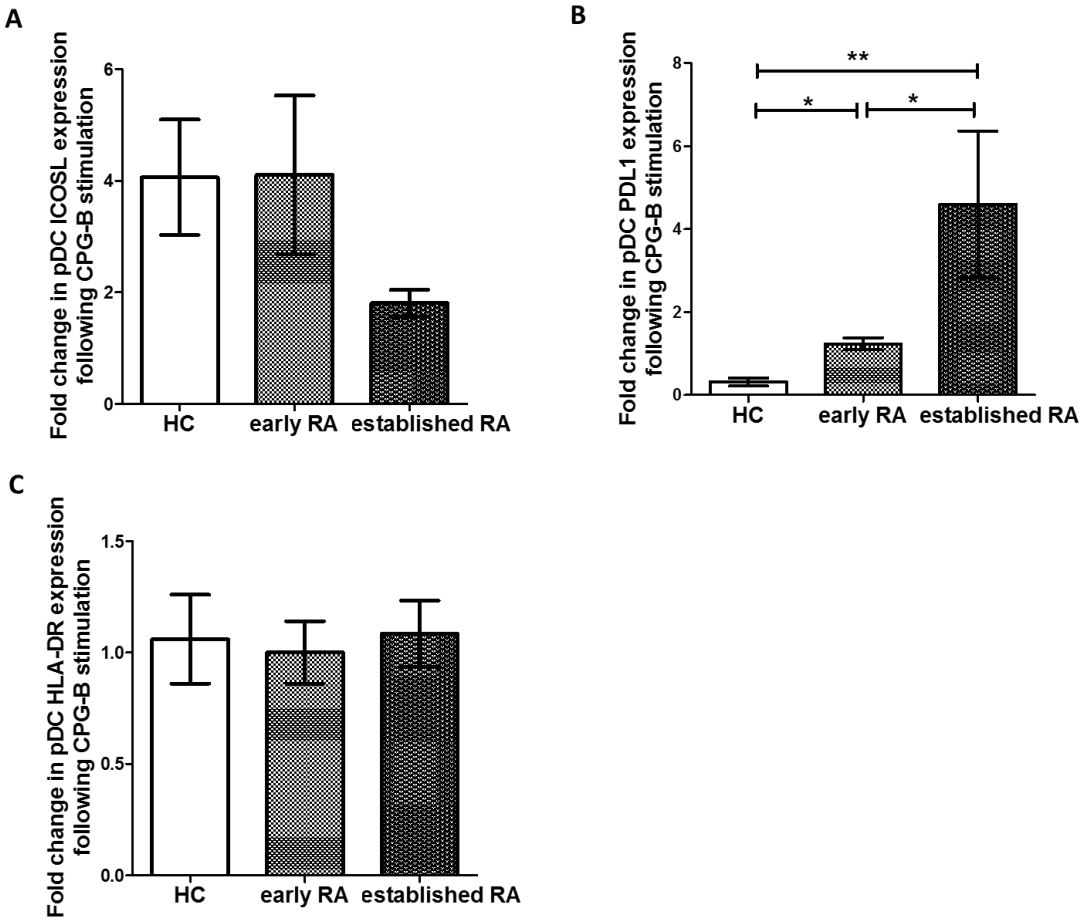


Figure 4.10 Surface marker expression in pDCs following stimulation via TLR9

PBMCS were cultured overnight with TLR9 ligand CPG-B (ODN 2006) at 5ug/ml. Using flow cytometry pDCs were identified as being CD20-, CD19-, CD20-, CD203-, HLA-DR+, CD123+ and their subsequent expression of ICOSL, PDL1 and HLA-DR quantified (median florescence intensity, MFI). **A:** Fold change in pDC ICOSL expression (unstimulated vs CPG-B stimulated) for healthy controls, early RA and established RA patients. **B:** Fold change in pDC PDL1 expression (unstimulated vs CPG-B stimulated) for healthy controls, early RA and established RA patients. **C:** Fold change in pDC HLA-DR expression (unstimulated vs CPG-B stimulated) for healthy controls, early RA and established RA patients. Mean and SEM depicted. Kruskal-Wallis tests used. *$p<0.05$, ** $p<0.01$

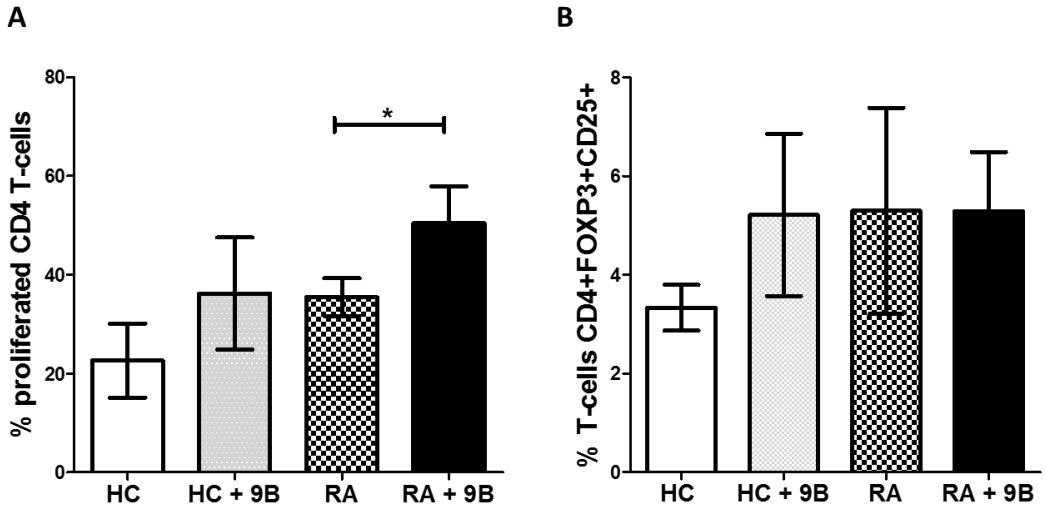


Figure 4.11 Proliferation and induction of $CD4^+FOXP3^+CD25^+$ T-cells following MLR with pDCs from RA patients and healthy controls

MLRs were performed using and pDCs from healthy controls (n=4) or established RA patients (n=4) and CFSE labelled naive allogenic $CD4^+$ T cells. PDCs were either matured (overnight stimulation with TLR9 CPG-B) or immature (overnight in culture media only). **A:** $CD4^+$ T cell proliferation (%) between the cohorts was calculated by CFSE dilution. **B:** Percentage of $CD3^+CD4^+$ T cells that were $FOXP3^+CD25^+$ compared between the cohorts. Mann-Whitney U test and paired t-tests used. Mean values with SEM are shown. *$p<0.05$

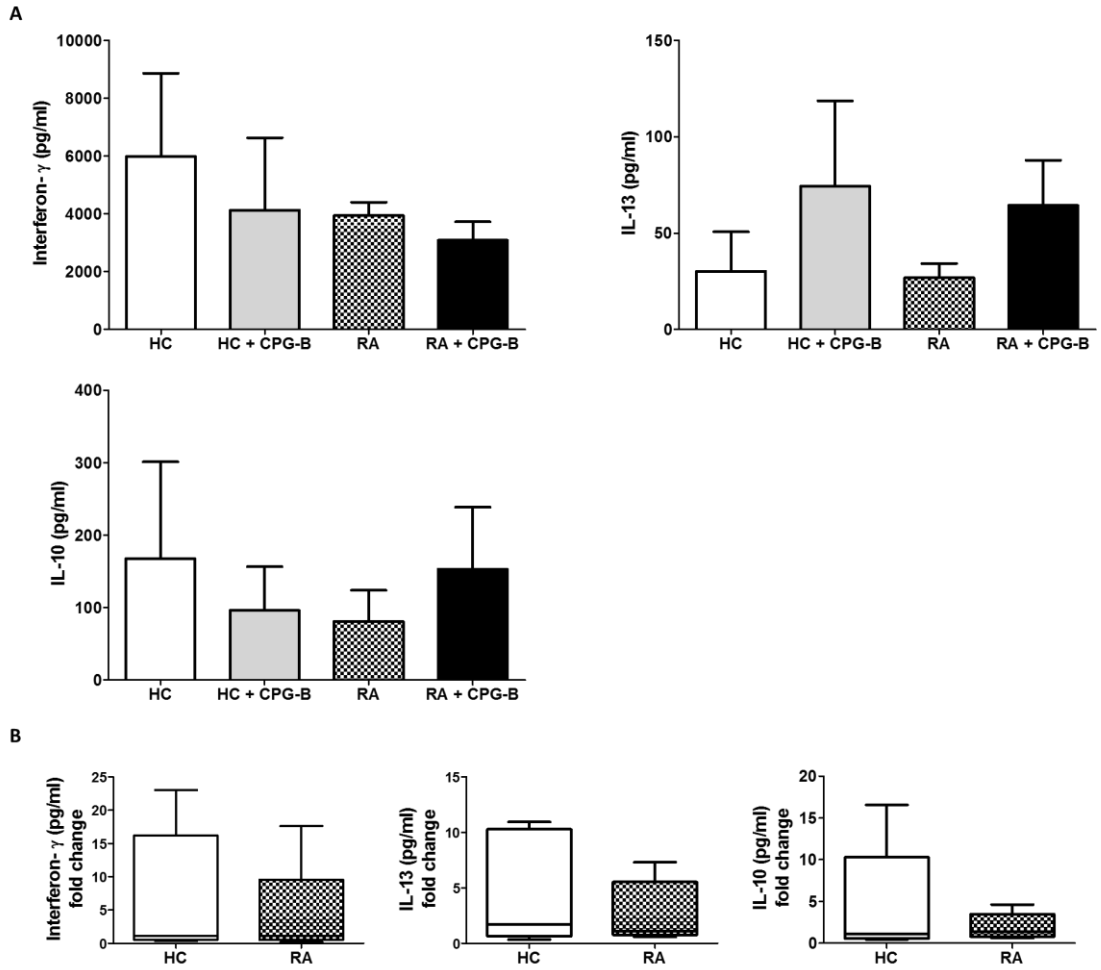


Figure 4.12 Cytokine secretion into supernatant of pDC: T-cell MLR using pDCs from RA patients and healthy controls

pDCs (+/- TLR9 ligand CPG B maturation) from healthy controls (n=6, HC) and RA patients (n=5) underwent a MLR with naive allogenic $CD4^+$ T cells. **A:** MLR cytokine production (interferon-γ, IL-13 and IL-10) was quantified from the culture supernatant and compared between cohorts. Mean values with SEM are depicted. **B:** Fold changes in cytokine production was examined between the cohorts. Box and whisker plots depicted which display median, quartile and range. Mann-Whitney U tests

4.4 Discussion

In this chapter I examined pDCs in the peripheral blood of early DMARD/glucocorticoid naive RA patients. PDCs were significantly reduced in early RA compared with healthy controls, but despite their type 1 interferon-producing potential their frequency/phenotype did not associate with the IGS. However CCR7 expression was increased in seropositive early RA patients; it inversely associated with pDC frequency but positively associated with anti-CCP titres. In a longitudinal early RA cohort there was a significant fall in pDC expression of activation markers, apart from CCR7 and pDC frequency remained static. Finally compared with healthy controls, pDCs from established RA had reduced ICOSL but increased PDL1 upregulation in response to maturing stimuli. However in a MLR there was no difference in their effect on allogenic naive $CD4^+$ T cells.

This was the first time that pDCs have been examined in early RA. Comparisons were made between healthy controls, established RA patients as well as ACPA+ arthralgia patients. These latter cohort were chosen for comparison as a "pre-RA" state, to explore whether any findings in early RA pre-dated the onset of inflammatory arthritis in a susceptible cohort (25). SLE was chosen as a positive disease model as pDCs are known to be important in SLE pathogenesis (314, 315). I observed a reduced frequency of peripheral blood pDCs, in early RA patients and SLE patients when compared with healthy controls. There was a similar identified for ACPA+ arthralgia and established RA patients which did not meet significance, although this may have been due to a question of power (figure 4.2). PDCs are known to be reduced in established RA, particularly in those with active disease (54, 87, 316, 317), so it is interesting to see a similar finding in early RA, potentially suggesting an involvement of pDCs in early RA pathogenesis. One suggestion for reduced pDCs in established RA is increased TNF-α levels inhibiting the differentiation of pDC progenitors (318). However I did not see any association between circulating TNF-α levels and pDC frequency in early RA. Others conversely report increased generation of pre-pDC in bone marrow of rheumatoid arthritis compared with OA patients (319). These data may therefore suggest normal pDC development but abnormal migration patterns. When dividing the early RA cohort by serostatus there was reduced pDC frequency in seropositive patients compared with seronegative patients and this was associated with increased CCR7 expression in the seropositive cohort (figures 4.4-4.5). In keeping with this I detected an inverse association between CCR7 expression and pDC frequency (figures 4.5-4.6). CCR7 is a chemokine receptor expressed on pDCs upon activation and it causes pDC migration towards its ligand CCL19/CCL21. This

CCR7-CCL19/CCL21 interaction is the main determinant of pDC migration and CCL19/CCL21 is normally found in lymph nodes or within the lymphatic system (320, 321). A number of groups have shown that pDCs are not normally found in the synovial compartment but migrate there from the peripheral circulation in inflammatory settings, such as RA, where they are relatively increased in number (85-88, 322). In keeping with this observation, CCL19/CCL21 is also significantly increased in the synovial fluid of RA patients compared with non-inflammatory OA controls (323) and established RA patients have increased pDC CCR7 expression when compared with healthy controls (317). I subsequently hypothesise that, in early RA, the pDC population is developing normally but migrating to the RA synovial compartment, at least in part by way of CCR7-CCL19/21 interactions. Supporting this theory was my subsequent observation that pDC CCR7 expression over the course of a year remained stable which was in keeping with a static frequency in the peripheral blood (figures 4.8-4.9).

The effect of serostatus on pDC frequency was corroborated by the significant positive association between anti-CCP titres and pDC CCR7 expression (figure 4.7). B cells produce anti-CCP antibodies and as more is understood about pDC biology their interactions with B cells are particularly intriguing; pDCs can influence B cell function by either cell-cell contact, such as via CD70-CD27 interactions (324, 325) or by the production of inflammatory cytokines, such as interferon-α or IL-6, which causes the generation of immunoglobulin secreting plasma cells (142). In RA pDCs have been shown to cluster with B-cells within the inflamed synovium and there is a positive association between the number of pDCs in the joint and circulating anti-CCP antibodies. Indeed RA patients who are seropositive had higher numbers of pDCs in their synovial fluid than those that are seronegative (88). There is evidence of raised type 1 interferon signalling in the RA joint (85, 89) and exposure to interferon-α can further promote the activation and proliferation of autoreactive B cells by stimulating BAFF/BLyS release (299). Indeed sustained interferon-α production from pDCs was sufficient to induce RA patients' B cells to produce autoantibodies *in vivo* (316). B cells, via a cross-talk mechanism, can also promote further production of type 1 interferons by pDCs and thereby cement pathological signalling pathways (326). Thus pDCs migrating to the joint space under the influence of CCR7 could modulate B cell function and autoantibody production. As to why this effect may occur intra-articularly comes from the increasing appreciation that in RA the synovial tissue can act as a secondary lymphoid organ. For example, ectopic lymphoid tissue forms germinal centres within the synovium in

approximately 25% of RA patients. Although there is debate as to their ultimate function, their number is increased in anti-CCP+ patients and they have been proposed as a site of autoantibody generation (105, 327). Pertinently cellular interactions from other cells within the synovial compartment can impact on the longevity of autoreactive B cell anti-CCP responses generated in these centres (328). I therefore hypothesise a model where in early RA pDCs express high levels CCR7 resulting in migration towards CCL19/CCL21 present in the inflamed RA synovial compartment. Once in the joint the pDCs cluster with B cells within ectopic germinal centres and, through both cell-cell contact and the production of inflammatory cytokines, promote the development of anti-CCP antibodies.

I did not examine additional chemotaxis/migration pathways (only CCR7) however CCL10 is more highly expressed in RA synovial fluid compared with crystal arthropathies, and pDCs have been shown to migrate to CCL10 (89, 321, 329). It is therefore possible that other signalling mechanisms are implicated in pDC migration in early RA but given my findings, these may be more important in seronegative disease.

CCR7 is normally increased upon pDC activation by TLR ligation and this process would classically also cause release of type 1 interferons (320). Furthermore pDCs exposed to type 1 interferons *in vitro* have increased migratory potential towards CCL19 (330). Therefore, with an increased IGS one could expect increased trafficking of pDCs out of the peripheral circulation. It was therefore counter intuitive that there was no association between the IGS and pDC frequency or phenotype (figure 4.3). Some suggest that pDCs are mainly interferon-α-producing in their immature state, and upon maturation develop antigen-presenting capacity and reduced interferon-α production (43). This would in part explain the lack of association between pDCs and the IGS however another explanation for these anomalies could be the site of exposure to the type 1 interferons. The serum of my early RA patients had undetectable interferon-α levels (Chapter 3) and this is similar to what has been demonstrated in established RA (187). The exposure to type 1 interferons could instead be occurring in lymphoid tissue during peripheral blood circulation. Thus the IGS would not accurately represent the contemporaneous pDC exposure to, or production of, type 1 interferons and would, in part, explain the disconnect between the IGS and pDC number or phenotype.

Following exposure to activating stimuli DCs undergo a process of “maturation”, exemplified by the up regulation of MHC, costimulatory molecules (CD80/86) and

activation markers (CD40). DCs present either self or foreign antigen, in a MHC complex to T cells. How these cells respond to that antigen depends on what additional signals are provided by the DC; if it is immature the T cell is skewed towards anergy and preservation of tolerance, however if the DC is mature the T cells are skewed towards a cytotoxic response against that presented peptide/protein. Thus presentation of self-antigen in the context of an inappropriately matured DC could lead to the breach of tolerance towards self and the development of autoimmunity (30, 81, 331). The main purpose of pDCs is frequently reported to relate to type 1 interferon production which ultimately has pleiotropic effects on other immune cells (43). However pDCs also have antigen presentation abilities and are involved in the homeostasis of effector and regulatory $CD4^+$ T cells (332). Given the DC's role in the balance between autoimmunity and tolerance I was interested in pDC phenotype in early RA and ACPA+ arthralgia. These early disease states are theoretically closer to that breach of tolerance and thus more likely to identify any abnormal DC function. I found that pDC baseline (pre-treatment) expression of HLA-DR (MHC class II), CD86 and CD40 was not increased in early RA compared with healthy controls, however there was a significant and sustained reduction in all of these surface markers' expression over 12 months following the initiation of disease modifying therapies (figure 4.9). This observation is in line with published data where established RA patients (on immunomodulatory therapy) had reduced pDC CD80 and CD83 expression compared with healthy controls (317) and pDC maturation markers were reduced following infliximab treatment (333). This is intriguing as this early comparatively activated phenotype may have generated inappropriate responses towards self-antigen or have contributed to early disease activity (331). Indeed intra-articular transfer of activated pDC and mDCs were able to propagate an inflammatory arthritis phenotype in mice (270) and pDCs from RA synovial fluid activated T cells and triggered the production of inflammatory cytokines (85).

Despite this, the relative role of pDCs in RA initiating or propagating an inflammatory phenotype remains unclear. Indeed some evidence suggests pDCs may actually promote tolerance; depletion of pDCs in a RA model resulted in breach of tolerance with development of autoantibodies (90) and RA patients with low disease activity had pDCs which were able to skew naive T cells towards a regulatory T cell phenotype (54). This suggests a beneficial role of pDCs in modulating RA activity and the tolerising potential of pDCs is being increasingly appreciated. For example they have recently been shown to be important in controlling autoreactivity to RNA-binding nuclear antigens by the

production of antigen-specific Tregs (334) and the cancer field comprehensively demonstrates that pDC expression of ICOSL primes $CD4^+$ T-cells to differentiate into IL-10-producing Tregs (56-59). ICOSL can be upregulated following TLR simulation (335) and this effect was reproduced in my data (figure 4.10). However pDCs from established RA patients did not upregulate ICOSL to the same degree as healthy controls (figure 4.10). Reduced expression/upregulation of ICOSL in pDCs has been reported in other human diseases. For example in asthma pDCs from patients with persistent disease had a significantly lower expression of ICOSL compared with cured patients (336) and in SLE pDCs had lower ICOSL mRNA expression in active SLE and an increased expression in inactive disease (261). Although some evidence on Treg frequency is contradictory this defect is in keeping with some *in vivo* observations where RA patients have fewer functional Tregs (75, 96-99). However this was the first time, to my knowledge, that a defect in ICOSL has been reported in RA. What is intriguing though is that while ICOSL upregulation in established RA was greatly reduced, programmed cell death 1 ligand (PDL1) expression was dramatically increased (figure 4.10). PDL1 is also known to promote a tolerogenic phenotype by regulating lymphocyte activation and promoting Treg development and function (337, 338). The net function of pDCs, in view of these differentially expressed markers, is therefore still unclear.

Given these gaps in the understanding of pDCs in RA I wished to further examine their functional capacity to skew T cell development by performing mixed leukocyte reactions (MLRs) with RA pDCs and allogenic naive T cells. By examining the MLR T cell phenotype and cytokine production I was able to examine the effects of the differential PDL1/ICOSL expression. Interferon-γ and IL-13 were chosen as typical Th1 and Th2 cytokines respectively and IL-10 due to its association with Tregs. As expected there was a trend towards increased T cell proliferation in the matured DCs and this was significant for the RA cohort (figure 4.11). Examining the phenotype of these cells CD25 and FOXP3 expression was quantified. Although both CD25 and FOXP3 can go up on cell activation, they have also been reported to be part of a tolerogenic or regulatory (Treg) phenotype, although notably this is normally $CD25^{hi}$ expression, not only $CD25^+$ which I report here (75). Nonetheless there was no real change in the expression of $CD4^+CD25^+FOXP3^+$ T cells in the RA pDC MLR when compared with healthy controls (figure 4.11). Concerning cytokine production, there was a trend in both healthy controls and RA patients to increased IL-13 production in the mature pDC MLRs, but this was non-significant and there were no similar changes noted for interferon-γ or IL-10. In fact

cytokine production was pretty comparable across all groups. Following pDC maturation there was a slight (non-significant) trend towards reduced fold-change induction of all cytokines in the RA cohort (figure 4.12), although given the similar absolute cytokine values this is unlikely to be relevant. Thus despite the differences in ICOSL there was no real difference in functional capabilities. One explanation for these findings is, given the importance of Tregs and tolerance to self, there may be a potential functional redundancy in pathways involved in their generation. For example IDO is also important in pDC derived Treg generation (47) although some argue the effects were partially dependent on PDL1 expression (339). Nonetheless mature pDCs from RA patients promoted differentiation of naive T cells into IL-10-secreting Tregs via IDO activity (54). Thus IDO/PDL1 may be compensating for any defect in ICOSL. Notably this assay used healthy donor T-cells which would remove any intrinsic abnormalities in the functional immunoregulatory abilities of RA derived Tregs; such as their relative resistance to IDO activation (96, 99). Thus whether the comparable effect noted between healthy and RA pDCs would effect would occur *in vivo* is unclear. It is conceivable that my observed reduced ICOSL upregulation in RA pDCs may therefore have pathogenic importance when occurring with concurrent RA T cell defects, i.e. IDO resistance.

A consideration when interpreting this data is that these functional studies were done with established RA patients and in this work I demonstrate a fall in activation and co-stimulatory marker expression over a year (figure 4.9) These markers are important in the interaction with T cells and therefore the phenotype induced by pDCs early on in disease may be different to that seen here and examined elsewhere (54). Another consideration is that the ages of the healthy controls and established RA patients were not matched. PDCs from older people have reduced TLR-7 and TLR-9-induced interferon-α production and have poorer T cell stimulatory capacities (340-342). Additionally a transcriptional analysis of PBMCs suggested ICOSL-ICOS activity was affected in older women (343) although this was not seen in old mice (344). These differences in ICOSL upregulation between the cohorts could therefore potentially be an artefact of immunosenescence. Furthermore I used established RA patients who were on disease modifying therapies thus any differences could have theoretically been medication derived. Further caveats include the fact that peripheral blood circulating dendritic cells and their phenotype may not be representative of what is happening in the inflamed RA synovial compartment. It is known that dendritic cells from at different anatomical sites have different phenotypes, derivative origins, cycling kinetics and are exposed to varying antigen loads (345). Indeed

when comparing the phenotype of dendritic cells between RA synovium and lymph nodes, there were differences in DC maturation and chemokine production (104). Therefore care should be taken when extrapolating these findings to synovial pathology. Finally I presented the percentage of pDCs in the lymphocyte population and older patients become lymphopenic as part of immunosenescence (346, 347). So while their absolute number of DCs may fall, their relative proportion amongst lymphocytes could remain stable. I could have therefore missed any potential absolute changes in DC numbers over the year, as well as falsely interpreted the data.

4.5 Conclusions

To my knowledge this is the first time that pDCs have been examined in early drug-naive RA. I show a decrease in pDCs in the peripheral circulation and hypothesise that they are migrating to the inflamed joint via increased CCR7 expression and subsequent intra-articular CCL19/21 interactions. Once in the joint they have the potential to potentiate the function of anti-CCP producing B cells - thus explaining the observed associations with serostatus. Anti-CCP antibodies themselves can cause bony erosions due to osteoclast activation (348), so understanding mechanisms potentiating their production is of therapeutic relevance.

Established RA pDCs do not appear to cause an activated T cell phenotype, but this could be due to therapy-induced modifications. Indeed I show the maturation phenotype of pDCs significantly falls 12 months after starting DMARD therapy. While conventional or myeloid DCs have been proposed to play a pathological role in early RA, potentially in the breach of tolerance (349-351), is not clear from my data if pDCs are also contributing to this phenomena.

There is emerging evidence that the early and aggressive treatment of RA has greater long-term beneficial outcomes (11, 352, 353), and therefore having a greater understanding of the pathological processes that are prevalent in early disease has future therapeutic merit. Additional work is however needed to elucidate these the potential contribution of pDCs to these pathological pathways.

4.6 Future work

The number of RA patients in my cohort could be increased. This would consolidate the findings regarding differences between seropositive and seronegative patients in relation to CCR7 expression as well as the effect of CCR7 expression on the percentage of circulating pDCs. I also would examine other markers involved in pDC migration and thus examine if CCR7 was exclusively responsible in early RA. I have only looked at DC percentage but number would also be beneficial. To accurately compare between groups however I would need to know the lymphocyte count from the healthy controls, which would be challenging.

Unfortunately due to patient sample limitations and time pressures we were not able to perform the MLRs on pDCs from early RA patients. The pDC-T cell MLRs would therefore ideally be repeated using peripheral blood and synovial pDCs from early RA patients and suitably age matched healthy controls. When analysing MLR T cells staining for CD25, CD39, CD152 (CTLA-4) as well as FoxP3 would allow me to more confidently state if the phenotype was tolerogenic or activated.

To address the recurrent caveat/issue about blood versus synovium I could examine pDCs within the synovial compartment and examine for correlations with peripheral blood parameters of interest, for example pDC CCR7 and autoantibody titres. The observation linking pDCs and anti-CCP status is intriguing and MLRs could be performed with pDCs and B cells from the synovial compartment of early RA patients. Cell numbers are likely to be challenging so immunohistochemistry on synovial biopsies could also be performed. In particular I would examine surface markers linked to activation status and those known to be important in the interaction of pDCs and B cells, such as CD70 (324).

Chapter 5: Early RA transcriptomics

5.1 Introduction

Gene expression profiling or transcriptomics is at the forefront of many advances in understanding the pathophysiology of multiple conditions. Applying these techniques to RA has been crucial in helping to delineate disease-associated pathways (354). However the pathological processes in early RA are distinct from established disease. This has been shown at both a clinical and a transcriptomics level where different signalling pathways are activated in early RA compared with established RA (5-8). Steroids and treatments used to treat RA have profound effects on the transcriptome and thus potentially mask the contribution of certain cellular pathways in treated disease (23, 355). To overcome these confounding effects the transcriptome of certain cell subsets, such as $CD4^+$ T cells (9), have been examined in early drug naïve RA. However the transcriptome of peripheral blood pDCs has not been analysed in early drug naïve RA. Indeed pDCs have only recently had their transcriptome examined in the context of autoimmunity, namely primary Sjogrens syndrome (pSS) (236), and have never been examined in established RA.

The role of pDCs in RA has been contradictory with ongoing debate if their net function is actually inflammatory or tolerogenic (54, 85, 89, 90). I therefore decided to focus on pDCs for in-depth transcriptional analysis comparing early RA patients with healthy controls. All immune cell types have the potential to produce type 1 interferons but given their prolific production of type 1 interferons pDCs have been the logical cell subset presumed to promote the generation of the IGS detected in autoimmunity (43). Indeed in SLE there have been therapies developed that target pDCs and their production of type 1 interferons (157). However it is not clear in RA if pDCs are the main source of type 1 interferons and generate the IGS. I therefore wished to compare type 1 interferon production across the 6 major peripheral blood cell subsets in the context of the IGS.

Type 1 interferons have pleiotropic effects on multiple immune cell subsets (145, 155, 260) and my findings in Chapter 3 demonstrated associations between the IGS and RF. Additionally in ACPA+ arthralgia a RA prediction model based on B cell phenotype was improved upon including the IGS (356). I decided therefore to examine the transcriptional profile of B cells IGS+ and IGS- patients thereby exploring any correlations between the IGS and B cell gene expression profiles.

This work would further dissect the role of pDCs in RA as well as the role of the IGS on B cell function.

5.2 Aims

The aims of this chapter are to address the following questions:

1. Is there a difference in the transcription profile of circulating pDCs between early RA and healthy controls?
2. Does the interferon gene signature associate with the transcription profile of pDC and B cells?
3. Which immune cell subset is responsible for producing type 1 interferons in both early RA patients and the healthy controls?

5.3 Results

5.3.1 Demographics

Patient and healthy control demographics for use in the subsequent transcriptomics analysis are shown in table 5.1. In brief, there were 8 early RA patients recruited, all double seropositive for RF and anti-CCP antibodies, 4 of whom had a positive IGS and the other 4 had a negative IGS. An additional 4 healthy controls were also recruited. There was no significant difference between the age or sex of any of the groups. Furthermore there was no significant difference in the IGS+ or IGS- cohorts with regard to DAS-28, ESR, CRP, anti-CCP or RF titres. As expected however there was a significant difference between the IGS score between the groups.

	Early RA			HC	Difference between IGA+, IGS- eRA & HC cohorts P value*	Difference between IGA+ & IGS- eRA cohorts P value±
	IGS +	IGS -	All			
Number in cohort	4	4	8	4	-	-
Age (years) Median [range]	56 [49-64]	54.5 [53-60]	55 [49-64]	53 [48-62]	0.905	0.771
Male: Female ratio	3:1	3:1	3:1	1:1	0.750	0.847
Median IGS Score	0.001156	0.000203	0.00071	0.000198	0.0003	0.028
RF titre (IU/ml) [Median and range]	263.5 [122-650]	384 [20-501]	339.5 [20-650]	-	-	1
Anti-CCP titre (U) Median [range]	684.5 [457-7156]	999 [14-13745]	684.5 [14-13745]	-	-	0.886
DAS-28 Median [range]	3.4 [2.63-4.28]	4.38 [1.63-6.18]	3.71 [1.63-6.18]	-	-	0.689
CRP mg/L Median [range]	6 [4-11]	7.5 [4-56]	7 [4-56]	-	-	0.661
ESR mm/hr Median [range]	17 [2-33]	26 [7-56]	22.5 [2-56]	-	-	0.384

Table 5.1 Demographics table of cohort used for cell sorted NanoString analysis

Flow cytometry cell sorting was performed on 8 early RA patients (eRA) and 4 heathy controls (HC). The early RA patients were further split into 4 IGS+ and 4 IGS- patients. The respective demographics for each are shown.* One-way ANOVA was used to compare age, male: female ratio and median IGS score between the IGS+, IGS- and healthy control groups. ± Mann-Whitney U tests were used to compare clinical and disease activity parameters between the IGS+ and IGS- early RA cohorts.

5.3.2 Technical validation

To address the aims of this chapter I flow cytometry cell sorted 6 immune cell subsets (pDCs, mDCs, B cells, $CD8^+$ T cells, $CD4^+$ T cells and $CD14^+$ monocytes). To validate that the cells sorted fitted the conventional morphology of those subsets, cytospins were performed for each sorted subset (figure 5.1).

A principal components analysis was performed on the gene expression data from the 6 immune cell subsets (figure 5.2). As expected there was clear separate clustering of each of the immune cell subsets separated by cell type. The $CD8^+$ and $CD4^+$ T cell populations clustered tightly as expected. The mDCs in turn were closer to the monocytes, which were also of myeloid lineage. Interestingly however the pDCs were more closely affiliated with B cells than the mDCs.

Notably however there was no obvious difference between the healthy control and early RA groups for each of the subsets.

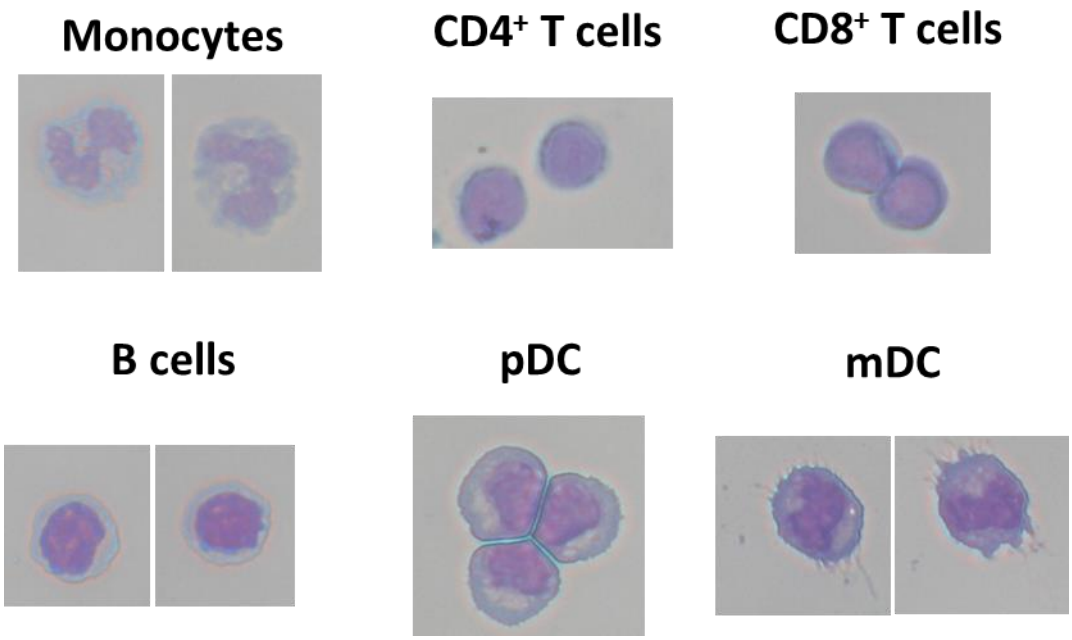


Figure 5.1 Cytospin images of flow cytometry sorted immune cell subsets.
Cytospins to validate morphology and appearance were performed on the 6 cell sorted subsets which included **pDCs** ($CD19^-$, $CD20^-$, $CD3^-$, $CD14^-$, $HLA\text{-}DR^+$, $CD1c^-$, $CD11c^-$, $CD123^+$), **mDCs** ($CD19^-$, $CD20^-$, $CD3^-$, $CD14^-$, $HLA\text{-}DR^+$, $CD1c^+$, $CD11c^+$), **B cells** ($CD19^+$, $CD20^+$, $CD14^-$, $CD3^-$), **$CD8^+$ T cells** ($CD19^-$, $CD20^-$, $CD14^-$, $CD3^+$, $CD4^-$, $CD8^+$, $CD56^-$), **$CD4^+$ T cells** ($CD19^-$, $CD20^-$, $CD14^-$, $CD3^+$, $CD4^+$, $CD8^-$, $CD56^-$) and **$CD14^+$ monocytes** ($CD3^-$, $CD14^+$). Cytospins were stained with May-Grunwald and Giemsa to visualise the cell nucleus.

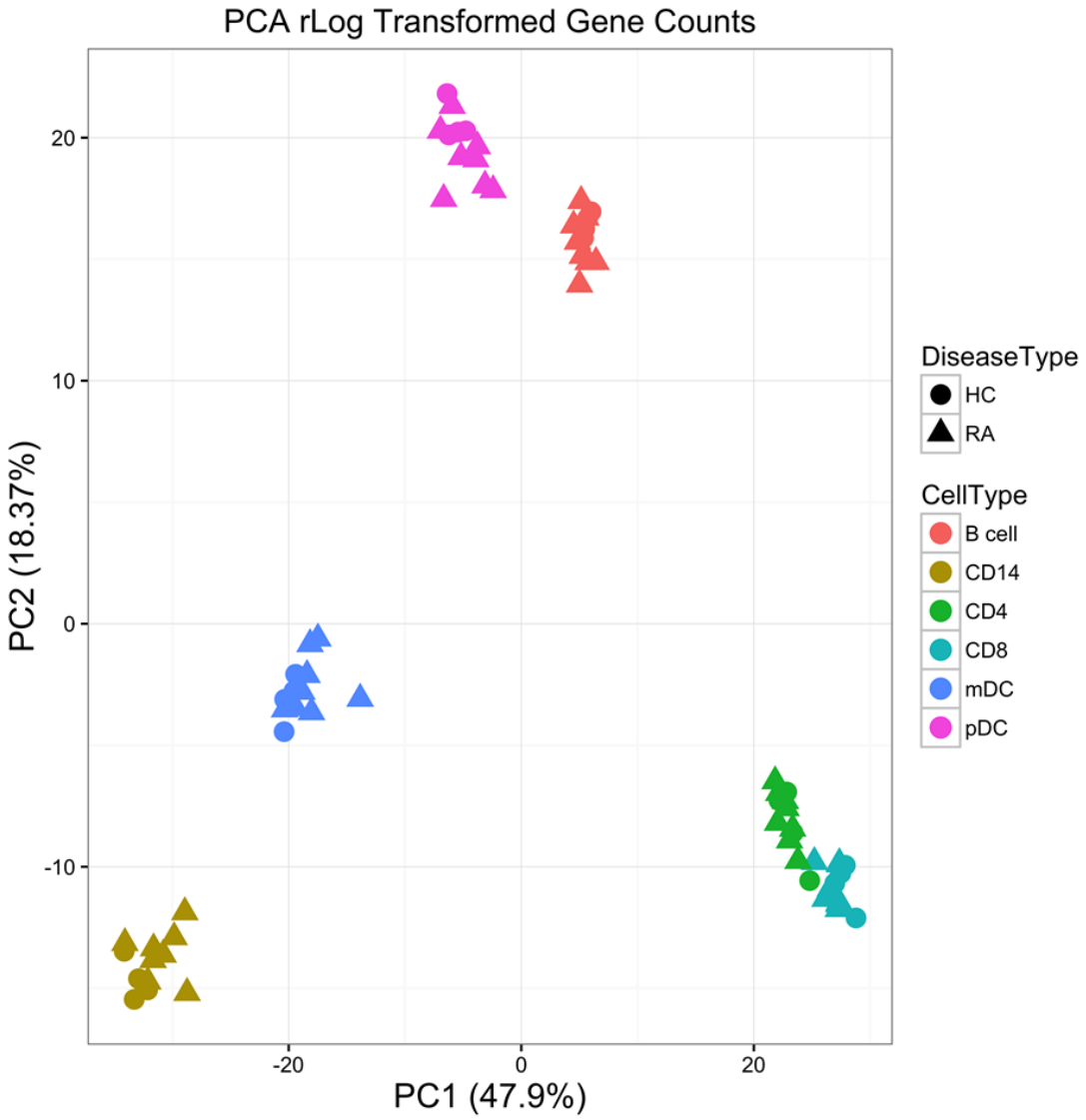


Figure 5.2 Principal components analysis (PCA) of immune cell subset gene expression

Immune cell subsets have clear, distinct gene expression profiles. Six separate immune cell subsets (pDCs, mDCs, B cells, $CD4^+$ T cells, $CD8^+$ T cells and CD14+ monocytes) were isolated from the peripheral blood of healthy controls and RA patients using flow cytometry sorting. RNA was extracted and run on a custom NanoString system. A principal components analysis was run using R *Core team (2016).* CD4 = $CD4^+$ T cell; CD8 = $CD8^+$ T cell; CD14 = $CD14^+$ monocytes; pDC = plasmacytoid dendritic cell; mDC = myeloid dendritic cell; HC = healthy controls; RA = early rheumatoid arthritis patients.

5.3.3 Differential gene expression in pDCs by disease and IGS status

A comparison of pDC gene expression between the RA patients and healthy controls was performed. Differences were termed significant when the false discovery rate (FDR) adjusted p-value was <0.05 and the fold change was >1.5. I first compared expression between all RA patients and healthy controls. There were 12 differentially expressed genes; in the early RA cohort 3 of these genes were upregulated and the remaining downregulated. The top upregulated genes included *PRDM1* and *IRF4* (figure 5.3)

Ingenuity® Pathway Analysis (IPA®) uses the differentially expressed gene lists to identify trends in expression that could indicate differential activation or involvement of known biological networks or pathways. These associations are based on the current published literature in the IPA® database. A p value and an activation Z score (or Z score) is generated; the Z score infers activation state, i.e. either an increase (positive score) or decrease (negative score) in associated biological functions. Scores > ±2 are deemed a strong association. For some biological networks there is insufficient published evidence to identify if gene involvement would denote activation or inhibition; for these scenarios no Z score is given.

IPA® was performed on the significantly differentially expressed pDCs genes observed when comparing early RA patients and healthy controls. This strongly (Z score ≥ 2) suggested early RA patients had increased pDC proliferation. It also suggested early RA pDCs had reduced apoptosis although this effect was less pronounced. Early RA pDCs also had differential gene expression linked to rheumatoid arthritis, inflammation of the joint and systemic autoimmune disease, although it was not clear if these pathways were activated or inhibited (no Z score) (figure 5.4).

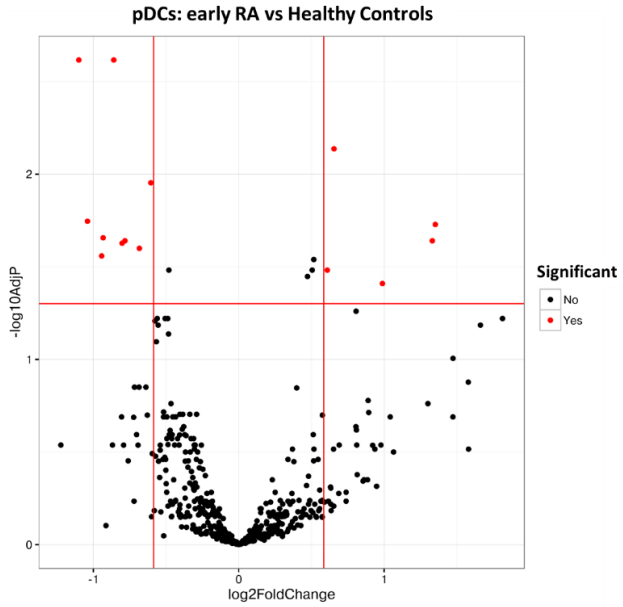
When comparing IGS+ and IGS- early RA patients, pDCs had only 2 genes that were significantly differentially expressed, one was significantly upregulated (*IFI44L)*, an IRG and the other downregulated (*S100A8)* (figure 5.5.A). However when looking at IGS+ early RA versus healthy controls, there were 25 significantly differentially expressed genes; 5 of these were upregulated and the remaining 20 down regulated. The top differentially expressed gene was *IFI44L* and other top genes included those seen earlier; *PRDM1* and *IRF4* (figure 5.5.B).

An IPA® was performed on the significantly differentially expressed pDCs genes observed when comparing IGS+ early RA and healthy controls. This strongly (Z score ≥ ± 2) suggested IGS+ patients had increased pDC proliferation and reduced lymphocyte

migration. It also suggested IGS+ pDCs had reduced adhesion and an increase in pathways concerning inflammation of an organ or anatomical region. These latter associations however were not as strong (figure 5.6)

In light of potential upstream triggers for type 1 interferons a specific examination of the gene list was performed for any abnormally expressed pDC genes relating to nucleic acid sensing pathways, such as TLR7, TLR9, MDA-5, RIG1, MAVS, cGAS, STING etc.

The only significantly differentially expressed gene was *TMEM173* which encodes STING, a cytosolic DNA senor. However counterintuitively expression was reduced in the IGS+ early RA cohorts when compared with healthy controls.



Gene	Fold Change	Adjusted p value	
CSF1R	2.553013	0.0186833	Relative expression increased in early RA vs healthy controls
PRDM1	2.516564	0.02288432	
IRF4	1.984029	0.03893869	
CD164	-1.520027	0.01113042	Relative expression decreased in early RA vs healthy controls
IL6R	-1.60581	0.02514847	
MAPK14	-1.719796	0.02288432	
IFNAR1	-1.743639	0.02360053	
CD48	-1.814137	0.00241879	
IL6ST	-1.908326	0.02205872	
TNFSF4	-1.922154	0.02764461	
CCR5	-2.056634	0.01795218	
TNFRSF17	-2.143103	0.00241879	

Figure 5.3 Statistically differentially expressed genes in pDCs between early RA patients and healthy controls.

PDCs were flow cytometry sorted from early RA patients (n=8) and age- and sex-matched healthy controls (n=4). PDC RNA was isolated and the transcriptome analysed using NanoString nCounter technology. Genes achieved differential expression significance when adjusted p-value <0.05 (FDR corrected) and fold change >1.5. Volcano plots show the Log_2Fold change against the $–Log_{10}$ Adjusted p value where genes plotted in red are termed significant. 2 are additional red dots shown as positively upregulated, these are spiked controls and an artefact of the technology.

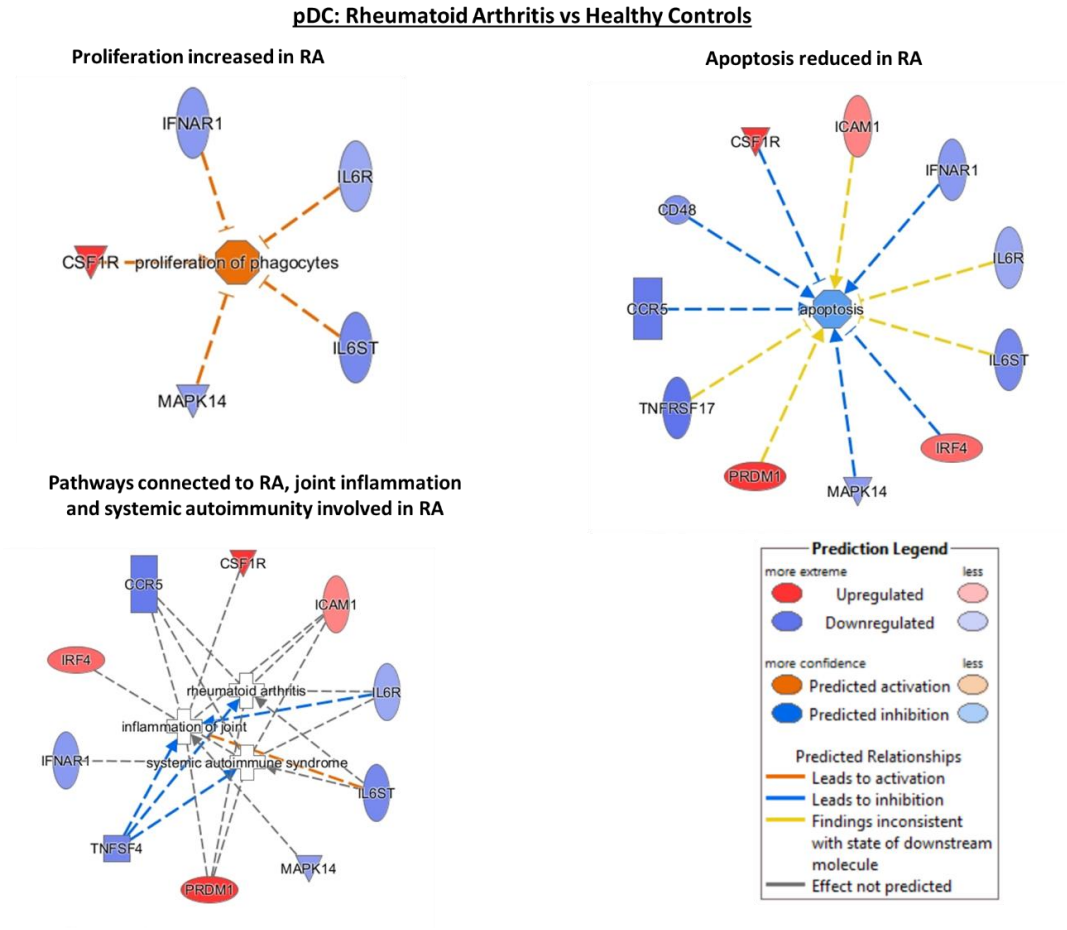
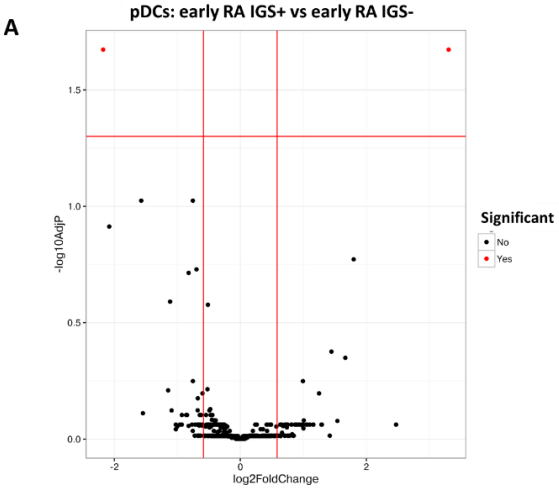


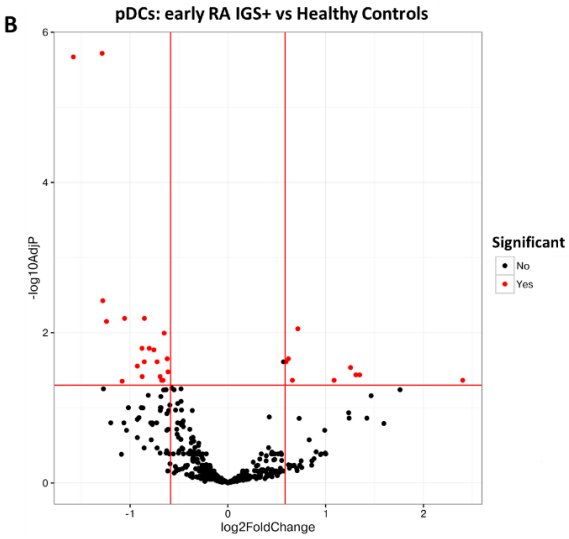
Figure 5.4 IPA® analysis for differentially expressed genes in pDCs between early RA patients and healthy controls

IPA® analysis was performed on pDC differentially expressed genes in early RA patients (n=8) and healthy controls (n=4). Red denotes genes upregulated in early RA and blue downregulated. Pathways identified in the early RA cohort include: increased proliferation ($p=1 \times 10^{-9}$, Z-score 2.17), reduced apoptosis ($p=1.02 \times 10^{-7}$, Z-score -0.97), rheumatoid arthritis ($p=2.49 \times 10^{-6}$), inflammation of the joint ($p=4.24 \times 10^{-8}$) and systemic autoimmune disease ($p=9.18 \times 10^{-8}$) in RA. For the latter 3 pathways no Z score was available.

- The colour of the shape in the centre of the network describes, based on the Z score, the predicted state of the associated biological network; orange, activated; blue, inactive; white, not known. The shape itself denotes the strength of that association; complete circle, high Z score; cross, low Z score. The colour of the gene denotes its relative expression; red, upregulated; blue, downregulated. The colour of the line from the gene to the centre denotes what is the predicted effect a gene has on a downstream pathway; orange, activation; blue, inhibition; yellow, variable or inconsistent findings; grey, not known. Finally the line's terminal shape indicates the ultimate effects of that downstream pathway on the central biological function, i.e. arrow, activation; flat line, inhibition.



Gene	Fold Change	Adjusted p value	
IFI44L	9.899	0.021	Relative expression increased in IGS+ vs IGS- early RA
S100A8	-4.522	0.021	Relative expression decreased in IGS+ vs IGS- early RA



Gene	Fold Change	Adjusted p value	
IFI44L	5.284023	0.04300632	Relative expression increased in IGS+ early RA vs healthy controls
PRDM1	2.544716	0.0364314	
CSF1R	2.478434	0.0364314	
CD247	2.386087	0.02910056	
IRF4	2.123662	0.04300632	
PTPN6	-1.528282	0.03322002	Relative expression decreased in early IGS+ early RA vs healthy controls
CD164	-1.536945	0.02225619	
CTNNB1	-1.569974	0.01013972	
PTK2	-1.583258	0.04300632	
ADA	-1.599569	0.04300632	
TMEM173	-1.616313	0.03837515	
FKBP5	-1.651401	0.0243994	
PYCARD	-1.688922	0.01694369	
LCP2	-1.745406	0.01613944	
IFNGR1	-1.806338	0.00644974	
PSMB5	-1.808176	0.0243994	
BTLA	-1.835535	0.03837515	
IL6R	-1.837473	0.01613944	
IFNAR1	-1.899368	0.0278069	
MAPK14	-2.078896	0.00644974	
CCR2	-2.114949	0.04421903	
CCR5	-2.363255	0.00707897	
IL6ST	-2.426055	0.00375155	
CD48	-2.438016	1.91E-06	
TNFRSF17	-2.988915	2.14E-06	

Figure 5.5 Statistically differentially expressed genes in pDCs by IGS and disease

PDCs were flow cytometry sorted from early RA patients (IGS+ n=4, IGS- n=4) and age- and sex-matched healthy controls (n=4). PDC RNA was isolated and the transcriptome analysed using NanoString nCounter technology. Genes achieved differential expression significance when adjusted p-value <0.05 (false discovery rate corrected) and fold change >1.5. Volcano plots show the Log_2Fold change against the $-Log_{10}$ Adjusted p value where genes plotted in red are termed significant. **A** Comparison between IGS+ and IGS- early RA pDCs **B** comparison between IGS+ early RA and healthy control pDCs. Note 4 additional genes are demonstrated in the volcano plot as being differentially expressed, these are control spikes and an artefact of the technology.

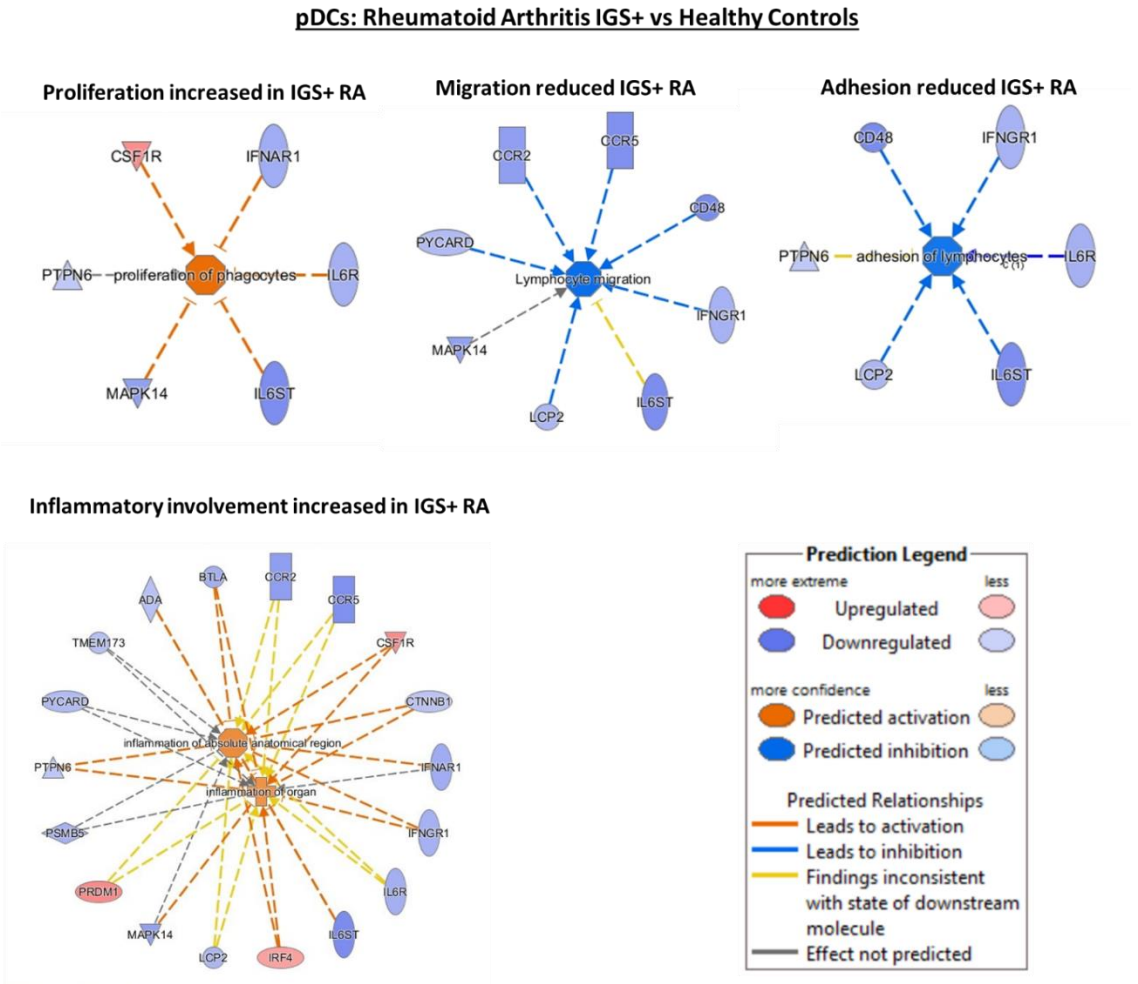


Figure 5.6 IPA® analysis for differentially expressed genes in pDCs between IGS+ early RA patients and healthy controls

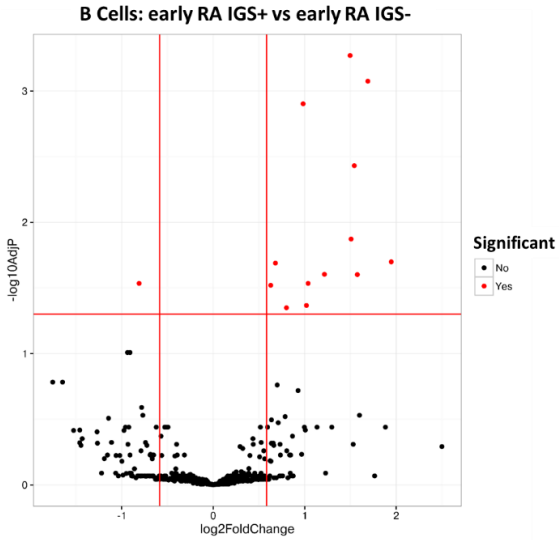
IPA® analysis was performed on pDC differentially expressed genes in IGS+ early RA patients (n=4) and healthy controls (n=4). Red denotes genes upregulated in IGS+ early RA and blue downregulated. Pathways identified in the early RA cohort include: increased proliferation ($p=6.32 \times 10^{-10}$, Z-score 2.17), reduced lymphocyte migration ($p=1.10 \times 10^{-8}$, Z-score -2.03), and adhesion ($p=2.06 \times 10^{-7}$, Z score -1.97). Pathways concerning inflammation of an organ ($p=3.07 \times 10^{-15}$, Z-score 1.05) or anatomical region ($p=7.38 \times 10^{-17}$, Z-score 1.57) were also activated.

❖ Interpretation and explanation of this figure format is described in Figure 5.4

5.3.4 B cell differential gene expression by IGS

In light of the effect of type 1 interferons on B cells (145) and the reported link between the IGS and RF titres (Chapter 3), comparisons were made between IGS+ and IGS- early RA patient B cells. There were 14 statistically significant differentially expressed genes (figure 5.7); 13 of these genes were upregulated in the IGS+ cohort and only 1 was downregulated (*PECAM-1*). Surprisingly none of these upregulated genes were clearly defined as IRGs. However they did associate with a more activated phenotype, e.g. CD80, which was the most differentially expressed in early RA with a fold change of 3.8. Other markers relating to specific B cell phenotypes were also identified, e.g. CD27. The highest significance (p=0.00008), and indeed one of the largest fold changes (3.226), was seen in *TNFRSF13B* which encodes the B cell receptor TACI. These examples all relate to proteins expressed on the cell surface, but differentially expressed genes also encoded transcription factors, such as *XBP1*.

IPA® was performed on these gene lists. Analysis showed that in IGS+ early RA there was a strong suggestion of increased B cell proliferation and evidence of pathways related to viral infection. There was also evidence of increased B-cell activation and reduced B cell number, although the association for these latter pathways was not as robust. There was once more involvement of pathways linked to RA pathology which included systemic autoimmunity and joint inflammation, although for these there was no Z score so the net involvement to biological function was unknown (figure 5.8).



Gene	Fold Change	Adjusted p value	
CD80	3.853	0.02	Relative expression increased in IGS+ vs IGS- early RA
TNFRSF13B	3.226	8E-04	
TCF7	2.981	0.025	
ITGAX	2.912	0.004	
CD27	2.843	0.013	
ITGB1	2.821	5E-04	
XBP1	2.323	0.025	
CD58	2.052	0.029	
TFRC	2.027	0.043	
CD99	1.977	0.001	
IL10RA	1.742	0.045	
GAPDH	1.602	0.02	
MAP4K1	1.546	0.03	
PECAM1	-1.753	0.029	Relative expression decreased in IGS+ vs IGS- early RA

Figure 5.7 Statistically differentially expressed genes in early RA B cells by IGS

B cells were flow cytometry sorted from early RA patients (IGS+ n=4, IGS- n=4). Cellular RNA was isolated and the transcriptome analysed using NanoString nCounter technology. Genes achieved differential expression significance when adjusted p-value <0.05 (false discovery rate corrected) and fold change >1.5. Volcano plots show the Log_2Fold change against the $-Log_{10}$ Adjusted p value where genes plotted in red are termed significant.

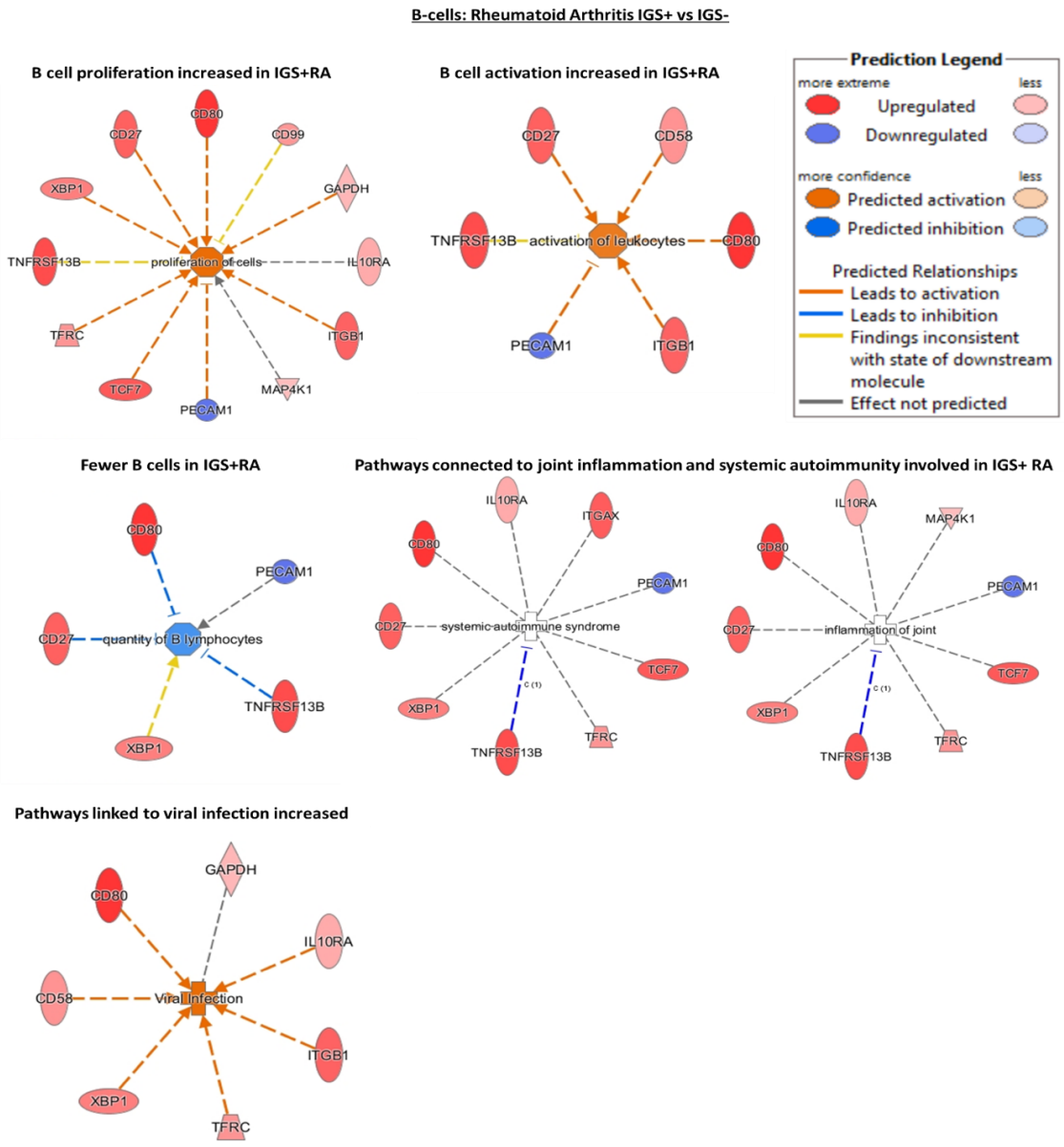


Figure 5.8 IPA® analysis for differentially expressed genes in B cells between IGS+ and IGS- early RA patients

IPA® analysis was performed on B cell differentially expressed genes in IGS+ early RA patients (n=4) and IGS- early RA patients (n=4). Red denotes genes upregulated in early RA and blue downregulated. Pathways identified in the IGS+ cohort include: B cell proliferation ($p=1.43 \times 10^{-6}$, Z-score 2.29), B-cell activation ($p=2.93 \times 10^{-6}$, Z-score 1.57), viral infection pathways ($p=1.76 \times 10^{-4}$, Z-score 2.39), Reduced B cell number ($p=3.22 \times 10^{-7}$, Z-score 1.08) and systemic autoimmunity ($p=7.89 \times 10^{-9}$) and joint inflammation ($p=3.29 \times 10^{-9}$). There was no Z score available for the latter 2 pathways.

❖ Interpretation and explanation of this figure format is described in Figure 5.4

5.3.5 Cellular origin of interferon-α in early RA

To question what cell subset was responsible for promoting the IGS the source of type 1 interferons was examined across the immune cell subsets in IGS+ early RA patients. Immune cell subsets included pDCs, mDCs, B cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD14^+$ monocytes. Of note these comparisons were not multiple test corrected however there was large variability between the donors which would have compromised significance; this was likely due to our small sample size.

There was no significant difference in *IFNA2* or *IFNA1/13* expression between any of the immune cell subsets in IGS+ early RA (figure 5.9).

To examine if the IGS was instead caused by uniform increase in immune cell expression of type 1 interferons I compared *IFNA2* or *IFNA1/13* expression between healthy controls, IGS+ and IGS- early RA patients for each immune cell subset (figure 5.10). $CD4^+$ T cells from IGS- patients had significantly increased *IFNA1/13* expression compared to CD4+ T cells from IGS+ patients and healthy controls. However no other significant differences between healthy controls, IGS+ and IGS- cohorts in the remaining immune cell subsets for either *IFNA2* or *IFNA1/13* expression. Thus there was no global increase in *IFNA1* or *IFNA1/13* in immune cell subsets from IGS+ patients.

I further examined *IFNA2* and *IFNA1/13* expression in the context of the IGS (continuous score) using linear regression for all the early RA patients. For both *IFNA* subtypes and for each cell subset there was no significant association between the IGS and cellular expression of type 1 interferons (data not shown).

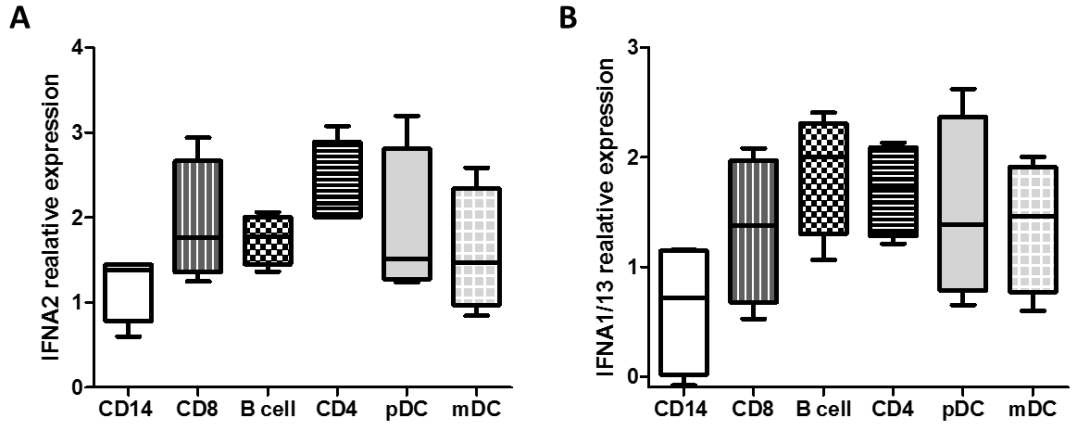


Figure 5.9 Immune cell *IFNA2* and *IFNA1/13* expression in IGS+ early RA patients
A *IFNA2* and **B** *IFNA1/13* mRNA expression as determined by NanoString nCounter technologies was compared across flow cytometry cell sorted immune cell subsets in IGS+ early RA patients (n=4). Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Kruskal-Wallis test.

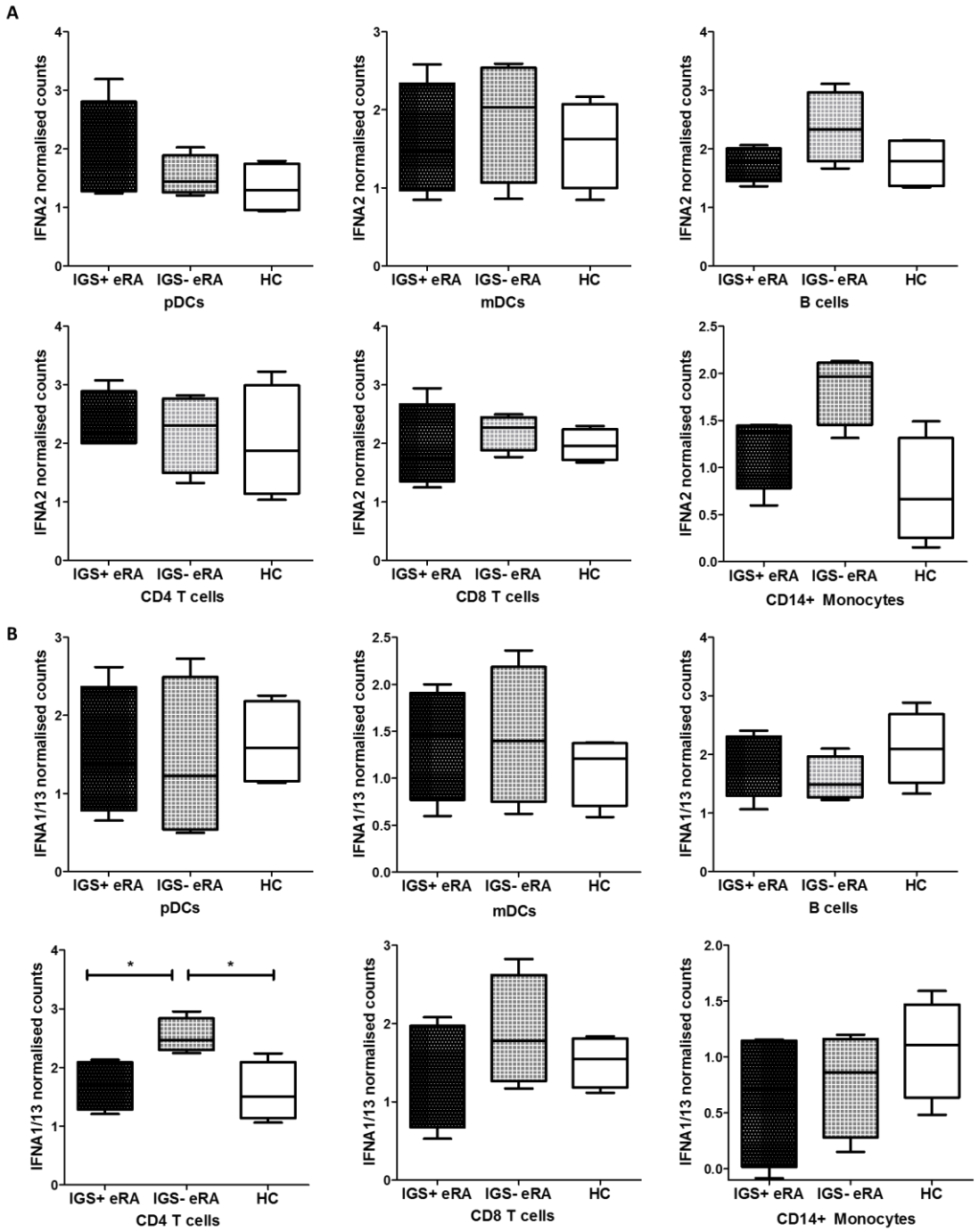


Figure 5.10 Immune cell *IFNA2* and *IFNA1/13* expression by disease cohort and IGS+ status

A *IFNA2* and **B** *IFNA1/13* mRNA expression, as determined by NanoString nCounter technologies, was compared across flow cytometry cell sorted immune cell subsets in healthy controls (HC, n=4), IGS+ early RA patients (IGS+ eRA, n=4) and IGS- early RA patients (IGS- eRA, n=4). Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Kruskal-Wallis tests. *$p<0.05$

5.4 Discussion

In this chapter I examined the transcriptome of pDCs in early RA and compared them with healthy controls. There were marked differences in gene expression between early RA patients and healthy controls, as well as between IGS+ and IGS- early RA patients. PDCs in early RA appeared to have pathways related to increased proliferation but reduced apoptosis in early RA. However there was no indication if pDCs in early RA had a more activated or tolerogenic transcript. Unexpectedly pDCs did not appear to be the main interferon-α producing subset in IGS+ early RA. Compared with IGS- early RA, B cells from IGS+ patients demonstrated increased expression of genes related to B cell activation, proliferation and plasma cell generation. This further confirms the relevance of B cell and IGS interactions in RA.

This is the first time the transcriptome of pDCs from patients with RA has been examined. IPA® pathway analysis demonstrated increased proliferation and reduced apoptosis in early RA pDCs (figure 5.4). This is counter intuitive with respect to the known reduced pDC number in the peripheral circulation of RA patients (54, 87, 316) (and as shown in Chapter 4). However there is a suggestion that the pDCs migrate to the synovial compartment in RA and numbers are increased in the inflamed joint (85-88). Therefore increased pDCs proliferation in the peripheral circulation could be relevant in synovial disease. Conversely when examining IGS+ early RA patients versus healthy controls there was increased proliferation but pathways relating to cell migration and adhesion were reduced. These latter features are contrary to what was shown in Chapter 4 where there was no effect of the IGS on pDC frequency or phenotype.

However one of the main caveats interpreting this IPA® data is the low patient sample size and the subsequent relatively small gene list generated. This is particularly relevant in the context of RA being a highly heterogeneous disease where large numbers are often required to overcome patient variability. In addition, much of the literature in the IPA® database used to generate these associations relates to other cell subsets, the functions of which may not be reproduced in pDCs. For example, one of the highest upregulated genes between early RA (all) or IGS+ early RA patients and healthy controls was *PRDM1*, which encodes B lymphocyte-induced maturation protein-1 (Blimp-1). Blimp-1 has been most extensively investigated in B cells where it promotes proliferation and differentiation of autoantibody secreting plasma cells (357). However the effect of Blimp-1 expression in DCs is less clear. It may promote DC survival as Blimp-1 knockout mice have reduced mDC and pDC numbers (358). Another mouse study demonstrated Blimp-

1 negatively regulating mDCs during homeostatic development and promoting DC maturation (359). However, Blimp-1 may have an anti-inflammatory role; Blimp-1 knockout in mDCs caused adult female mice to spontaneously develop a lupus-like phenotype (360) and in human monocyte derived DCs Blimp-1 inversely correlated with HLA-DR expression (361). As demonstrated these are mainly murine mDC studies, and little work has been done looking at the role of Blimp-1 in human pDCs. Thus the effect of increased Blimp-1 expression in early RA pDCs remains unknown and IPA® conclusions involving its expression are weakened. As highlighted by this example, the paucity of literature relating to pDC-specific function of the differentially expressed genes identified makes interpretation of the global function of pDCs in early RA challenging. Nonetheless *IFI44L* was most differentially expressed gene in pDCs from IGS+ early RA patients when compared with both healthy controls and IGS- patients (figure 5.5). The function of *IFI44L* is unknown, however it is an IRG and makes up part of an anti-viral response (362). Although it was not similarly increased in the IGS+ B cells (figure 5.7), its increased expression in pDCs supports increased type 1 interferon exposure the IGS+ cohort. IRF4 was also increased in the same pattern as *PRDM1*. IRF4 is an interferon regulatory factor which modifies interferon production following viral exposure (363). However its expression in pDCs has recently been shown to skew T cells towards a Th2 response (364). In early RA there is evidence of an increased Th2 response with increased IL-13 in synovial fluid which falls with time (95). It is possible that early RA pDCs may be involved in this process. Examining the effect of the IGS on pDCs from early RA patients there were only 2 differentially expressed genes; increased *IFI44L* as discussed above, and significantly reduced *S100A8*. The protein product of *S100A8* forms a heterodimer with S100A9 and this complex has many pro-inflammatory properties, such as TLR4 ligation. Increased circulating levels of S100A8/A9 have been detected in autoimmunity (365-367) and its secretion by pDCs is increased in SLE upon immune complex stimulation (366). The reduced *S100A8* expression therefore conversely suggests a less inflammatory phenotype in the IGS+ cohort. Overall, when analysing these data, it ultimately remains unclear what is the overall effect of the IGS on pDC function as well as whether the net role of pDCs in early RA is pro- or anti-inflammatory. The only conclusion that can be drawn is their role in RA is likely to be complex and potentially alters during the different stages of disease.

Pathogen-derived nucleic acids are the main trigger for the production of type 1 interferons (260). A recent study showed increased expression of RNA sensors TLR7,

RIG1 and MDA-5 in pDCs from pSS IGS+ patients and the authors suggested increased detection and exposure to either host- or pathogen-derived RNA as a potential promotor of the IGS (236). However there was no evidence of RNA sensing pathways being involved in the IGS+ early RA cohorts. Autoantibodies to self-RNA, such as anti-ribonucleoprotein (anti-RNP) which include anti-Ro/SSA and anti-La/SSB are commonly found in pSS (368). Their related immune complexes can directly bind to FcγIIa expressed on pDCs, resulting in internalization and interaction with intracellular RNA sensing pathways causing interferon-α production (369). These RNP autoantibodies are not as widespread in RA, indeed antibodies against nuclear antigens are relatively rare in RA. This might explain the differences in pDC expression of RNA sensing pathways between IGS+ patients from pSS and early RA. When examining DNA sensing pathways between the cohorts I unexpectedly detected reduced STING expression, a cytosolic DNA sensor, in both the IGS+ cohort and the early RA cohort. Ligation of STING causes an increase in its expression as well as interferon-α release and this pathway is known to be functional in human pDCs (370). Type 1 interferons further promote increase in STING expression, thereby promoting a positive feedback mechanism (120). This suggests that pDC sensing of cytosolic DNA is not promoting the IGS in early RA and is in fact not following the normal physiological response to type 1 interferon exposure.

However pDC pathways involved in the triggering of type 1 interferons could be irrelevant as it is unknown what cell type is primarily responsible for generating the IGS in early RA. Due to the known function of pDCs and their potential to produce vast quantities of type 1 interferons (43) it was expected that their *IFNA2* and *IFNA1/13* transcripts would be increased in IGS+ RA and associate with the IGS. However this was not the case and my data did not show which cellular subset was ultimately responsible for the IGS (figures 5.9-5.10). In SLE there is a suggestion that pDCs become "fatigued" and actually produce less interferon following repeated stimulation (279). Furthermore herpes virus induced interferon-α production is decreased in SLE PBMCs and SLE patients exhibit a reduced number of BDCA-2 expressing pDCs; this is the pDC subset generally thought to be primary interferon producing (371). All cells have the ability to produce type 1 interferons and it is presumed that with time other cell subsets take over as primary producers of type 1interferons in SLE. Potentially similar processes may have occurred in early RA although none of the other immune cell subsets examined had an association between expression of *IFNA2* and *IFNA1/13* transcripts and the IGS (figure 5.10). Another potential explanation for these observations is the anatomical origin of the

cells examined. There is no detectable interferon-α in the serum of RA patients (187) (which I have also shown – see Chapter 3) and activated pDCs migrate to lymph nodes and areas of local inflammation such as the synovial lining in RA (320). PDCs also require a critical density to initiate a spontaneous type I interferon response which can have an autocrine effect on promoting further interferon-α production (372); this is more likely to occur in lymphoid tissue/synovium. Therefore in RA those pDCs, and indeed any other cell, involved in type 1 interferon production may not be in the peripheral circulation but rather clustered in either lymphoid or inflammatory tissue. They would therefore not have been identified in this peripheral blood analysis. One final consideration is the complexity of type 1 interferons themselves. There are 12 different functional interferon-α products (159) as well as ligand-specific differences in induced expression; TLR9 agonists elicited expression of all type I interferon subtypes by pDCs but TLR7 agonists did not (308). I only examined 2 members of the interferon-α family (*IFNA2* and *IFNA1/13)*. It may be that other pDC derived *IFNA* subsets are implicated in generating the IGS or triggered from pathological stimuli in RA.

The effect of the IGS on the B cell transcriptome in early RA was also examined. IPA® databases examining the differential gene expression profile in B cells were more robustly supported by B cell relevant literature. Identified pathways included increased proliferation and activation in the IGS+ early RA cohort (figure 5.8). This is consistent with what is known about the effect of type 1 interferons on B cell function (145). However type 1 interferons can also promote B cell differentiation into antibody-producing plasma cells; low concentrations of interferon-α cause B cell differentiation into plasmablasts, whereas higher concentrations cause plasma cell maturation (373). Furthermore $IFNAR^{-/-}$ knockout mouse models of SLE (with resultant loss of interferon-α signalling) do not develop autoantibodies (374). In keeping with this I saw increased *XBP1,* a crucial transcription factor in the development of plasma cells, in the IGS+ B cells (375, 376). There was also a higher expression of *CD27* in the IGS+ cohort. CD27 belongs to the tumor necrosis factor receptor family and is involved in the late-stage differentiation of B cells into plasma cells, and plasma cells have markedly increased CD27 expression ($CD27^{++}$). In addition increased expression of CD27 on memory B cells suggests ongoing differentiation into plasma cells with the acquired ability to secrete high levels of immunoglobulin (377). *CD80* was also increased in the IGS+ cohort. Increased surface expression of CD80 is associated with B cell activation but also been implicated in plasma cell generation (378). This has further pathogenic relevance as RA B cells have

increased CD80 expression and often also co-express receptor activator of nuclear factor kappa-B ligand 9 (RANK-L). This has implications for bone reabsorption and erosion development (379). *TNFSRF13B* encodes the B cell transmembrane activator and CAML interactor (TACI) and its expression was also increased in the IGS+ cohort. TACI is expressed on the cell surface and is a receptor for APRIL and BAFF/BLyS. It is highly expressed on marginal zone and $CD27^{+}$ memory B cells and upregulated following TLR9 or BCR ligation (184, 380). Its function has been contradictory and enigmatic when comparing across mouse and human studies but nonetheless activation of TACI results in B-cell differentiation into plasmablasts, isotype switch and antibody production which includes T cell-independent antibody production (298, 380, 381). TACI also promotes sustained Blimp-1 expression on B cells, which limits the clonal expansion of B-cells and facilitates the maintenance and/or generation of long-lived antibody secreting cells (382); a phenotype potentially involved in autoantibody production (383). Increased TACI also has further relevance to RA as increased BAFF/BLyS-TACI signalling may cause the escape of autoreactive B cells and promote breach of tolerance (73). Indeed in very early RA patients there was increased TACI mRNA expression in the PBMCs when compared with healthy controls (384) and TACI polymorphisms in immunodeficiency syndromes predispose to autoimmune disease and lymphoid hyperplasia (385) (all figure 5.7).

Although not directly related to plasma cell generation, the significant downregulation of *PECAM1* (CD31) in the IGS+ early cohort (figure 5.7) may also promote autoantibody generation. PECAM-1 is a member of the immunoglobulin superfamily and is expressed on the surface of all of hematopoietic cells, including B cells (386). B cell $PECAM\text{-}1^{-/-}$ knockout mice have an activated and hyper responsive B-cell phenotype and develop autoantibodies. These mice also have increased numbers of B1 cells, which are the subset in mice associated with IgM production, and have increased serum IgM but normal levels of other immunoglobulin isotypes (387). This downregulation of PECAM-1 is likely to have implications *in vivo* as rheumatoid factor (RF) is an IgM autoantibody. Together these findings all suggest that B cells in the IGS+ cohort are primed towards plasma cell differentiation and potentially the production of IgM subclass of antibody.

These demonstrated *in vivo* effects of IGS on B cells gene expression in early RA further corroborates findings relating to the effect of the IGS in longstanding disease. There has been a large body of evidence in established RA demonstrating that a positive IGS negatively influences the clinical response to rituximab, an anti-CD20 B cell depleting therapy (23, 186, 200-202). Furthermore early reconstitution of $CD27^{+}$ B cells (388), as

well as high numbers of $CD27^{++}$ plasmablasts pre–treatment (389) is a negative predictor of remission following rituximab. I showed increased *CD27* expression in the IGS+ group (figure 5.7) and promotion of this B cell phenotype may be one of the mechanisms whereby the IGS negatively influences rituximab response.

Finally the connection between the IGS and an abnormally activated or proliferative phenotype is interesting in the context of the increased risk of B cell malignancies in RA patients (390-394). *CD58* was increased in the IGS+ B cells and B cell expression of this marker is normally only seen in malignant plasma cells, such multiple myeloma, where is it widely expressed (395). There was also upregulation of *CD99*, *IL10RA* and *IGBT1* (CD29) in the IGS+ cohort which are also overexpressed in B cell malignancies, such as diffuse large B cell lymphoma (DLBCL) (396-398). Finally *TCF7* was also increased in the IGS+ cohort and this mediates Wnt signaling by associating with β-catenin in the nucleus. In mice Wnt signaling promotes proliferation of bone marrow pro-B cells (399) and many tumors, including chronic lymphocytic leukemia, DLBCL and multiple myeloma display increased Wnt/β-catenin signaling (400-404). Cumulatively this suggests that normal pathways regulating plasma cell differentiation may be deranged in IGS+ early RA allowing for inappropriate expansion of a potentially pathological subset.

A caveat of transcriptomics is that mRNA levels do not always equate to protein levels. There is therefore not always a definitive link between the level of gene expression and the relative impact on biological functions. Nevertheless, the application of this technique is primarily hypothesis generating rather than hypothesis testing and is useful to identify trends for further investigation. I used NanoString to analyse the transcriptome in the cell subsets. This uses a dual probe hybridization to directly enumerate specific RNA molecules with no RNA amplification thus minimising variability and error. Reactions are also performed with excess transcript-specific probes to avoid hybridization and measurements are made on a digital instead of analog scale to reduce fluorescence scale biases (256). NanoString data is highly robust to different normalisation methods providing reassurance that computational processing biases are not skewing my findings (405) and results have been shown to be comparable to other more established methods, such as microarray (406). Nonetheless there are some considerations; I ran each cell subset on an individual chip meaning that, despite data normalisation, batch effect differences could occur. This would compromise any comparisons made between cell subsets and this caveat must be fully considered.

5.5 Conclusions

The transcriptome of peripheral blood pDCs was examined in early RA and compared with healthy controls. This is the first time the transcriptome of pDCs has been examined in RA but the net inflammatory vs tolerogenic function remains unclear. Unexpectedly there was no clear evidence that pDCs were the primary interferon-α subset in the peripheral blood. It may be that the other interferon-α subtypes are more important for RA, the subtypes I measured have relatively less relevance, or that the interferon-α producing pDCs have migrated out of the peripheral blood (320). Nonetheless given the distinct nature of early RA compared with established RA there may be a separate function of pDCs in early disease compared with established disease. As treatment increasingly is targeted towards early RA (11-20) the relevance of these findings is increased.

B cells were compared between IGS+ and IGS- early RA patients and IGS+ patients demonstrated increased gene expression related to pathways involving proliferation, activation, plasma cell differentiation and autoantibody production. I also detected upregulation of genes previously identified in B cell malignancies. This suggests that B cells may be pathologically relevant in IGS+ early RA and thus are a potential cellular target of any type 1 interferon targeting therapies. My findings also support the recognised observation in established RA where a positive IGS negatively impacts on outcomes related to B cell depletion (186, 200) and propose mechanistic pathways that may be potentially involved.

This work is preliminary and performed on a very small number of individuals. Nevertheless it findings raises some intriguing questions about the role of the IGS in early RA and suggests that B cells are pathologically affected. Consolidation of this work may therefore propose novel therapeutics options and applications of interferon targeting therapies in early RA.

5.6 Future work

This was a small pilot study and clearly further work and increased sample numbers is required to improve the power and validate these preliminary findings.

One of goals of this work was to identify what cell subset was most responsible for the production of type 1 interferons in IGS+ early RA patients. There was no clear answer but this may have been due to the fact I only measured two subtypes of interferon-α due to a limitation of space on the NanoString technologies customised panel. Ideally this work would be repeated with an additional customised panel to quantify all interferon-α subtype transcript expression in the above cell subsets but also additional immune cells if possible, such as NK cells or neutrophils.

There was clear upregulation of some known IRGs in the IGS+ pDC population (e.g. IFI44L). However this was not clearly seen in the B cells. I would therefore like to analyse in depth if the differentially expressed genes in the B cells were mainly IRGs. Furthermore I would like to identify which cell subset had the highest increased expression of IRGs which may suggest increased sensitivity or increased proximity to interferon-α production. This could potentially involve the use of modelling techniques or online platforms/databases that allow in depth analysis of interferon related genes, such as the Interferome V2.0 (http://interferome.its.monash.edu.au/interferome/) (407).

Nonetheless the B cell transcriptome of IGS+ early RA patients compared with IGS- patients was distinctly different. A crucial first step would be to validate the expression of these genes using qRT-PCR. I would also need to increase patient numbers, especially including patients that were seronegative. If these genes were increased using qRT-PCR a next step would be to study the protein level either using flow cytometry or western blot.

I used NanoString in this series of experiments mainly due to the low numbers of dendritic cells available and thus low RNA yield, and as a way of avoiding subsequent amplification bias normally required when using other transcriptomic technology. However if I were to focus primarily on B cells there would be adequate material for next generation sequencing technology, such as RNA sequencing. This would widen the number of genes examined and provide a more comprehensive transcriptional comparison.

B cells cultured with interferon-α could be examined specifically for changes in those genes identified on the NanoString as well as examine B cell functional characteristics and surface marker expression. This would validate if the gene expression data translated into B cell phenotype and if interferon-α alone was responsible. If the proteins/genes related to B cell malignancies were confirmed to be upregulated an additional step would be to recruit patients with a new diagnosis of a RA related B cell malignancy and measure their IGS and B cell phenotype.

Chapter 6: Retroelements in early RA

6.1 Introduction

There is increasing evidence that prior to the development of autoimmunity there is a series of events, both genetic and environmental, that prime and direct the immune system towards self-reactivity. One proposed mechanism for the development of autoimmune disease is the presence and activation of retrotransposons. This so called "junk" virus-derived DNA (retroelements) makes up to 40% of the human genome and, rather than being inert as previously thought, some repeat insertions have retained ability to replicate within a cell (213, 230). There are distinct types of retroelements: 1) Those that are autonomous and are able to encode all the proteins necessary for replication include endogenous retroviruses (ERVs) (often detected as long terminal repeats, LTRs) and long interspersed elements (LINEs); 2) Those that are non-autonomous include short interspersed elements (SINEs), of which Alu is the most common. These require the presence of active LINEs for their mobility and hijack the LINEs replication machinery during replication (121). These separate classes of retroelement are depicted in figure 6.1

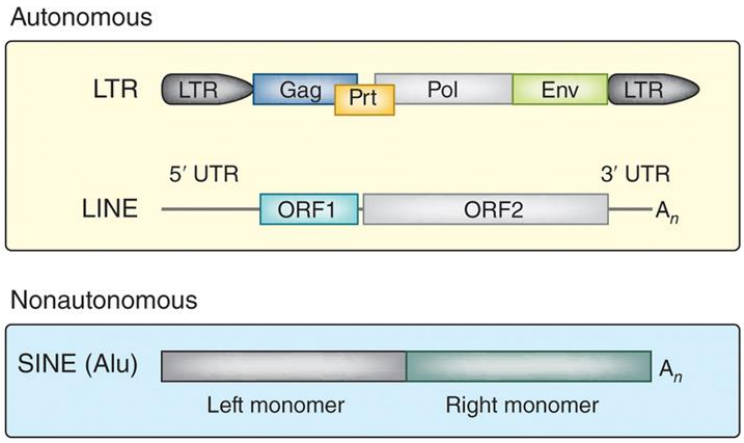


Figure 6.1 Types of retroelements in a mammalian genome

Autonomous retroelements include endogenous retroviruses (ERV) - detected as long terminal repeats (LTRs) - as well as long interspersed nuclear element 1 (LINE-1). These both encode all proteins necessary for their replication and retrotransposition. Non-autonomous retroelements, such as short interspersed nuclear element 1 (SINE), the most common of which is Alu, are much smaller and hijack LINE-1 machinery to facilitate their mobility. Gag, group-specific antigen; Prt, protease; Pol polymerase; Env, envelope; UTR untranslated region. Figure adapted from Volkman *et al.*, 2014 (121).

Retroelements are viral nucleic acids and during their replication process they are exposed to intracellular nucleic acid sensors which triggers downstream anti-viral pathways. Activation of these pathways frequently culminates in type 1 interferon production and

contributes to generalised immune activation. This would subsequently promote more effective presentation of antigen, potentially of self-components, and thus promote autoimmunity (121, 230). This clearly has relevance to diseases where there is a marked IGS, primarily SLE but also RA. Indeed LINE-1 elements are increased in the synovium of RA patients (237, 239). Furthermore human ERVs are increased in RA synovium and evidence of their transcriptional activity, as well as viral protein products, are detected in the peripheral circulation of RA patients (217, 238).

Given this background I was interested in the role of retroelements in generating the IGS. I hypothesised that increased activity in RA would be driving the IGS. Retroelement activity is determined by transcript expression and can be detected by standard mRNA detection methodology, such as qRT-PCR. I wished to look at whole blood but also pDC specific retroelement activity. This was due to the pDC potential of prolific type 1 interferon production (43) as well as a recent study linking the IGS in pSS to increased pDC expression of cytosolic RNA/DNA sensing pathways, and thus potential retroelement activity (236).

Retroelements, however, may contribute to the development of autoimmunity independently of interferon-α induction. Some ERVs retain their ability to produce viral protein and molecular mimicry may occur where foreign retrovirus derived proteins share sequence homology with peptides on self-reactive cells, and therefore cross-activate these cells (216, 217). ERV proteins may also present as antigens and this could be a mechanism for the development of antibodies in autoimmunity. Interestingly both serological and molecular assays showed significant increases in the endogenous retrovirus HERV-K (HML-2) *gag* activity in RA patients compared to disease controls and these *gag* and e*nv* proteins shared the same peptide sequences with rheumatoid factor epitopes on IgG1Fc (242, 243). Antibody responses can also develop to cell components associated with retroelement nucleic acid and their virus like proteins; anti-Ro60 binds a RNA motif derived from Alu retroelements (232). This association could direct immune responses against these self-derived elements resulting in the production of autoantibodies.

In light of these associations between retroelements and B cell function I wished to specifically examine any associations between whole blood retroelement activity and autoantibodies in my early RA cohort. Additionally I wished to focus on the relative expression of retroelements in B cells between early RA patients and healthy controls.

6.2 Aims

In this chapter I wished to address the following questions:

1) Are whole blood LINE-1 elements increased in early RA patients compared with healthy controls?
2) Do whole blood LINE-1 elements associate with the IGS or any other circulating cytokines?
3) Do whole blood LINE-1 elements associate with autoantibodies?
4) Does pDC and B cell retroelement activity vary between early RA patients and healthy controls?
5) Do pDC retroelements associate with actively transcribed genes?

6.3 Results

6.3.1 Whole blood LINE-1 analysis: c-DNA and qRT-PCR optimisation

cDNA was initially generated using the standard protocol described for IGS analysis. Whole blood LINE-1 activity was measured by transcription of the LINE-1 5 prime untranslated region (L1-5'UTR) and the LINE-1 open reading frame 2 (L1-ORF2). However, during qRT-PCR it became clear that, although the water control amplified L1 5'UTR at >35C_T, the –RT control (i.e. no superscript II) amplified at a similar level to the test samples (figure 6.3.A). As the retroelement sequences present in cDNA and genomic DNA (gDNA) are identical the primers used in qRT-PCR can amplify both cDNA and gDNA. The amplification in the –RT control suggested that there was gDNA contamination. Furthermore there was no successful amplification of L1-ORF2 (figure 6.3.B).

I therefore optimised a new method of cDNA generation ("super-clean" method) which had additional DNAse steps within the protocol to remove any gDNA. In addition, I used gene specific primers to amplify up only the desired mRNA (L1-5'UTR, L1-ORF2 and housekeeper gene TBP). To test the efficiency of the gDNA removal by the additional DNase step a PCR was performed which used primers spanning an exon-intron-exon region in the *HPBI* gene. If pure cDNA is present only one short band will be formed (152bp) as the intron regions are not present in cDNA, whereas if there is gDNA contamination an additional large band (570bp) will be seen due to the added presence of intron regions. This is demonstrated in the following schematic (figure 6.2)

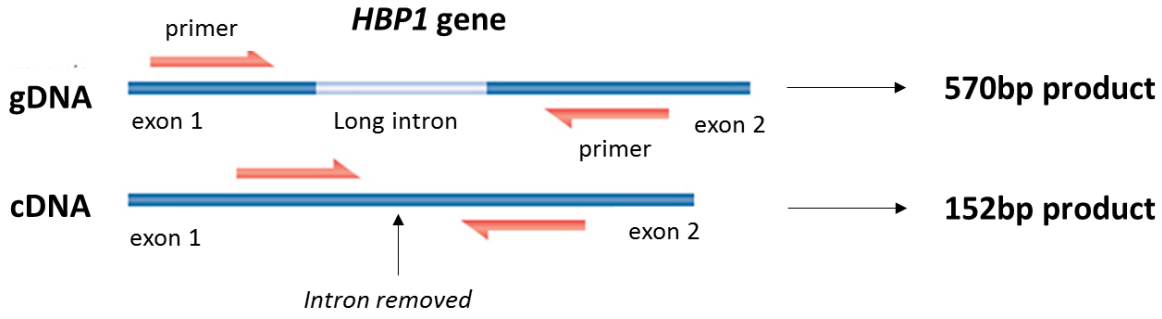


Figure 6.2 Schematic of different PCR product size generated from either genomic DNA or c-DNA when using intro-exon spanning *HBPI* primers

PCR primers spanning the intron-exon regions of the *HBPI* gene will generate different sized product depending on if genomic DNA (gDNA) or c-DNA is used; gDNA will retain an intron section and have a larger band size (570bp) compared with intron-less c-DNA (152bp).

A gel electrophoresis was subsequently performed to validate the purity of my c-DNA (figure 6.4). I could see for the c-DNA samples generated using the "super-clean" method there was only one band detected at 152bp (indicating c-DNA generated from intron-free mRNA), whereas for our positive gDNA control there was an additional band seen at 570bp indicating presence of intronic gDNA.

As there was no amplification of L1-ORF2 using a TaqMan assay I tested a SYBR green assay, which does not rely on probes in addition to primers to amplify the message. I found using this method there was successful amplification of both L1-5'UTR and L1-ORF2 (figure 6.5). Furthermore, in for both transcripts, the –RT control amplified at the level of the water control confirming the success of the gDNA elimination step.

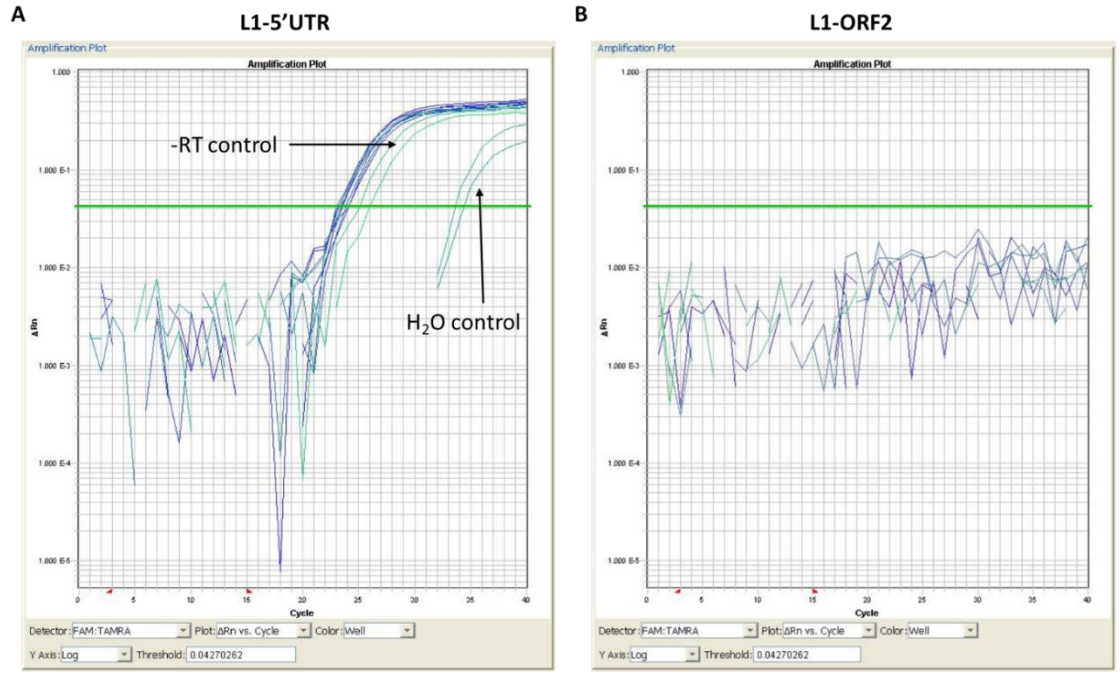


Figure 6.3 Genomic DNA contamination is observed in c-DNA generated from whole blood RNA

RNA was extracted from whole blood using the Tempus RNA system. C-DNA generated using SuperScript II reverse transcriptase following the manufacture's protocol. A TaqMan assay was used to quantify LINE-1 5'UTR **(A)** and ORF2 **(B)**. A representative of the amplification plots is shown for each assay.

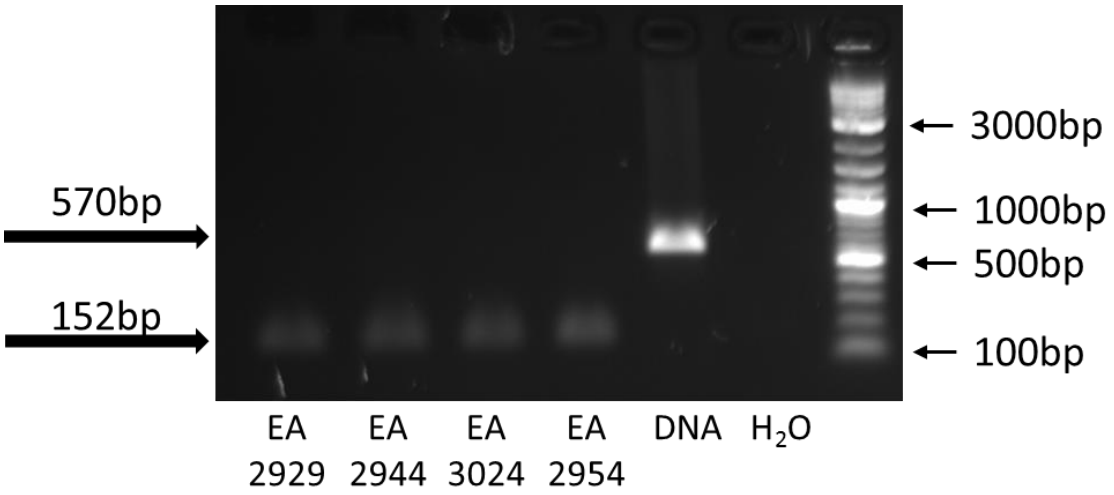


Figure 6.4 An optimised "superclean" cDNA generation method eliminates gDNA contamination

RNA was extracted from whole blood using the Tempus RNA system. cDNA was generated using the "superclean" optimised method and a PCR performed using primers which spanned an intron and exon region the HBPI gene. cDNA with contaminating genomic DNA (gDNA) will produce 2 bands (570bp and 152bp), while uncontaminated cDNA will have just one product (152bp). I show here representative findings using patient samples (EA2929, EA2944, EA3024 and EA2954) as well as a positive DNA control.

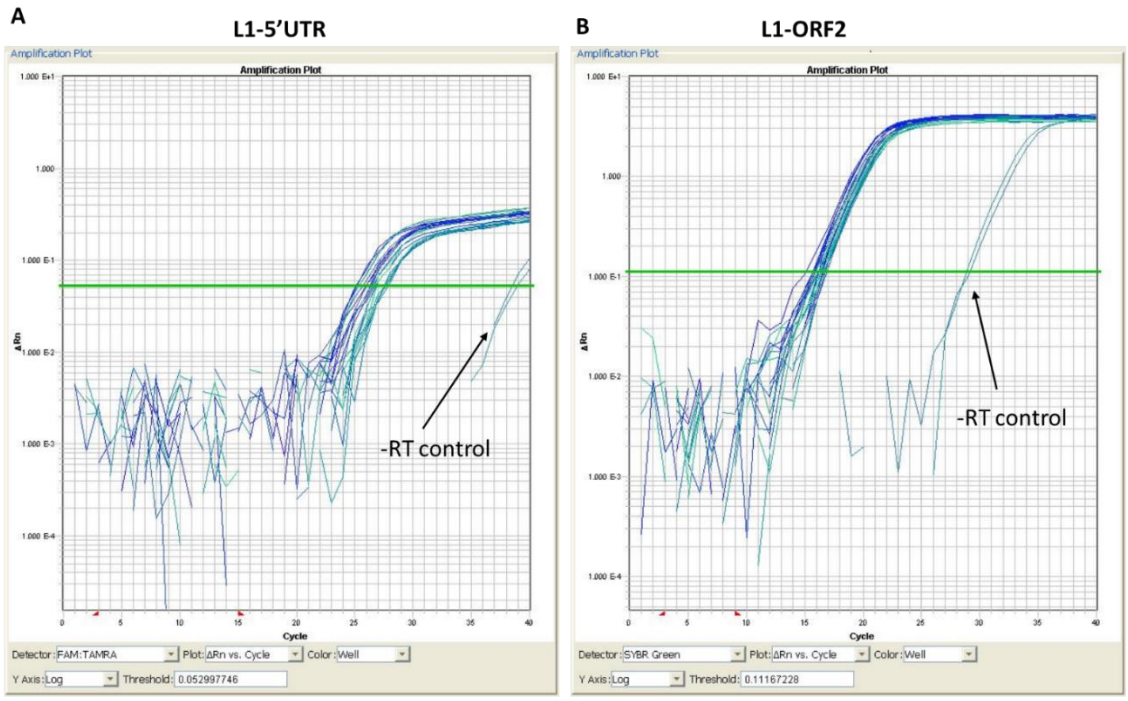


Figure 6.5 Both LINE-1 5'UTR and ORF2 successfully amplify using a SYBR green assay

RNA was extracted from whole blood using the Tempus RNA system. C-DNA generated using the "superclean" optimised method. A SYBR green assay was used to quantify LINE-1 5'UTR **(A)** and ORF2 **(B)**. A representative of the successful amplification plots is shown for each assay

6.3.2 Whole blood LINE-1 analysis: Associations with age or gender

Early RA patients and healthy controls had their peripheral whole blood L1-5'UTR and L1-ORF2 expression quantified using the optimised SYBR Green assay.

The demographics of each group are shown in table 6.1. There was a significant difference between the groups regarding age where the early RA cohort were older, but there was no difference between the groups regarding gender.

Correlations were examined between age and the LINE-1 elements for the whole cohort. There was no significant association between age and L1-5'UTR or L1-ORF2 expression (figure 6.6.A). However due to the age differences between the cohorts the association with age was looked at separately for each cohort; again there was no association with age detected for either cohort (figure 6.6.B-C).

I next examined the effect gender may have on LINE-1 expression. Again for the whole cohort, there was no effect of gender on L1-5'UTR or L1-ORF2 expression (figure 6.7.A). In early RA patients alone there were no significant differences in expression of either L1-5'UTR or L1-ORF2 (figure 6.7.B). However, healthy controls demonstrated a significant increase in L1-ORF2 expression in females compared to males, although there was no significant difference in L1-5'UTR expression (figure 6.7.C).

In light of these findings when comparing whole blood LINE-1 activity between the cohorts age and sex corrections were performed, whereas within a cohort it was not.

	Early RA	Healthy control	Difference between groups (p value)
Number	56	23	-
Age (years) Median [range]	58 [30-87]	39 [23-62]	<0.0001
Male: Female ratio	1:1.8	1:1	0.325
% seropositive (either anti-CCP+ or RF+)	77%	-	-
DAS-28 Median [range]	4.3 [1.3-7.6]	-	-

Table 6.1 Demographic data for early RA and healthy control cohorts

Early RA patients (n=56) and healthy controls (n=23) had their peripheral whole blood LINE-1 retrotransposon activity measured (as described in figure 6.5). The demographics for the two cohorts are shown here. Differences between groups were calculated using a Mann Whitney U test.

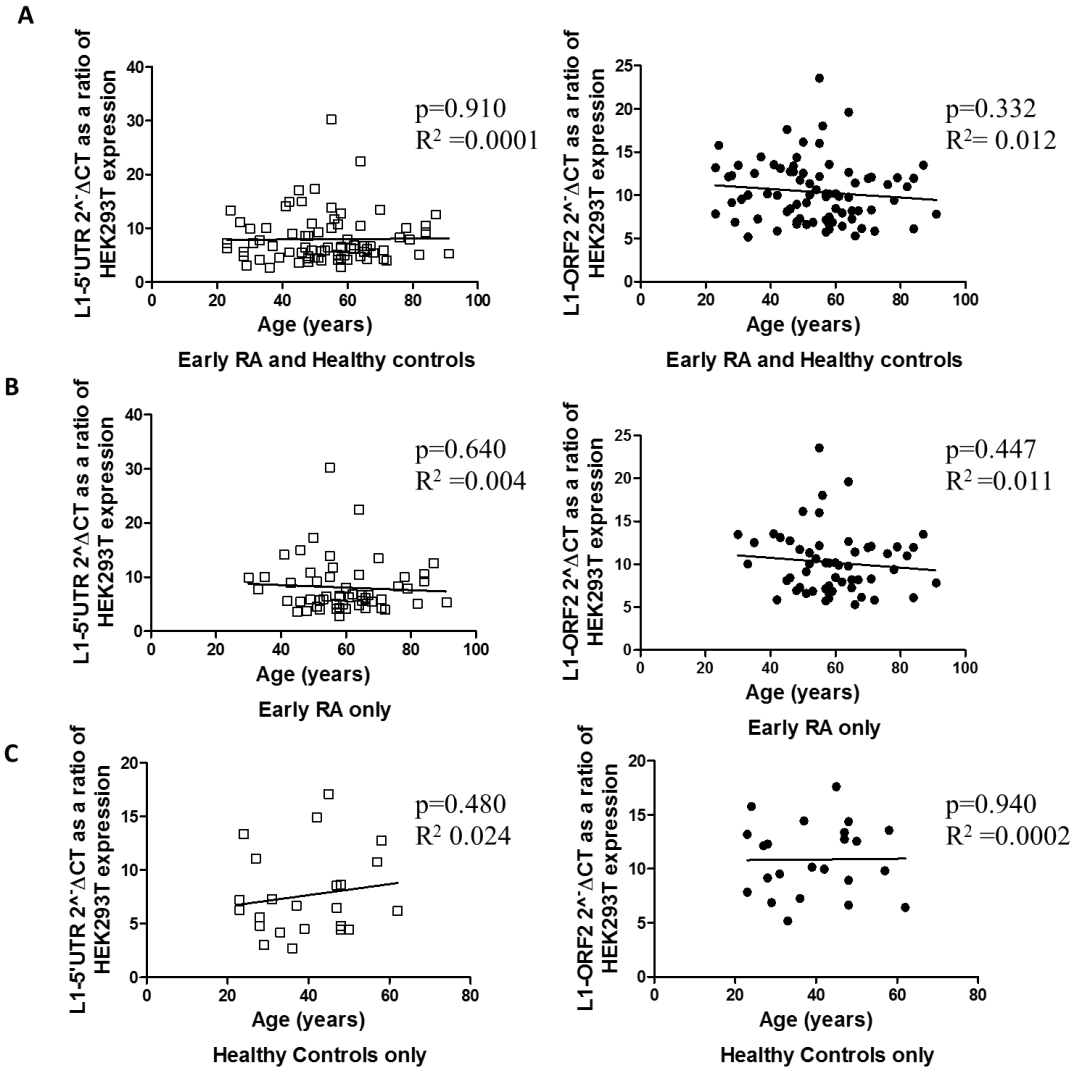


Figure 6.6 Age and whole blood LINE-1 expression

Whole blood LINE-1 expression was analysed (as described in figure 6.5) in early RA patients (n=56) and healthy controls (p=23). Associations with age were examined. **A:** Plots depict linear regression between age and L1-5'UTR and L1-ORF2 expression in pooled early RA and healthy controls. **B:** Plots depict linear regression between age and L1-5'UTR and L1-ORF2 expression in early RA patients. **C:** Plots depict linear regression between age and L1-5'UTR and L1-ORF2 expression in healthy controls.

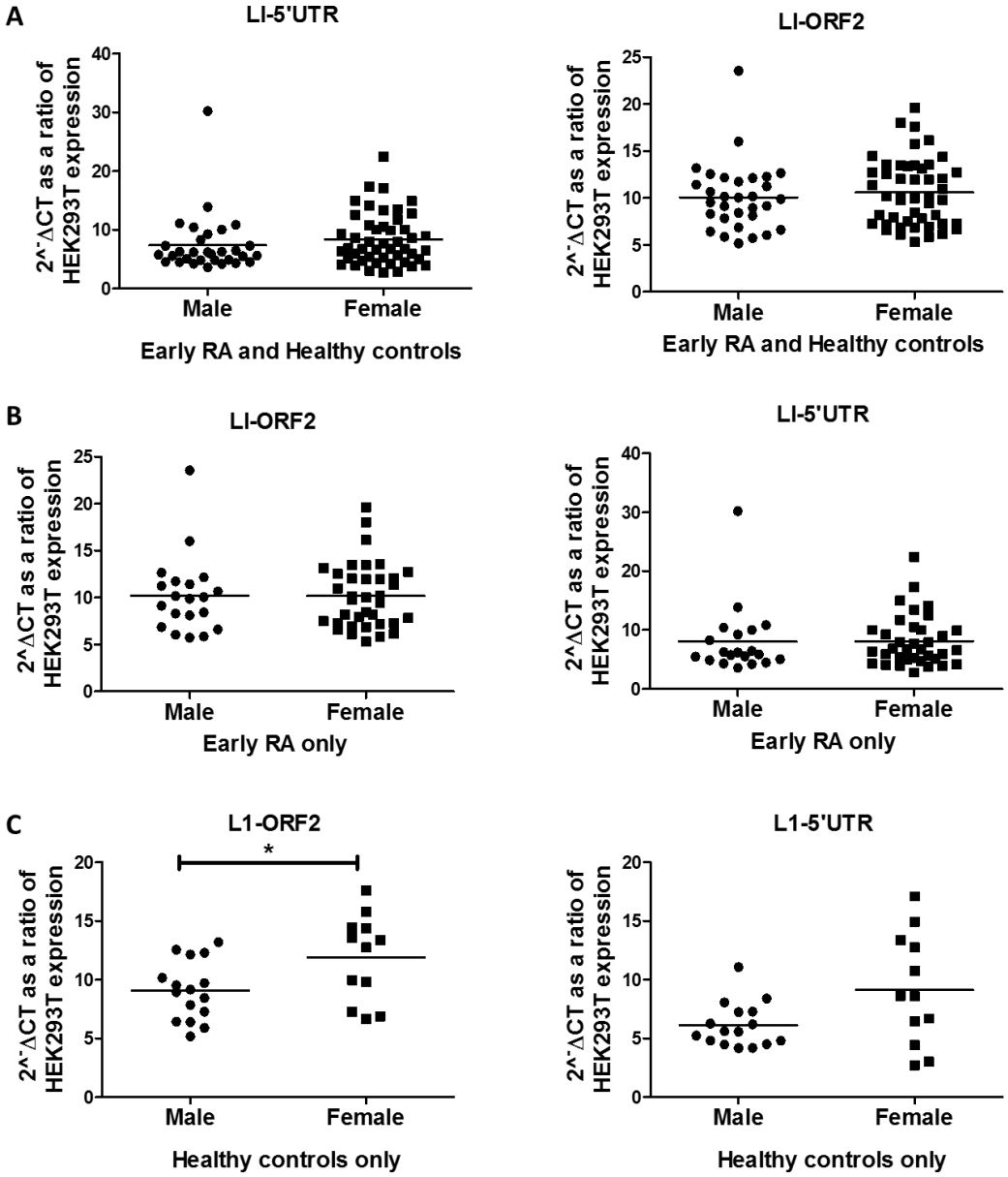


Figure 6.7 Gender and whole blood LINE-1 expression

Whole blood LINE-1 expression was analysed (as described in figure 6.5) in early RA (n=56) and healthy controls (n=23). Associations with gender were examined. **A:** Pooled early RA and healthy control LINE-1 activity divided by gender. **B:** Early RA patient LINE-1 activity divided by gender **C:** Healthy control LINE-1 activity divided by gender. Horizontal lines depict median values. Mann-Whitney U tests. *$p<0.05$

6.3.3 Whole blood LINE-1 analysis: LINE-1 activity, the IGS and clinical phenotype

LINE-1 activity was compared between the early RA and healthy control cohorts (corrected for age and gender). No significant difference between the cohorts for either L1-5'UTR or L1-ORF2 expression was detected (figure 6.8).

Published literature suggests a possible link between autoantibody production and retroviral activity. I therefore sought to see if such a link existed in my RA cohort. The early RA cohort was split into seropositive (either anti-CCP+ or RF+) or seronegative (double anti-CCP- and RF-) but there was no difference in either L1-5'UTR or L1-ORF2 expression between these sub-groups (figure 6.9.A). I next looked at patients that were either RF+ or RF- and again there was no significant difference between the groups for either L1-5'UTR or L1-ORF2 expression (figure 6.9.B). This was the same when splitting the cohort into anti-CCP+ or anti-CCP- (figure 6.9.C). However when I examined absolute anti-CCP titres there was a significant positive association between both L1-5'UTR and L1-ORF2 expression and anti-CCP titres (figure 6.10.A). This effect however was not seen with RF titres (figure 6.10.B). Smoking can cause both an increase in anti-CCP titres and retroelement activity (408, 409), so smoking history was examined as a potential confounding variable. However there was no significant difference in whole blood LINE-1 activity between those who had never smoked and those that were actively smoking. Furthermore there was also no difference in anti-CCP titres between these cohorts either (data not shown).

In view of the ability of retroelements to trigger interferon-α production the early RA cohort was next divided by IGS. I detected significantly increased L1-5'UTR activity in the IGS-cohort compared to the IGS+ cohort (figure 5.11.A) and although a similar trend was seen in L1-ORF2 expression, it was not significant (figure 5.11.B). I next examined the IGS (as a continuous variable) and correlated this with the whole blood LINE-1 elements but found no significant correlation between the two (data not shown).

High levels of inflammatory cytokines, including IL-6, can inhibit ERV activity (242). I therefore examined circulating cytokines levels and LINE-1 activity in the early RA cohort. There was no association between retroelement activity and cytokines (interferon-γ, IL-6, IL-12 p70, TNF-α, IL-1β, IL-2, IL-13, IL-4 and IL-10) (data not shown). There was also no significant association between LINE-1 activity and CRP or ESR (data not shown).

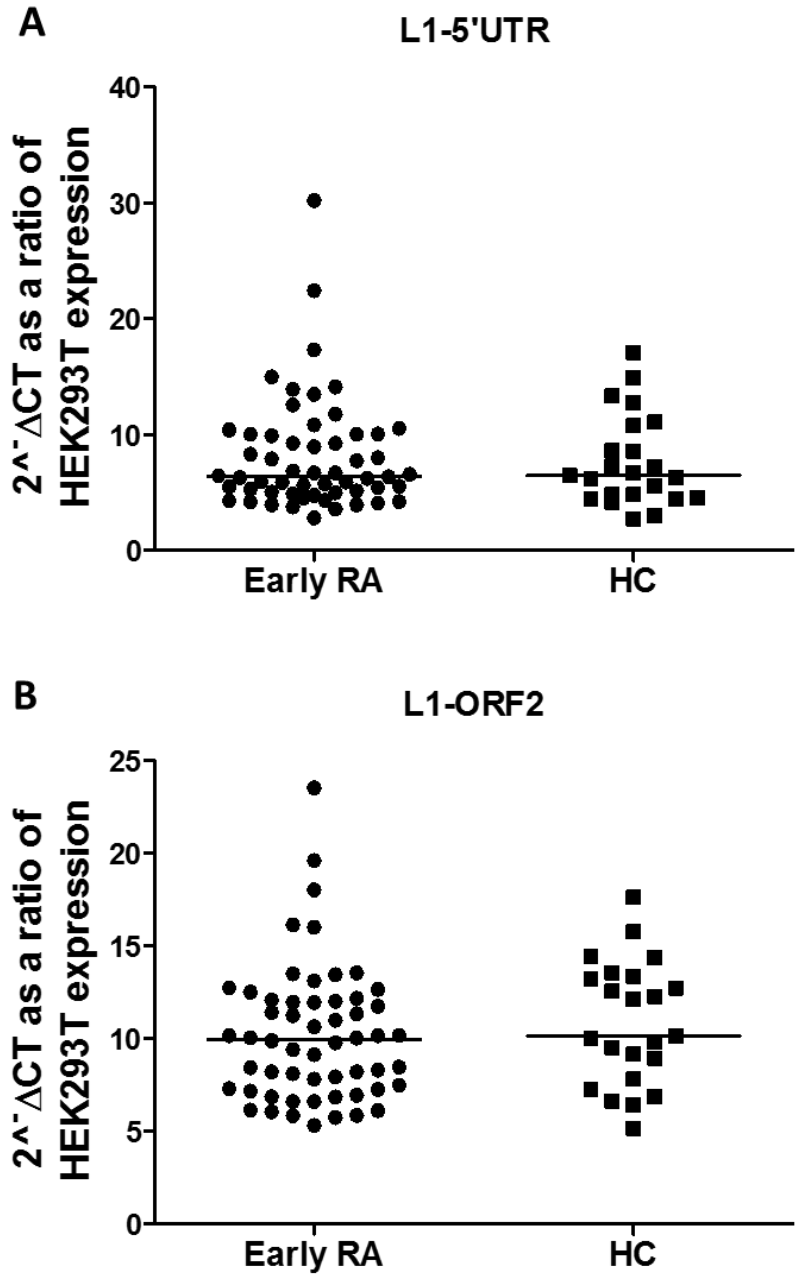


Figure 6.8 Disease cohorts and whole blood LINE-1 expression

Whole blood LINE-1 expression was analysed (as described in figure 6.5) early RA patients (n=56) and healthy controls (HC, n=23). **A.** L1-5'UTR expression between cohorts **B.** L1-ORF2 expression between cohorts. Horizontal lines depict median values. Univariate generalised linear model (age and sex corrected).

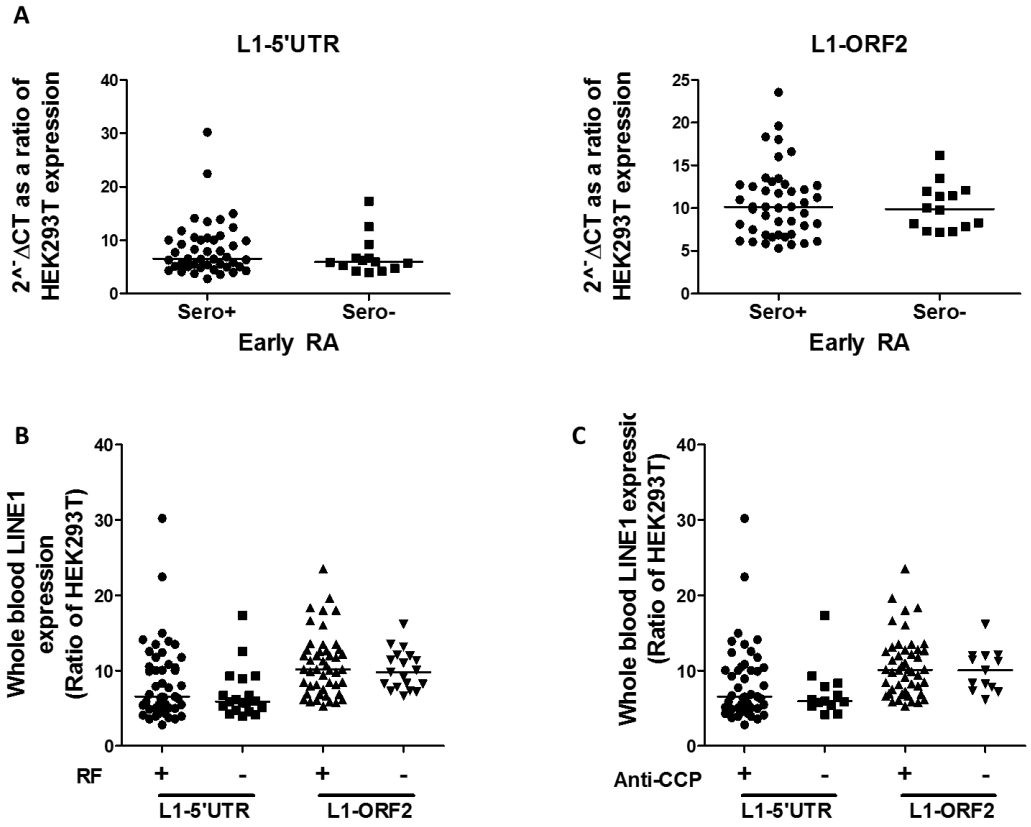


Figure 6.9 Early RA autoantibody status and whole blood LINE-1 activity

Whole blood LINE-1 expression was analysed (as described in figure 6.5) in early RA patients (n=56) and compared according to antibody status. **A** L1-5'UTR and L1-ORF2 activity between seropositive early RA (either RF+ or anti-CCP+, n=43) and seronegative early RA (both RF- and anti-CCP-, n=13). **B.** L1-5'UTR and L1-ORF2 activity in RF+ (n=37) and RF- (n=19) patients **C.** L1-5'UTR and L1-ORF2 activity in anti-CCP+ (n=43) and anti-CCP- patients (n=13). Horizontal lines depict median values. All Mann-Whitney U tests.

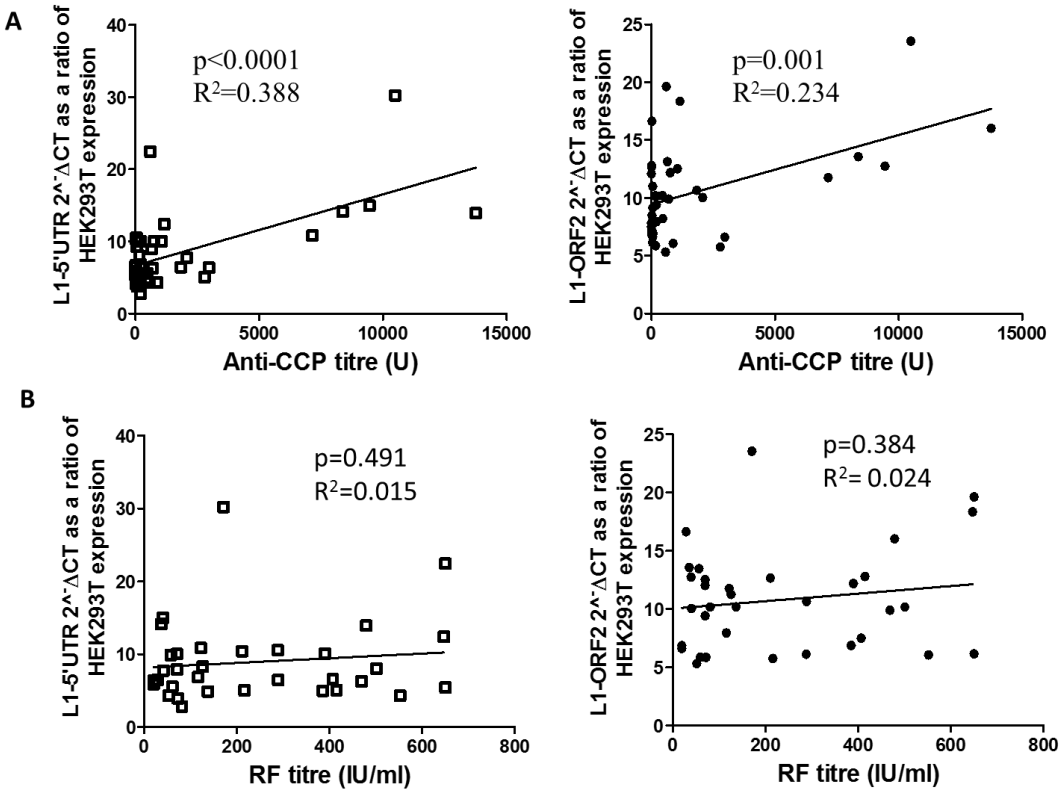


Figure 6.10 Autoantibody titres and whole blood LINE-1 activity

Whole blood LINE-1 expression was analysed (as described in figure 6.5) in early RA patients and compared according to circulating antibody titres. **A.** Plots depict linear regression between early RA patient (n=39) anti-CCP titres and whole blood L1-5'UTR and L1-ORF2 expression. **B.** Plots depict linear regression between early RA patient (n=34) RF titres and whole blood L1-5'UTR and L1-ORF2 expression. Linear regression for all.

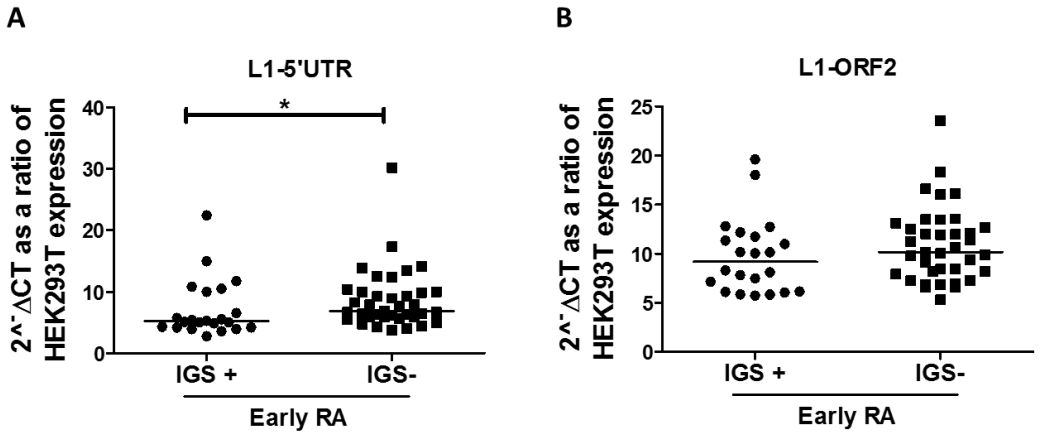


Figure 6.11 Whole blood LINE-1 expression and the IGS

Whole blood LINE-1 expression was analysed (as described in figure 6.5) in early RA patients who were divided by their IGS status; IGS + (n=21) and IGS- (n=35). **A** L1-5'UTR expression between the IGS+ and IGS- cohort. **B** L1-ORF2 expression between the IGS+ and IGS- cohort. Horizontal lines depict median values. Mann-Whitney U tests. *$p<0.05$

6.3.4 Early RA retrotransposon transcriptomics: Technical validation

I wished to examine retrotransposon expression in individual immune cell subsets rather than in whole blood. Rather than looking only at LINE-1 elements (L1-5'UTR and L1-ORF2) as had been done in whole blood, Alu sequences (AluYa5 and AluYb9) as well as an ERV (LTR5) were also examined. Early RA patients and healthy controls had 6 immune cell subsets cell sorted by flow cytometry; pDCS, mDCs, B cells, $CD4^+$ and $CD8^+$ T cells and $CD14^+$ monocytes. The sorting method and cohorts used for this for this analysis are the same as described in Chapter 5. In summary, 4 age and sex matched healthy controls were compared with 8 early RA patients who were further split into IGS+ and IGS-. The full demographics for this cohort are shown in Chapter 5, table 5.1.

The lack of transcription in NanoString nCounter technology means that the sample is not heated to a level that could cause genomic DNA to denature and cause contamination; thus avoiding gDNA hybridization to oligonucleotide probes and potential background noise. However despite this I wished to optimize gDNA removal from the RNA to prevent findings being compromised.

Healthy control CD14+ monocytes and CD8+ T cells were sorted by flow cytometry and split into two equal fractions. RNA was isolated either without or with the incorporation of a gDNA spin column to remove any residual contaminating gDNA. To validate that gDNA removal step was effective a PCR was performed on the RNA product using primers for the *HBP1* gene, which would only amplify from a gDNA template, not RNA. The subsequent products were run on a gel (figure 6.12). Any contaminating gDNA would produce a PCR product at 1011bp. As well as sorted immune cell subsets with/without use of the gDNA spin column, positive controls of gDNA as well as a standard curve of decreasing amounts of gDNA were used. After use of the gDNA spin columns there was <0.05ng of contaminating gDNA in the RNA preparation. Therefore, use of a gDNA spin column enables sufficient elimination of gDNA for subsequent downstream assays.

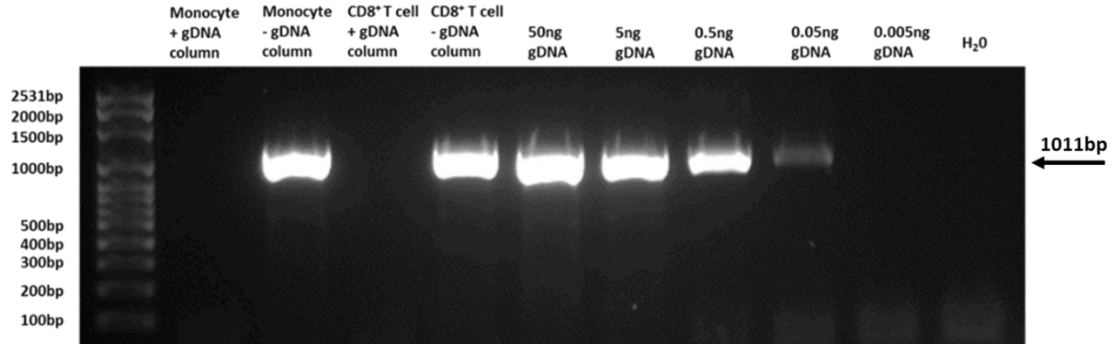


Figure 6.12 Successful gDNA removal from cell subset RNA to less than 0.05ng using gDNA spin columns

$CD14^+$ monocytes and $CD8^+$ T cells from healthy controls were sorted using flow cytometry. The RNA was extracted with or without a genomic DNA (gDNA) eliminator spin column. A PCR was then performed with primers designed to only amplify gDNA (*HBPI gene*, band size 1011bp). The PCR products underwent agarose gel (1%) electrophoresis along with a reducing titration of gDNA (positive control) and water (negative control). This gel is pictured.

6.3.5 Early RA retrotransposon transcriptomics: Retroelement activity in early RA

Retroelement expression was examined in pDCs and B cells from early RA patients and healthy controls. These subsets were chosen in view of the type 1 interferon producing potential of pDCs and the potential association between retroelement and autoantibodies (B cells). In pDCs there was significant reduction in all the classes of retroelement (SINE, LINE-1 and ERV) activity in the early RA cohort compared to healthy controls (figure 6.13). This pattern was not seen in B cells where there was instead comparable expression between the two cohorts for all retroelement subsets (figure 6.14).

These data demonstrate that in RA retroelement expression may differ from healthy controls in only certain immune cell subsets. I therefore questioned if the extent of retroelement activity was in fact distinct across multiple immune cell subsets in early RA and so compared retroelement expression in pDCs, mDCs, B cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD14^+$ monocytes from early RA patients. There were marked differences in all immune cell subsets when comparing SINE, LINE-1 and ERV activity (figures 6.15-6.17). For all retroelements there was reduced expression in pDCs, mDCs and $CD14^+$ monocytes but increased expression both T cell subsets and B cells. For Alu and LTR5 retroelements sequences the median expression in B cells tended to be the highest, but this was only significantly so when examining LTR5 expression (figure 6.17.B). When the RA patients were grouped by IGS status the same pattern was seen with no additional differences between IGS- and IGS+ patients (data not shown). A similar pattern was also noted when examining healthy controls in isolation (data not shown).

I next questioned what could be precipitating the difference in retroelement expression between immune cell subsets. Although, retroelement activity can induce type 1 interferons, there is a suggestion that type 1 interferons inhibit retroelement propagation (410). Sensitivity to type 1 interferons can be measured by IFNAR expression (248), therefore expression of *IFNAR1* and *IFNAR2* was compared across the immune cell subsets in healthy controls (figure 6.18). Given the significant difference in the IGS between healthy controls and RA patients (table 5.1) healthy controls were chosen to minimise any confounding change in expression that may have occurred following exposure to type 1 interferons. I found *IFNAR1* expression was significantly higher in pDCs and B cells than T cells and borderline significantly higher than $CD14^+$ monocytes. *IFNAR2* expression was significantly highest in pDCs. Taken together this suggests that pDC may have intrinsically increased sensitivity to type 1 interferons. Finally, as pDCs in healthy controls demonstrate the highest level of interferon receptor expression (both

IFNAR1 and *IFNAR2*) I sought to compare expression of both receptor subunits between RA patients and healthy controls. *IFNAR1* expression in pDCs was significantly reduced in early RA but this pattern was not repeated with *IFNAR2* expression (figure 6.19).

Finally in view of the earlier association between anti-CCP titres and whole blood LINE-1 activity associations between B cell retroelement activity and autoantibody titres were examined. However, there were no significant associations seen for any of the retroelements, including LINE-1 (data not shown).

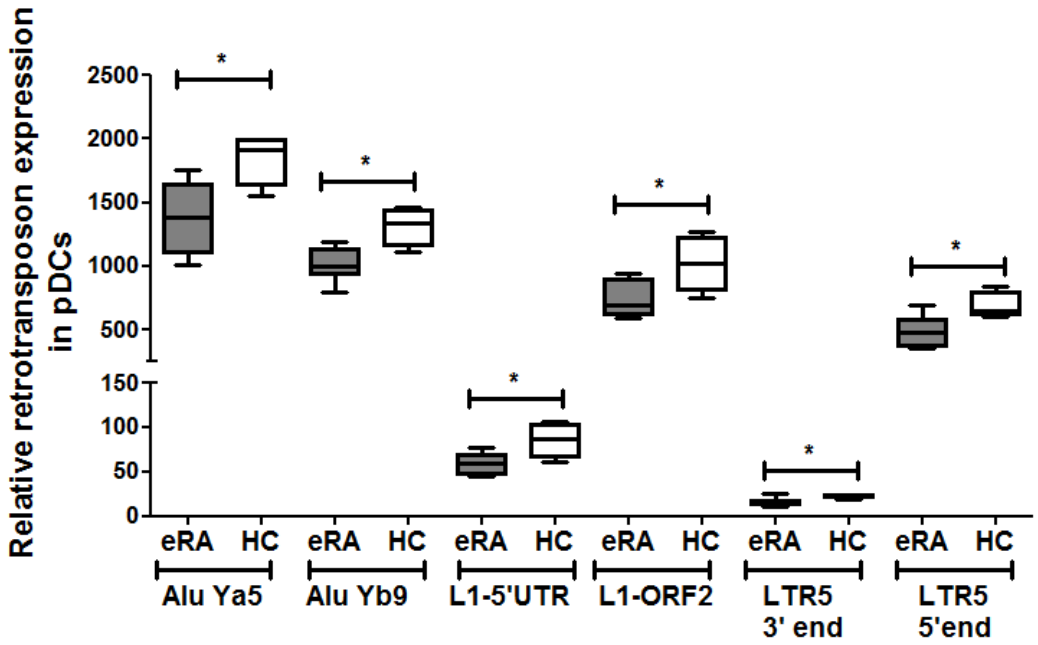


Figure 6.13 Retroelement expression in pDCs from healthy controls and early RA patients

Retroelement expression (SINE: Alu Ya5, Alu9b9; LINE-1: L1-5'UTR, L1-ORF2; ERV: LTR5) was examined using NanoString nCounter technology in flow cytometry sorted pDCs from early RA patients (eRA, n=8) and healthy controls (HC, n=4). Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Mann-Whitney U tests. *$p<0.05$

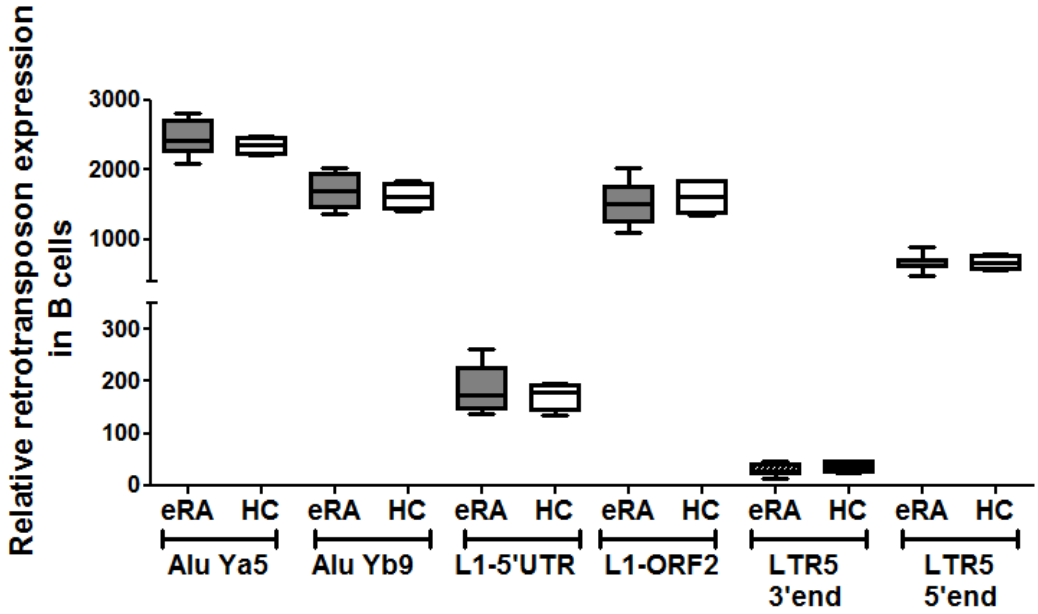


Figure 6.14 Retrotransposon expression in B cells from healthy controls and early RA patients

Retroelement expression (SINE: Alu Ya5, Alu9b9; LINE-1: L1-5'UTR, L1-ORF2; ERV: LTR5) was examined using NanoString nCounter technology in flow cytometry sorted B cells from early RA patients (eRA, n=8) and healthy controls (HC, n=4). Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range.Mann-Whitney U tests.

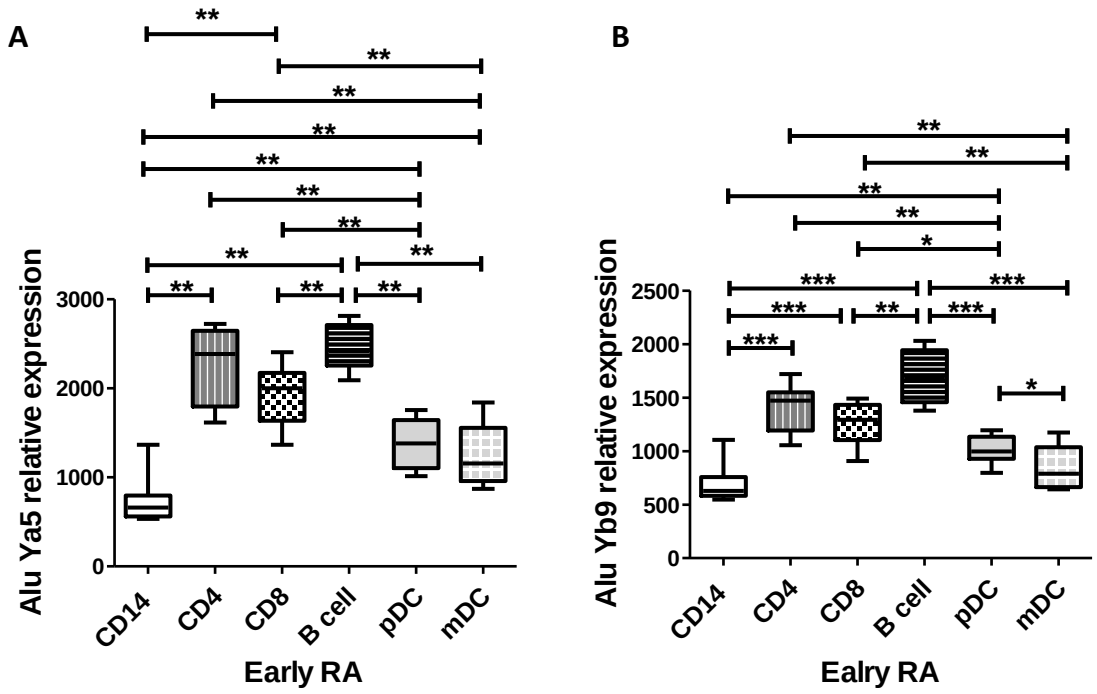


Figure 6.15 Alu expression in different immune cell subsets in early RA

Early RA patients (n=8) had their peripheral blood immune cells (plasmacytoid dendritic cells, pDCs; myeloid dendritic cells, mDCs, B cells, $CD4^+$ T cells, CD4; $CD8^+$ T cells, CD8; $CD14^+$ monocytes, CD14) flow cytometry cell sorted and cellular RNA was analysed using NanoString nCounter technology for SINE activity: **A:** AluYa5 and **B:** AluYb9. Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Kruskal-Wallis tests. *p<0.05, ** p<0.01, ***p<0.001.

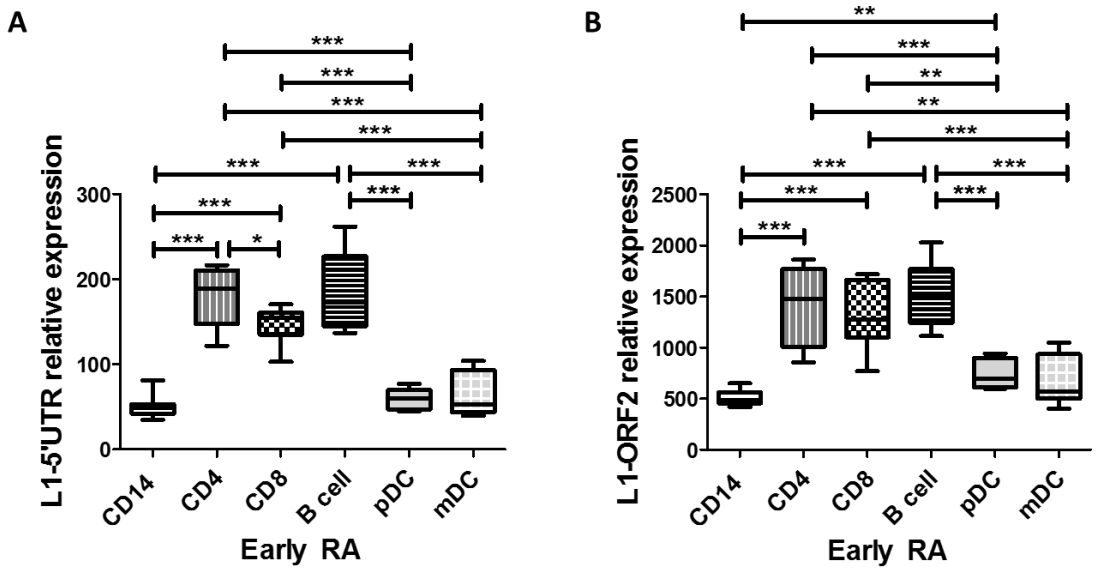


Figure 6.16 LINE-1 expression in different immune cell subsets in early RA

Early RA patients (n=8) had their peripheral blood immune cells (plasmacytoid dendritic cells, pDCs; myeloid dendritic cells, mDCs, B cells, $CD4^+$ T cells, CD4; $CD8^+$ T cells, CD8; $CD14^+$ monocytes, CD14) flow cytometry cell sorted and cellular RNA was analysed using NanoString nCounter technology for LINE-1 activity: **A** L1-5'UTR and **B** L1-ORF2. Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Kruskal-Wallis test. *$p<0.05$, ** $p<0.01$, ***$p<0.001$.

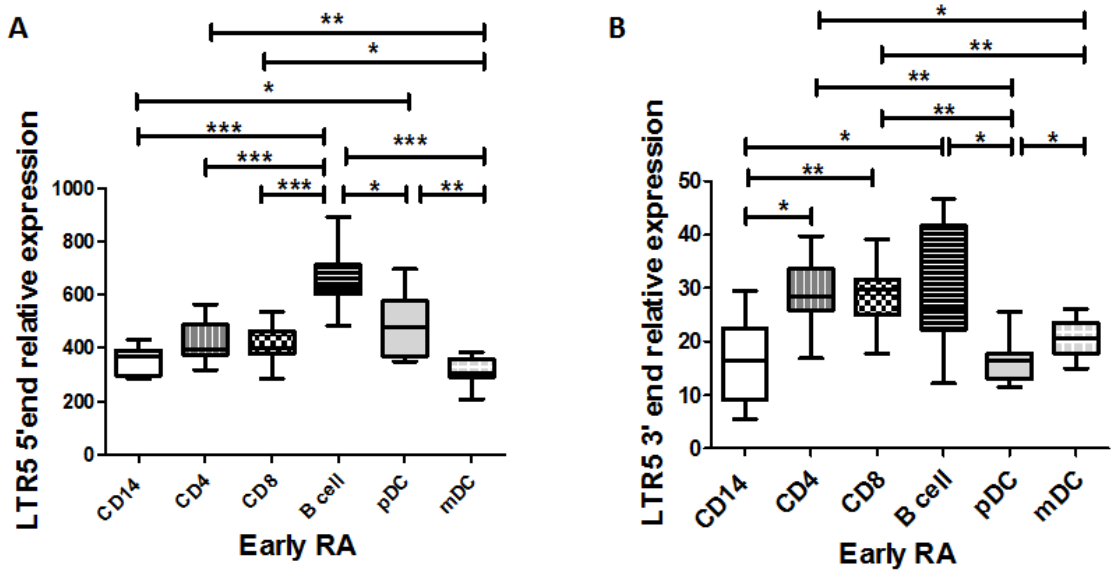


Figure 6.17 LTR5 expression in different immune cell subsets in early RA

Early RA patients (n=8) had their peripheral blood immune cells (plasmacytoid dendritic cells, pDCs; myeloid dendritic cells, mDCs, B cells, $CD4^+$ T cells, CD4; $CD8^+$ T cells, CD8; $CD14^+$ monocytes, CD14) flow cytometry cell sorted and cellular RNA was analysed using NanoString nCounter technology for ERV LTR5 activity: **A:** LTR5 5'end and **B:** LTR5 3'end . Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Kruskal-Wallis test. *p<0.05, ** p<0.01, ***p<0.001.

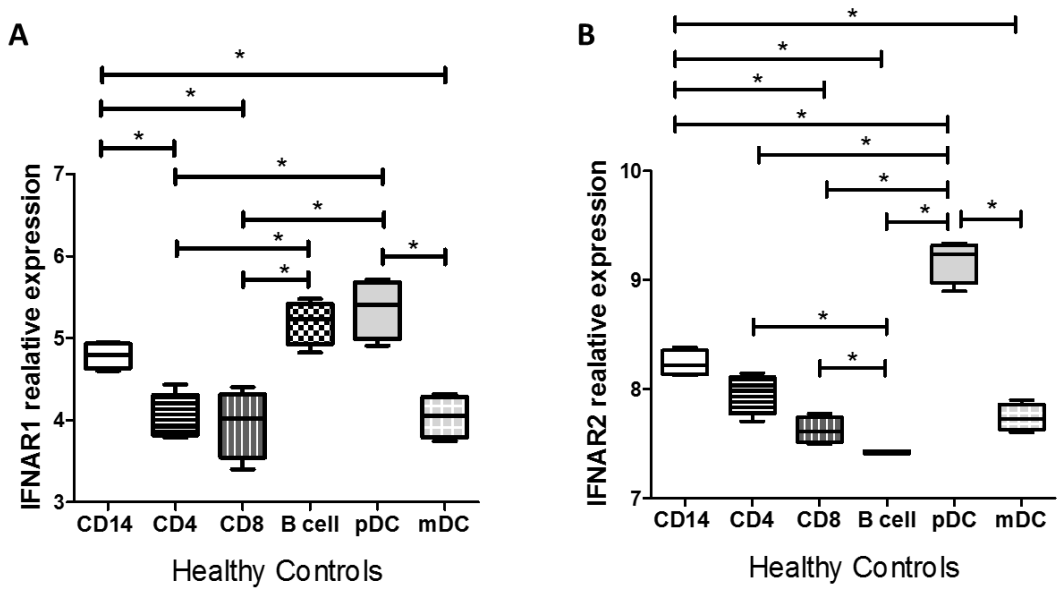


Figure 6.18 *IFNAR1* and *IFNAR2* expression in immune cell subsets from healthy controls

Healthy controls (n=4) had their peripheral blood immune cells (plasmacytoid dendritic cells, pDCs; myeloid dendritic cells, mDCs, B cells, $CD4^+$ T cells, CD4; $CD8^+$ T cells, CD8; $CD14^+$ monocytes, CD14) flow cytometry cell sorted and cellular RNA was examined using NanoString nCounter technology for **A:** *IFNAR1* and **B:** *IFNAR2* expression. Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Kruskal-Wallis test. *$p<0.05$

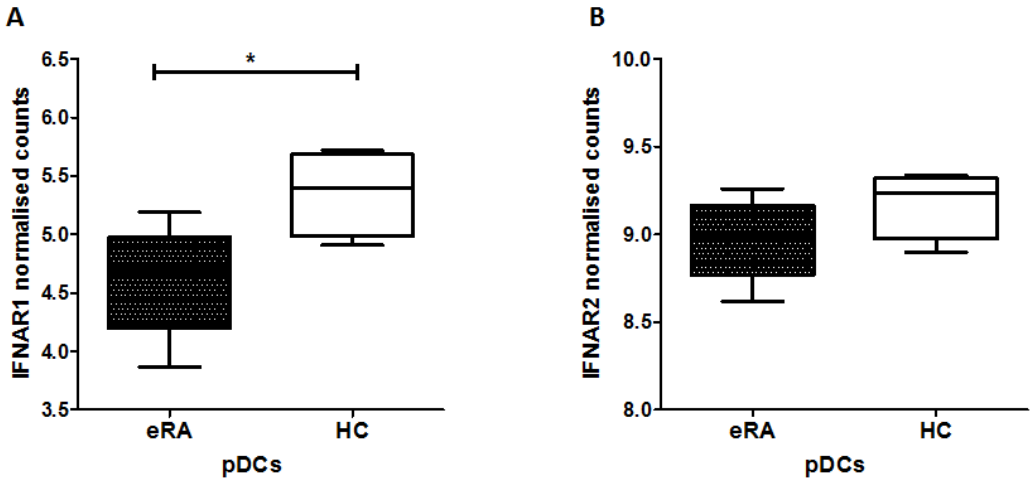


Figure 6.19 *IFNAR1* and *IFNAR2* expression in early RA pDCs

Healthy controls (n=4) and early RA patients (n=8) had peripheral blood pDCs flow cytometry cell sorted and the respective cellular RNA examined, using NanoString nCounter technology, for **A:** *IFNAR1* and **B:** *IFNAR2* expression. Data are presented as box and whisker plots, in which the horizontal line represent the median value, the box represents upper and lower quartiles and the error bars represent range. Mann-Whitney U tests. *$p<0.05$

6.3.6 Early RA retrotransposon Transcriptomics: Retroelement expression and signalling pathways

I had concurrently performed an extensive gene expression analysis on the immune cell subsets used for retroelement analysis (See Chapter 5). To compare retroelement activity and cellular type 1 interferon production I compared concurrent *IFNA2* and *IFNA1/*13 expression and retroelement activity in all immune cell subsets. There was no significant association between *IFNA2* and *IFNA13* and any of the retroelements for either pDCs or the other immune cell subsets examined (data not shown).

I next compared the expression of all genes (n=593) analysed in the NanoString nCounter with each of the retroelements in all the immune cell populations. This analysis was corrected for FDR, i.e. multiple test corrected, with significance when the FDR adjusted p value was <0.05. Only 2 significant associations were found. In pDCs there was a highly significant positive association between *MAVS* expression and LTR5 expression (figure 6.20.A). There was also a positive association between *ILF3* and L1-5'UTR expression in $CD4^+$ T cells. In view of L1-5'UTR and L1-ORF2 activity being connected I sought to see what the association was between *ILF3* and L1-ORF2. There was a similar pattern seen for *ILF3* and L1-ORF2 expression, but upon FDR adjustment the p value was far from significant (figure 6.20.B).

There were no significant associations with any of the other pathways linked to nucleic acid or retroelement sensing, retrotransposition or degradation (e.g. *Trex1, RNaseH2, STING, cGAS, RIG-1, MDA5, SAMHD1, TLR3, TLR7-9*) in any of the immune cell subsets (data not shown).

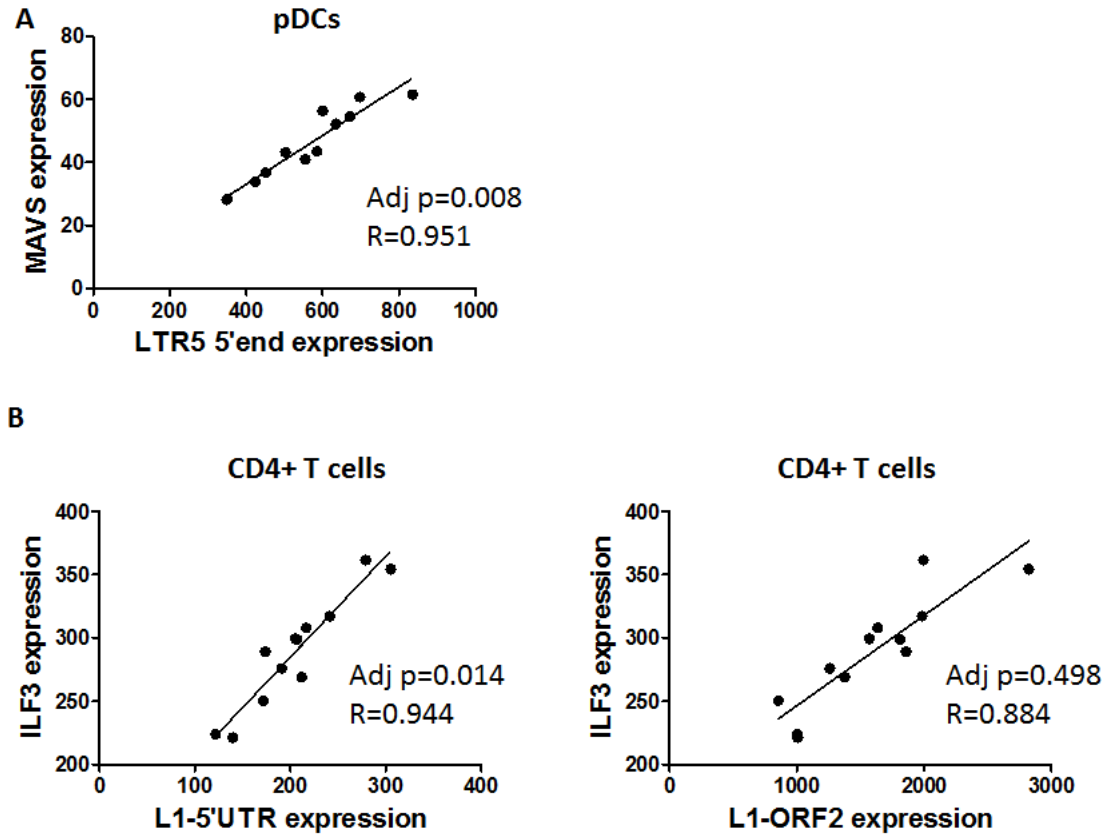


Figure 6.20 Retroelement activity is screened for any associations with other genes: associations noted for pDCs and CD4$^+$ T cells

In the whole cohort (both healthy controls and early RA, n=12) retroelement expression in flow sorted peripheral blood subsets was screened for any associations with other genes (n=593) examined during the NanoString nCounter analysis **A:** pDC LTR5 (5'end) expression significantly associates with MAVS expression. **B:** CD4$^+$ T cell ILF3 expression significantly associates with L1-5'UTR expression but not L1-ORF2. Pearson's Correlation tests.

6.4 Discussion

I examined for the first time retroelement activity in both peripheral whole blood cells and isolated peripheral blood immune cell subsets of early RA patients. There was no difference in whole blood LINE-1 activity (mRNA transcription) between early RA patients and healthy controls. In addition, there was no association between retroelement expression and biochemical markers of disease activity. However, whole blood LINE-1 activity positively associated with anti-CCP titres in RA patients. Interestingly, this trend was not reproduced when examining B cell specific retroelement activity. Unexpectedly IGS+ RA patients had reduced whole blood LINE-1 activity compared to IGS- RA patients. Furthermore, early RA pDCs had consistently reduced retroelement expression when compared with healthy controls, although there was no difference between the IGS+ and IGS- cohorts. Early RA retroelement expression varied between immune cell subsets with increased expression in T cells and B cells and reduced expression in DCs and monocytes. Finally there was a clear positive association between pDC LTR5 and MAVS expression as well as between $CD4^+$ T cell L1-5'UTR and ILF3 expression. There were however no other associations between retroelement activity and pathways related to their sensing or degradation.

When examining age and gender in the cohort there was no association between whole blood LINE-1 activity and these parameters in the RA cohort, however healthy controls females had increased LINE-1 expression (figure 6.6-6.7). There are several potential explanations; MECP2 is encoded on the X chromosome and its product binds the LINE-1 promoter (411); the testis-determining factor gene SRY can also modulate LINE-1 promoter activity (412) and Piwi proteins, expressed in the ovary, can regulate LINE-1 activity (413). Why this effect was not seen in the early RA cohort is unclear. Hypothetically regulatory mechanisms are potentially deranged in early RA allowing for relatively higher expression in men, or reduced expression in women. Retroelement activity has been shown to increase with age (414), however effect was not seen in either of my cohorts. This could have been due to insufficient power.

There was comparable whole blood LINE-1 activity in both the early RA and healthy control cohorts (figure 6.8). Notably most published literature has focused on increased retroelement expression in RA synovium and SLE or pSS disease affected tissue (217, 231, 237, 239, 240, 415). Given the anatomical origin of RA pathology is predominantly synovial, it is possible that peripheral blood was the wrong place to identify pathological associations. Nevertheless I did detect a positive association between anti-CCP titres and

whole blood LINE-1 activity (figure 6.10). Retroelements may be involved in generation of autoantibodies from either associating with self-peptides, molecular mimicry or by producing a *de novo* antigen (216, 217, 232, 416). However another possible explanation involves the contribution of smoking to both retroelement activity and anti-CCP antibodies. Smoking causes hypomethylation of LINE-1 thereby promoting its transcription (408) but I did not detect any significant difference in LINE-1 activity based on smoking history. Furthermore I did not see any impact of smoking status and anti-CCP antibodies. This was unexpected as their generation has been extensively and robustly linked to smoking (417). However this latter effect is predominantly in those who carry the shared epitope (409, 417) which I did not examine. Furthermore the association with anti-CCP antibodies was identified using large RA population studies, whereas my cohort size is relatively small. Therefore although LINE-1 hypomethylation is unlikely to depend on the underlying genotype, this experiment was unlikely to pick up similar effects with LINE-1 due to comparatively lack of power. Moreover the cohort was divided into "currently smoking" and "never smoked" so nuances related to duration or heaviness of smoking history would have been missed. It is therefore possible that the association between anti-CCP titres and LINE-1 elements is still confounded by smoking but a larger study is required to examine this more robustly.

One of my main questions was if retroelements were driving the IGS in early RA. However despite the ability of retroelements to trigger type 1 interferons, there was reduced whole blood LINE-1 activity (L1-5'UTR) in IGS+ early RA patients (figure 6.11). This is counterintuitive, but while retroelements trigger type 1 interferon production, type 1 interferons themselves can trigger negative feedback mechanisms which inhibit further retrotransposon replication (410). This may explain why IGS+ RA patients have a low retroelement expression/activity despite having a high type 1 interferon response. Interestingly, however when examining the individual immune cell subsets there was varied modulation of retroelement activity in pDCs and B cells (figures 6.13-6.14). In early RA pDCs displayed reduced expression of SINEs, LINE-1 and ERVs but this was not seen in B cells. One explanation may be the relative sensitivity of each cell subset to type 1 interferon exposure. As mentioned type 1 interferon signalling can negatively affect LINE-1 propagation (410), and I show that pDCs have the highest expression of both IFNAR1 and IFNAR2 (figure 6.18). IFNAR1 and IFNAR2 expression have been proposed as a surrogate marker of interferon-α sensitivity (248). Upon ligation IFNAR1 is downregulated (418), and I further show reduced pDC *IFNAR1* expression in

the early RA cohort as compared to healthy controls (figure 6.19) suggesting increased type 1 interferon signalling occurs in early RA. Overall and contrary to expectations, these data suggest that in early RA retroelements are negatively regulated by interferon-α exposure and pDCs are most sensitive to this effect due to their high sensitivity to type 1 interferons. Since pDCs are also primary producers of type 1 interferons in a viral setting (43), having these cells modulated first would make evolutionally and biological sense. As to why I do not detect a corresponding fall in *IFNAR2* expression may be explained by the variability in affinity of different interferon-α proteins for the interferon receptor subunits (419).

Additional differences were observed when comparing retroelement activity across immune cell subsets in early RA. Expression was higher in B cells and T cells and lower in DCs and monocytes (figures 6.15-figure 6.17). Although sensitivity to type 1 interferons may contribute to this effect, there is known to be variation in retroelement expression in healthy tissues depending on the spectrum of retroelements supported by individual cell types (420). One reason for this differential cell expression is methylation. This is an important factor controlling retroelement activity where hypomethylation causes increased transcription and activity (421, 422). Human peripheral blood subsets have different DNA methylation status (423). Furthermore when compared with T cells, B cell LINE-1 methylation was increased in both established RA and healthy control patients (424). Methylation is an epigenetic modification and these phenomena have been of increasing interest with regards to RA disease susceptibility and phenotype (425). Indeed chronic exposure to inflammatory cytokines, such as IL-1, can inhibit DNA methyltransferase enzymes, which maintain methylation, potentially resulting in hypomethylation (426). It is interesting therefore that the cell subsets (T cells and B cells) with the most retroelement activity, and potential hypomethylation, are known to be highly pathogenic in RA.

I also saw significantly increased LTR5 (an ERV) expression in B cells when compared with all other subsets. In mouse models T cell independent antigen immunisation causes upregulation of ERVs in antigen-specific B cells (427). Potentially a similar phenomenon may have occurred in early RA causing increased LTR5 in B cells. LTR5 expression denotes HERV-K (HML-2) activity (428) and some of the most compelling evidence of the involvement of retroelements in RA has been regarding this ERV; a viral load is detected in plasma samples from RA patients, with higher levels observed for those with active disease (241). However in relation to specific B cell function and autoantibodies

there was also upregulation of HERV-K mRNA, anti-HERV-K antibodies and *gag* protein in RA patient PBMCs compared with healthy controls. Furthermore of these viral proteins shared the same peptide sequences with rheumatoid factor epitopes on IgG1Fc (216, 241, 242). My small cohort size would undoubtedly have limited the subsequent power of any downstream associations between B cell retroelement activity and autoantibody titres. Nonetheless the B cell specific increase of an ERV known to be associated with antibodies in RA is intriguing.

Retroelements use both RNA and DNA intermediates for retrotransposition, and these can both trigger the production of type 1 interferons. The most widely known nucleic acid sensors are the TLRs. However, other cytosolic sensors are important, such as RIG-1 and MDA-5, which recognise short dsRNA and long dsRNA, respectively. DNA intermediates within the cytosol are recognised by STING. In addition, the enzyme cGAS binds immunostimulatory DNA and catalyses cyclic GMP-AMP formation, which also binds to STING. From work in the HIV field and Aciardi-Goutieres syndrome (AGS), additional sensing pathways in the detection and degradation of retrotransposition intermediates have been identified and include Trex1, RNaseH2 and SAMHD1 (121). These, along with the above cytosolic sensing pathways, were examined here for any association with retroelement activity in the immune cell subsets. Trex1 was of particular interest as RA synovial fibroblasts have been shown to have a deficiency in Trex1, which permits a longer half-life of gene products encoded by active LINE-1 retrotransposons (240). However, there was no association with Trex1 nor any difference in its expression between the cohorts. Nonetheless there was a highly significant association between LTR5 activity and MAVS expression in pDCs (figure 6.20.A). MAVS is a mitochondrial transmembrane protein which serves as an adaptor between RIG-1/MDA5 and downstream signalling pathways. MAVS activates TBK1 and transcription factors IRF3 and IRF7 which ultimately leads to the release of type 1 interferons. This strongly suggests that ERV LTR5 activity in pDCs results in activation of pathways downstream of RIG-1 and/or MDA-5 thereby promoting type 1 interferon production. In SLE patients increased MAVS aggregation in PBMCs was associated with type 1 interferons and autoantibody production (429). In another study, although MAVS activity was not assessed, pDCs from IGS+ pSS patients, when compared with IGS- patients, had upregulated RIG-1 and MDA-5 expression. This profile is believed to in part explain the increased pathogenic type 1 interferon production detected in these patients (236). As mentioned LTR5 or HERV-K mRNA levels are increased in the circulation of established

RA patients and have been linked with disease activity (216, 241, 242). Why there was an association with MAVS and not RIG-1 or MDA-5 may relate to a cumulative increase in MAVS expression due to both these signalling pathways feeding into its expression. With increased power additional associations with these sensors could also potentially be detected. My data therefore suggests that through the signaling of cytosolic RNA sensors MDA-5/RIG-1 and subsequent downstream MAVS activation, pDC HERV-K LTR5 activity may also be involved in type 1 interferons production. However I actually saw lower pDC HERV-K expression (as detected by LTR-5) in my early RA cohort. Furthermore there was no differential expression of MDA-5, RIG-1 or MAVS when comparing pDCs from IGS+ or IGS- early RA patients (Chapter 5). This may suggest that the HERV-K upregulation reported in established RA (216, 242) is a later event driven by more established inflammatory processes (426) or an acquired resistance to the negative effects of type 1 signalling (410).

When screening across the whole cohort for any other associations between retroelements and any other genes (as examined in the nCounter, NanoString, see Appendix A 2.0 for full list of genes), I saw a significant positive association between *ILF3* and L1-5'UTR in $CD4^+$ T cells (figure 6.20.B). The *ILF3* gene can generate 2 distinct proteins depending on splicing; interleukin enhancer-binding factor 3 (Ilf3) and nuclear factor 90 (NF90). These are both double-stranded RNA-binding proteins (DRBPs) that regulate RNA metabolism including RNA recognition, RNA processing, and RNA stability (430). In HIV, an exogenous retrovirus, NF90 increased the half-life of the HIV RNA as well as promoting HIV infection, gene expression, and virus production (431). Why this is most evident in $CD4^+$ T cells is unclear, however this is the cell subset most affected by HIV and potentially this cell-specific effect may make $CD4^+$ T cells more susceptible to also harbouring endogenous retroviruses. I hypothesise that this protein may be playing a similar role in retroelement replication by stabilizing LINE-1 RNA thereby promoting the observed association.

6.5 Conclusion

This highly novel work examines retroelement activity in whole blood and in isolated peripheral blood immune cell subsets from patients with early RA as well as healthy controls. I found that there was no clear difference in whole blood retroelement activity between the RA patients and healthy controls, and that, contrary to expectation, it was actually reduced in IGS+ RA patients compared to IGS- RA patients. Furthermore pDC retroelement activity is reduced in early RA and does not appear to be a trigger for interferon-α production. I propose type 1 interferon driven negative feedback mechanisms as a potential reason for these counter intuitive observations (410). I also observed differences in retroelement expression between immune cell subsets in early RA with increased expression in T cells and B cells. Methylation status can vary between peripheral subsets (424), and this epigenetic variation can affect retroelement activity which may account for my observations. Given the increasing importance of epigenetic modifications in RA pathogenesis, this increased retroelement expression in known pathogenic cell subsets, is intriguing.

This is a pilot study and many of my findings are likely to be compromised by the small sample number. Nonetheless it hints towards intriguing differences in activity and regulation of retroelements across cell subsets which, given their ability to either influence or modulate immune function, may be relevant to RA pathology.

6.6 Future work

There was a relatively small sample number when looking at the individual immune cell subsets. Ideally I would increase the sample size (in context of a power calculation) as well as examine more disease relevant tissue, i.e. synovium. Previous caveats relating to the NanoString technology discussed in Chapter 5 remain relevant to this interpretation. In particular, despite normalisation procedures, the running of each cell subset on individual chips could introduce the possibility of batch effects.

One of the main factors regulating retroelement expression is methylation status. Therefore I would isolate DNA from each of the immune cell subsets and, using bisulphate sequencing, determine each subset's relative methylation. This could then be correlated with respective activity of the retroelements.

I observed reduced retroelement activity in patients with a positive IGS. A potential theory for this is a negative feedback mechanism which has been shown for LINE-1 elements. This has not been examined previously in SINEs (Alus) or ERVs (LTRs). A logical step would be to validate this effect by culturing either cancer cell lines expressing constitutively high levels of retroelements or primary PBMCs with interferon-α and recording any change in retroelement activity. In addition, the ERV HERV-K (LTR5) has been most strongly linked with RA with evidence of *gag* and *env* protein in the peripheral circulation. Examining potential ERV activity at the protein level as well as at the mRNA level in early RA would be beneficial. Associations with autoantibodies could then be examined more extensively.

Chapter 7: General discussion

7.1 General discussion

RA is a highly heterogeneous disease and, whilst most patients present with a symmetrical, inflammatory polyarthritis there is diversity in patterns of onset, autoantibody status, as well as responses to therapies. One example of this heterogeneity is the interferon gene signature (IGS), which is present only in a subset of RA patients (155, 184-186). It is currently unclear what are the full implications of this phenomenon but, for example, the IGS can affect response to certain biological therapies (153, 185-187, 191, 200). Pathological mechanisms in early disease are increasingly being shown to be distinct from those in established disease (6-9). I therefore wished to investigate the IGS in early RA as well as attempting to delineate the origin and triggers of type 1 interferon production, with a particular focus on plasmacytoid dendritic cells (pDCs). I used a variety of techniques to address these questions and the 4 main strands of interest to emerge from this work are discussed below.

7.1.1 The IGS as a predictor of response to initial treatment

An IGS has been identified in numerous autoimmune conditions. It is most extensively studied in SLE, where it is present in 50-70% of patients and associates with disease activity and certain presentations, such as lupus nephritis (156, 157). An IGS is also present in 20-30% of established RA patients where current evidence demonstrates no correlation with either disease phenotype or activity (153, 185-187, 191). Type 1 interferons are proposed to be important at disease onset in other autoimmune conditions (148, 265, 266, 270) and, indeed, in an ACPA positive population a positive IGS was a predictive factor for the development of RA (182, 183). Furthermore some individuals receiving interferon-α therapy for viral infections developed a symmetrical inflammatory polyarthritis consistent with RA (180). Certain therapies, such as glucocorticoids, can modulate the IGS which could confound analyses in established RA (23). I therefore examined the prevalence of the IGS, as well as any effects on disease activity or therapeutic response, in early drug naive RA.

I found the IGS was significantly higher in early RA than established RA. Corroborating this was a significant fall in the IGS from baseline when examining a longitudinal cohort of early RA patients. This fall was sustained at up to 12 months independently of any glucocorticoids administration. This supports the observation that patients with a longer duration of RA had a lower IGS (191) and, as seen in other autoimmune diseases (148, 265, 266, 270), emphasises the importance of type1 I interferon signalling early in RA. Uniquely in this early drug naïve cohort I also demonstrated an association between the

baseline IGS and disease activity (DAS-28) both at baseline as well as 6 months after initiation of treatment. Furthermore the baseline IGS had a strong predictive effect on 6 month clinical outcomes whereby the higher the baseline IGS the poorer the outcome. These outcomes included DAS-28, number of additional glucocorticoid injections required and EULAR response. Importantly the IGS outperformed current conventional clinical markers of disease activity; thus baseline CRP, ESR or indeed DAS-28 did not predict these outcomes. An IGS has been shown to predict poorer response to certain biological therapies in established RA, most particularly rituximab (23, 186, 200-202), however it has never previously been shown to associate with disease activity or predict response to DMARDs.

I was particularly interested to see whether hydroxychloroquine (HCQ) could modulate the IGS associations, because patients who developed a RA phenotype following exogenous interferon-α administration responded well to HCQ (180). HCQ reduces the number of circulating immune complexes in RA (432) and, in SLE, RNA/DNA containing immune complexes induce interferon-α production from pDCs (147, 149-151, 433). Furthermore HCQ modulates endosomal acidification thereby modifying TLR ligand induction of type 1 interferons (280). The association between IGS and poor outcome was weaker when patients were also taking HCQ but the study was not adequately powered to draw definite conclusions. The effect of the IGS on the apparent poor response to glucocorticoids was also interesting. In SLE pDCs become relatively resistant to the effect of glucocorticoids due to concurrent TLR7 and TLR9 signalling mediated by immune complexes (276). A similar process may occur in early RA although this remains to be elucidated. The main findings for this section are summarised in figure 7.1

These data proposes that the type 1 interferon axis is more important than hitherto appreciated in RA pathophysiology. This in turn suggests therapeutic potential in modulating type 1 interferon production and signalling in early RA treatment. Prompt treatment of early RA leads to better long term outcomes (11, 352, 353), thus modulating the type 1 interferon axis in early RA could be a valid therapeutic goal. Type 1 interferon targeting therapies are being developed for SLE, the most successful of which is the IFNAR1 blocker, anifrolumab, which intriguingly also improved inflammatory joint pain symptoms (161, 162). Furthermore JAK1/3 inhibitors, recently licenced for the treatment of RA in some jurisdictions, inhibit type 1 interferon signalling (309) and, as mentioned previously, HCQ could also modulate the IGS. There is therefore a range of available

approaches for the modulation of an IGS in early RA. Their application could allow for better management of patients in the crucial early stages of disease and provide not only a biomarker for response to treatment, but also a more personalised approach to management.

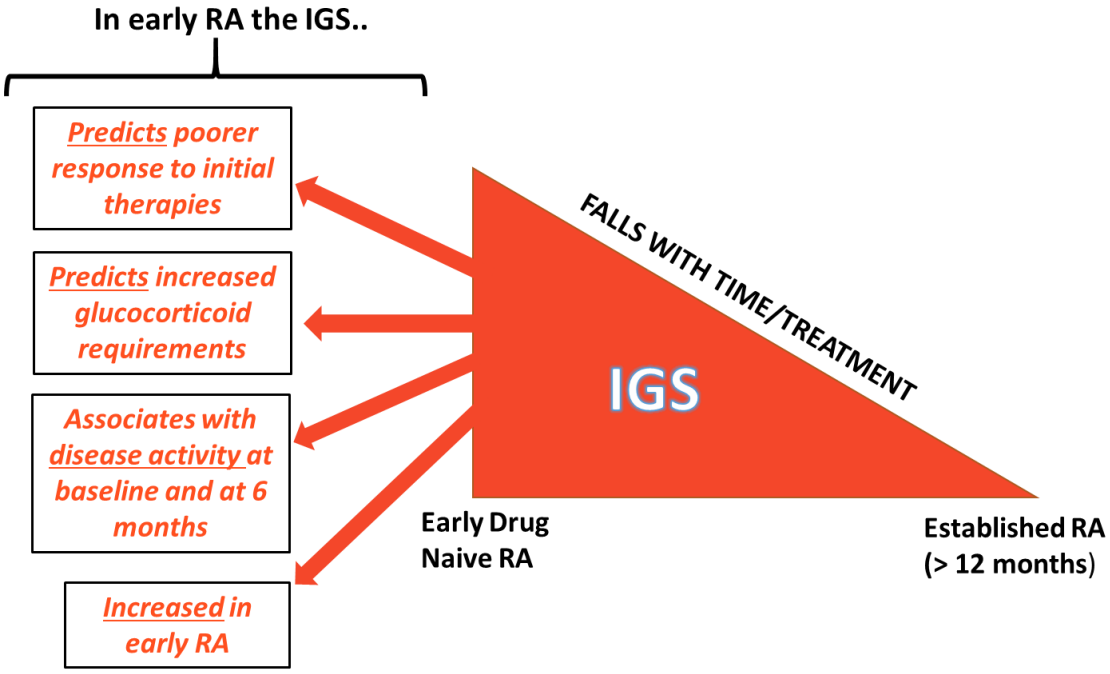


Figure 7.1 Summary of findings relating to the IGS prevalence in early RA as well as its predictive effect on disease severity and response to initial therapies

The interferon gene signature (IGS) in early RA falls with the initiation of treatment, however the baseline IGS predicts clinical outcome measures at up to 6 months.

7.1.2 The IGS and autoantibodies

Extensive evidence confirms the permissive effects of type 1 interferons on B cell activation, class switch and antibody production (139-142, 145, 296). I therefore sought associations between a raised IGS and autoantibodies in early RA. I observed a clear association between the IGS and RF titres, but not anti-CCP titres. However my data could not clearly determine if the IGS was a primary event or a secondary phenomenon, for example, RF containing immune complexes interacting with pDCs (147, 149-151, 433). Nonetheless B cells from RA patients stimulated with interferon-α *in vitro* produce IgM RF (290) and RF developed *de novo* in 34% of individuals given interferon-α therapy, whereas anti-CCP antibodies were much rarer (180). RF is predominantly a non-switched IgM antibody against self-IgG and some have proposed that, contrary to anti-CCP generation, RF is generated following TLR signaling in a T cell independent manner

(291). Like pDCs, B cells express TLR 7 and 9 (292) so it is conceivable that triggers for pDC interferon-α production could also trigger RF generation in parallel. Additionally B cells from IGS+ early RA patients had increased *TACI* gene expression. TACI is a B cell surface receptor which is important in T cell independent B cell activation and causes increased TLR7 expression upon ligation (434, 435). I also demonstrated a positive association between circulating BAFF/BLyS and RF but not anti-CCP titres. BAFF/BLyS binds to TACI (as well as to other receptors) and, in doing so, promotes B cell activation, immunoglobulin production and survival of autoreactive B cells (297, 298, 380, 381). I hypothesise that, in the IGS+ cohort, increased TACI results in increased sensitivity to BAFF/BLyS as well as promoting T cell independent B cell activation. This could culminate in RF production, thus providing a mechanistic link for my observations associating the IGS with RF titres. The differential association between the IGS, and RF and anti-CCP autoantibodies development was corroborated when examining the B cell transcriptome of IGS+/- early RA patients. *PECAM-1* was reduced in the IGS+ cohort and mouse knockouts of this gene had activated and highly proliferative IgM producing B cells (386, 387). This is the same immunoglobulin class as RF (when detected by conventional assays) whereas anti-CCP antibodies are mainly IgG. Other differentially expressed genes in the IGS+ cohort included *XBP-1*, *CD27* and *CD80*, suggesting both an activated B cell phenotype as well as increased plasma cell differentiation (375-378). Plasma cells are the subset of B cells which produce antibodies.

All this evidence suggests that in early RA a positive IGS is associated with the generation of a B cell phenotype that is abnormally active and skewed towards plasma cell development, IgM production and potentially T cell independent activation. This may culminate in a pathogenic B cell phenotype, with potential implications for disease progression, as well as increased RF titres. Conversely in established RA there was no association between autoantibody status (either RF or anti-CCP) and the IGS (294). However I showed that the IGS falls with treatment, as does the proportion of IgM anti-CCP (4) which may potentially confound previously published work examining associations between the IGS and autoantibodies (294). This further emphasises the benefit of examining drug naive early RA.

This interaction in early RA between the IGS and B cells is supported by the prediction model in ACPA+ arthralgia where the use of B cell phenotype to predict development of RA was improved after including the IGS (356). My findings also support the established clinical observation that IGS+ RA patients have a poorer response to rituximab, a CD20

depleting therapy (23, 186, 200-202). Poor responders also have early expansion of $CD27^{+}$ B cells (388), as well as high numbers of $CD27^{++}$ plasmablasts pre–treatment (389). My findings of high *CD27* expression in the IGS+ cohort and a highly proliferative phenotype skewed towards development of plasmablasts, provides mechanisms whereby a positive IGS could materially influence response to rituximab. This also suggests that the response to rituximab might be optimised by targeted co-administration of type 1 interferon blocking therapies. As these are already in phase 3 trial for SLE (162), this is an intriguing possibility in the development of a personalised approach to the use of biologics in the treatment of RA.

The IGS+ cohort also had increased expression of genes normally associated with B cell malignancy, particularly multiple myeloma and diffuse large B cell lymphoma (DLBCL). In RA there is a 5 times increased risk of developing non-Hodgkin's lymphoma (NHL), and these are predominantly of a DLBCL phenotype (390, 391). This risk is highest amongst RF positive patients (436). This would correlate with my findings of a raised IGS, and thus potentially malignant phenotype, in RF positive patients. Although not as established there is also a suggested link between RA and an increased risk of developing multiple myeloma, a plasma cell malignancy (392-394). Notably numerous genes associated with plasma cell development were increased in the IGS+ cohort, as were genes more specifically associated with multiple myeloma. Conversely however interferon-α has been used as a therapy for some forms of relapsed non-Hodgkin's lymphoma (437). Nonetheless chronic exposure to type 1 interferons in an at risk population, i.e. RA, may promote different B cell phenotypes when compared with an acute short exposure. Although this is very preliminary it is nonetheless intriguing. If exposure to type 1 interferons promotes this pathological phenotype in RA, there may be scope to prevent these rare complications with interferon-α targeting therapies.

These findings are summarised in figure 7.2.

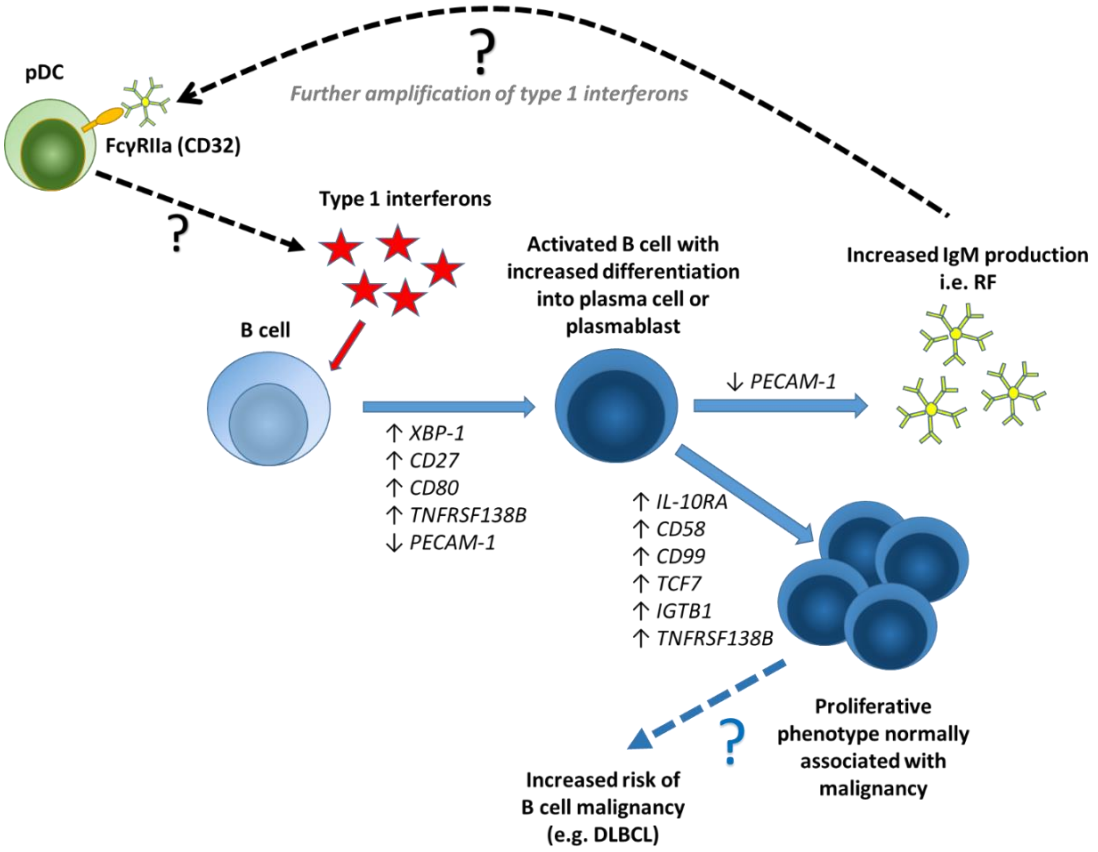


Figure 7.2 Summary schematic regarding the IGS and B cells in early RA

Type 1 interferons cause increase in B cell gene expression which is normally associated with B cell activation and differentiation into plasma cells or plasmablasts. There is also a suggestion of increased IgM production, which may promote further interferon-α production by immune complexes associating with pDCs. Furthermore genes related to malignancy are also upregulated, particularly those relating to diffuse large B cell lymphoma (DLBCL).

The "malignant" weighting of the B cell gene expression data in the IGS+ cohort is also interesting with respect to retroelement expression. In early RA there was most retroelement expression in B cells and T cells but LTR5 (HERV-K) expression was greatest in B cells. Retroelements have long been connected with malignancy (226) and there is evidence of increased ERV activity, such as HERV-K (which has also been associated with RA), in lymphoma and DLBCL (438, 439). Indeed there was a single case report of a patient with HIV and refractory DLBCL who had clinical improvement (along with immune reconstitution) upon treatment with anti-retroviral therapy which would have concurrently reduced ERV replication (440). The relevance of this observation to early RA is unclear, although there was an increase in gene expression pathways related to viral infection, and by proxy potential ERV activity, in IGS+ patients.

A potential scenario envisages retroelement activation being promoted by a concurrent and persistent infection (242), which also causes upregulation of type 1 interferons. This increased retroelement activity ultimately results in a malignant cell phenotype and, in a susceptible genetic background, potentially the development of lymphoma. Whilst this is highly speculative there is currently minimal understanding of the role of retroelements in RA and, as a potential risk factor for autoimmune-associated malignancy, they may warrant further investigation.

My data suggests that the IGS pathologically affects B cell gene expression and, potentiall, function in early RA. They raise many intriguing avenues for future research, including the application of interferon-α blocking therapies to a subset of RA patients in order to optimise current therapeutic strategies.

7.1.3 pDCs in early RA

PDCs are the primary interferon-α producing cell (43) however their role in established RA is contradictory (87, 90). I therefore attempted to dissect any potential pathological contribution in early drug naïve RA. The frequency of circulating pDCs was significantly reduced in early RA compared with healthy controls and comparable to the reduced level seen in established RA (54, 87, 316). Additionally pDC frequency was significantly reduced in seropositive early RA patients where it inversely associated with the concurrently increased CCR7 expression. CCR7 expression is increased upon pDC activation and normally facilitates lymph node migration (320, 321) but its ligand, CCL19/21, is increased in RA synovial tissue and fluid (323). PDCs are increased in the RA synovial compartment (85-88, 322) and I hypothesise they migrate there via CCR7-CCL19/21 interactions. The link with serostatus is supported by my observation that CCR7 expression positively associated with anti-CCP titres as well as published work linking synovial pDC number with anti-CCP titres (88). PDCs cluster with B cells in the RA synovium (88) and the potential for activation of B cells, including autoreactive B cells, by pDCs is increasingly appreciated; this can be via either cell-cell interactions or inflammatory cytokines, such as interferon-α (142, 324, 325, 373). Some suggest ectopic germinal centres within the synovium are partly responsible for the production of anti-CCP antibodies (105) and I hypothesise that this process could be potentiated by pDC-B cell interactions. One could envisage a potential scenario whereby pDCs are activated by anti-CCP autoantibody immune complexes, causing CCR7 upregulation (147, 149-151, 433); pDCs then migrate to the synovial compartment (via CCR7-CCL19/21) and activate autoreactive B cells within ectopic germinal centres. This promotes further anti-CCP

autoantibody generation thereby initiating a vicious cycle. This hypothesis is pictured in figure 7.3.

This process could theoretically be further potentiated by the effects of B cells on pDC function. It has been shown that the interferon-α production of pDCs is increased by proximity to B cells by both cell-cell contact as well as release of B cell derived soluble factors (326). Since interferon-α can further affect B cell function (139-142, 145, 296) a positive feedback mechanism may also occur. Thus the effect of simultaneous pDC and B cell presence in the synovial compartment, may have hitherto unappreciated effects on rheumatoid pathogenesis.

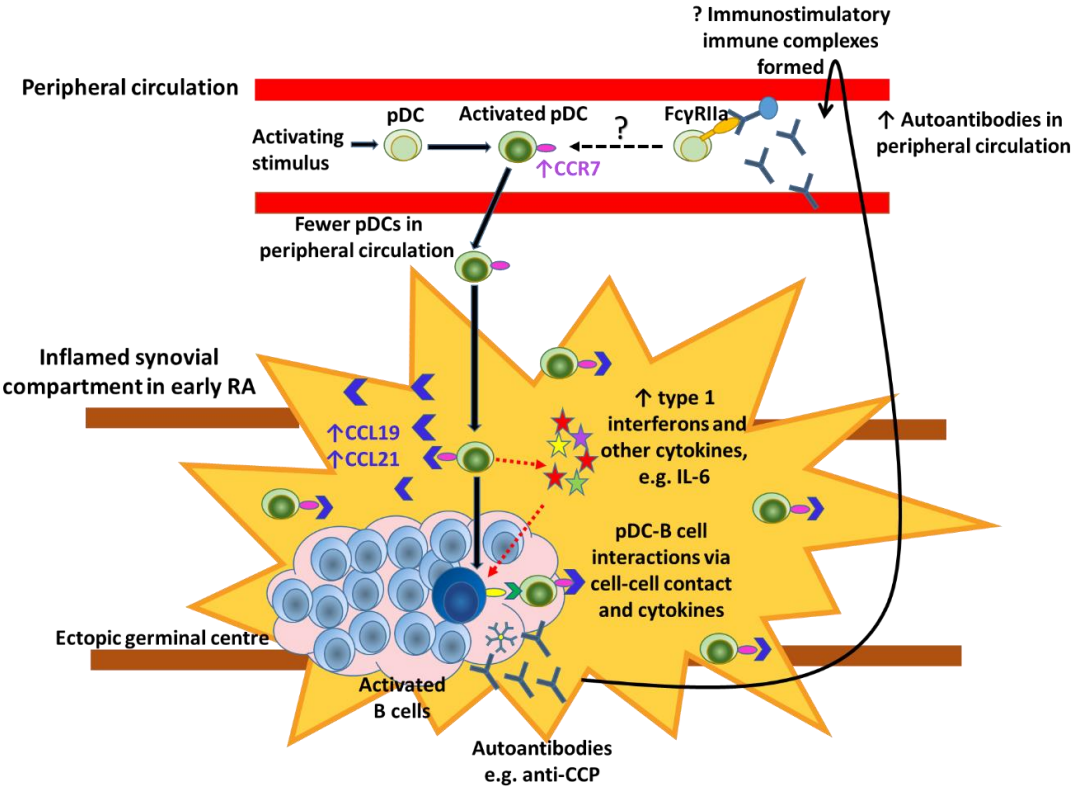


Figure 7.3 Schematic of hypothesis linking pDC number and CCR7 expression with anti-CCP autoantibody titres

Seropositive RA pDCs have increased CCR7 expression causing increased migration to CCL19/21 expressed in the RA joint. Once in the joint pDCs cluster with B cells within ectopic germinal centres and following cell-cell interactions and inflammatory cytokines production promote anti-CCP antibody production. These autoantibodies return to the peripheral circulation where they form immune complexes which activate pDCs causing further enhanced CCR7 expression, thereby promoting a positive, and potentially pathogenic, feedback mechanism.

I demonstrated that although pDC had no baseline difference in maturation status between healthy controls and early RA patients, activation markers fell with time and the initiation of therapy. This is in keeping with published evidence where pDCs have a less activated phenotype in established RA than healthy controls (317) and may suggest that pDCs have a relatively more activated, or pathogenic phenotype, in early disease. Conversely, when examining early RA drug naïve and healthy control pDC transcriptomes there was no clear upregulation of genes associated with a matured or pathogenic state in the early RA cohort. However notably these interpretations were limited by the relative paucity of human pDC literature. Contrary to expectation pDCs were not the main interferon-α producing subset in IGS+ early RA patients: all immune cell subsets had comparable interferon-α production in IGS- and IGS+ early RA patients. Consistent with this there was no detectable interferon-α in the circulation of early RA patients, similar to established RA (153, 185-187, 191), suggesting that interferon-α producing pDCs may have migrated to the lymph nodes or other inflamed tissues. Indeed there is a suggestion of increased type 1 interferon signalling within the RA synovial compartment (85, 89). This highlights the importance of microenvironment when examining pDCs; examining peripheral blood pDCs is unlikely to truly define their role in synovial pathology.

In conclusion the role of pDCs in RA is likely to be both time- and site-specific. Nonetheless my findings, and the work of others, hint of an interplay between B cells, autoantibodies and pDCs. However whether the net role of pDCs is pro-inflammatory or immunomodulatory remains to be further clarified.

7.1.4 Retrotransposons and early RA

Recently retroelement activity has been linked to autoimmunity. There are increased LINE-1 transcripts in RA synovium, lupus nephritis tissue and in the salivary glands of pSS patients (231, 237, 238, 240). ERV protein products are also increased in RA and associate with disease activity (242). Retroelements undergoing retrotransposition are essentially viral nucleic acids. They activate the immune system by triggering widely expressed cytosolic RNA/DNA sensing pathways resulting in type 1 interferon production (121). In RA there is likely to be an event which triggers the development of RA on a susceptible genetic and environmental background. As mentioned (Section 7.1.1), type 1 interferons may be relevant in the breach of tolerance (148, 182, 183, 265, 266, 270) and it is therefore possible that retroelement activity could be an initial trigger for the development of autoimmunity in RA (242, 245, 441). I therefore examined

retroelement activity in early RA peripheral blood, including individual immune cell subsets.

There was no difference in whole blood retroelement activity between early RA patients and healthy controls but unexpectedly there was reduced retroelement expression in IGS+ early RA patients. One explanation is that while retroelements can trigger type 1 interferons, negative feedback mechanisms exist whereby type 1 interferon signalling itself limits retroelement activity (410). I showed that pDCs had the highest *IFNAR1/IFNAR2* expression which would make them most sensitive to the negative feedback effects of type 1 interferon exposure. Indeed all retroelement activity (ERV, SINE and LINE) was reduced in early RA pDCs.

Anatomically RA pathology is predominantly articular and increased retroelement activity has been mostly shown in synovial tissue. I examined peripheral blood and so could have missed key differences between the cohorts by not examining the synovium. In addition my data may point to differences in modulatory feedback mechanisms between SLE and RA, despite the presence of an IGS in both diseases.

This was the first time multiple classes of retroelements were compared across immune cell subsets in the peripheral blood of early drug naïve RA patients. There was relatively increased expression in T cells and B cells and lower expression in DC subsets and monocytes. One potential explanation for this is differential methylation between the cell subsets. Methylation is an epigenetic modification and retroelement hypomethylation increases their transcriptional activity (421, 422). Epigenetic modifications are of increasing interest in RA (99, 426, 442), thus the potentially differentially methylated state of cell subsets known to be pathogenic in RA is intriguing and downstream effects may have relevance to disease.

7.2 Strengths and weaknesses

The strengths and weaknesses relating to the specific scientific approaches are summarised in table 7.1. However there are additional global considerations when considering the experimental design employed. This type of work comes under the umbrella of translational research, which aims to define human disease and unmet clinical need. This involves rigorous characterisation of patients and disease mechanisms, thereby defining potential therapeutic targets, such as the IGS. It frequently, as I have done, uses human samples to dissect pathological processes. However the early phases of this work are frequently performed *in vitro*, or using tissue which may not be relevant, such as in my case examining the peripheral blood instead of synovial tissue. Furthermore RA is highly heterogeneous and genetic risk factors are polygenic and can be subject to environmental interference. This variability is challenging requiring sufficiently sized cohorts to both discover and validate observations, which is not always possible. Finally, even if associations are identified they cannot robustly link cause and effect. Instead some argue that reductionist mouse models of disease, particularly with "humanised" characteristics, provide more tractable models to examine knockout particular pathways *in vivo*. There are considerations, however, that limit the value of mouse models for my investigations. For example, although there is interferon-α homology between mouse and man (443) there is no direct equivalent of a human pDC in mice (444). Furthermore, mice have increased basal retroelement activity when compared with humans (215) and although PRRs, such as TLRs are present in both mice and humans, the distribution, number, ligand specificity and in some cases function varies across species (445, 446). Finally an interesting aspect of this work was the link between RF and the IGS. Although some RA mouse models result in spontaneous disease with autoantibodies, the subtle relationships between RF, anti-CCP and the IGS may be lost. Similar caveats in other studies have been overcome by designing specific transgenic mouse models or using humanised mice. However in view of the above considerations the merit of these findings at this preliminary stage would be debated. Ultimately, human and murine experiments can inform one another, but generally human experimentation is required to provide the most relevant insights into human disease.

Strengths	Weaknesses
• Drug naïve early RA patients • Novel population for IGS examination • 5 IRGs used to calculate IGS • Disease controls included (SLE positive control) as well as healthy controls (negative control) • Longitudinal and cross sectional analysis of early RA patients • pDCs examined in early RA – unique • Intrinsic and extrinsic triggers of interferon-α analysed • Multiple retroelement classes examined simultaneously and across different immune cell subsets • NanoString technology allowed examination of rare subsets while avoiding any amplification bias • Rigorous QC controls in transcriptome technology	• Whole blood, not synovia examined • No validation of predictive model • Insufficient power to identify role of DMARDs on IGS prognostic effect • Low patient number in transcriptome work – potential for disproportionate skewing of data • Transcriptome only examined in seropositive patients • No validation by qRT-PCR of transcriptome findings • Comparison between cell subsets – potential batch effects • Only 2 *IFNA* subtypes examined • No MLR performed on early RA pDCs

Table 7.1 Outline of specific experimental design strengths and weaknesses

7.3 Future work

The findings discussed here are intriguing and suggest multiple avenues for further investigation. One of the first directions would be to increase participant numbers; this would help validate the IGS signal as a predictor of response. If confirmed I would attempt to modulate the adverse effects in IGS+ early RA patients by the prompt initiation of type 1 interferon modulating therapies, such as JAK1 inhibitors, potentially HCQ or ideally more specifically IFNAR1 blockers. To provide robust data this would need to be in a prospective clinical trial design which, when using the above oral therapies, would need to be double blinded and placebo controlled. Outcome measures would include the clinical parameters reported here, longitudinal IGS and, given the link between the IGS and TJC/SJC, ultrasonography would be a beneficial adjunct. HCQ is already a first line treatment in early RA and use of biological therapies in early disease is being increasingly trialled, making this an ethically sound proposal. One could envisage development of a chip based assay which could rapidly detect a raised IGS prompting additional IGS modifying therapies at RA diagnosis and thus optimisation of rapid disease control.

A highly interesting aspect of my findings relates B cell gene expression and the IGS. Correlations with B cell protein expression would need to be performed in conjunction with gene expression data and validation of associations with autoantibodies would again be achieved by increasing cohort size. Next generation sequencing on a larger cohort of B cells from early RA and ACPA+ arthralgia patients would provide more extensive pathway analysis, as well as exploring further the potential link between the IGS and malignancy in RA patients. This latter aim would also involve quantifying the whole blood IGS in RA patients with new onset B cell malignancies as well as examining the B cell transcriptome and retroelement activity (including methylation) in relevant tissue. Finally I would like to examine the IGS and serum immunoglobulins (IgM and IgG) to assess for specific or global B cell activation in both my current cohort and in any potentially larger future cohort.

Finally anti-CCP antibodies have been implicated in osteoclast activation and bone erosions (348), therefore if contribution of pDCs to their generation was validated, the pDC may provide a new therapeutic target. Examination of the appropriate tissue, i.e. blood vs synovium, is a caveat that has existed for both my retroelement and pDC work. What occurs within the synovial compartment is likely to be distinct from the peripheral blood and I would like to repeat these analyses using matched peripheral blood and synovium and, where possible, lymph node biopsies as well. Given the link with smoking

and retroelement hypomethylation (408, 414), as well as the smoke exposure in lungs being reported site for breach of tolerance in RA (447), an intriguing option would be to also examine retroelement activity in RA lung and oral mucosal tissue.

7.4 Final conclusions

In conclusion the IGS is increased in early RA and falls with time from diagnosis, and I propose a potential role for type 1 interferons in the initiation of disease. Furthermore in early RA the baseline IGS predicts the response to initial therapies at 6 months better than any conventional markers of disease activity and inflammation. Given the emerging importance of early and prompt disease control, and the fact that interferon-α targeting therapies are in development for the treatment of SLE, I suggest that inhibiting interferon-α signalling in a subset of early (IGS+) RA patients may deliver a useful therapeutic adjunct. The effect of the IGS is most marked on B cells where genes associated with activated phenotype and plasma cell differentiation are upregulated. The potential downstream effects of this are also demonstrated *in vivo* as the IGS positively associates with RF titres in early RA. The source of type 1 interferons was examined, but contrary to expectations pDCs in early RA were not the primary interferon-α producer in the peripheral blood. Despite this their numbers were significantly reduced in early RA and displayed an activated phenotype which fell with time and treatment. As a potential trigger of type 1 interferons I analysed retroelement activity in whole blood and peripheral immune cell subsets but there was no clear evidence that they were responsible for triggering type 1 interferon production. However there was differential activity between immune cell subsets which suggests epigenetic modifications are present, which may also impact cellular function.

Overall I offer an intriguing insight into the IGS in early RA and suggest that, rather than being an incidental finding, it has implications for disease pathogenesis and prognosis as well as generation of autoantibodies. I believe understanding these mechanisms further will allow optimisation of early RA treatment by the administration of concurrent interferon-α modulating therapies.

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Appendix A: Additional data

ACR/EULAR (2010) Classification Criteria for Rheumatoid Arthritis

Requirements: patients who have at least 1 swollen joint, and not better explained by another disease to be applied. A score ≥ 6 points is required for classification as definite RA.

Symptom Duration (as reported by patient)	**Points**
▪ < 6 weeks	0
▪ > 6 weeks	1

Joint Distribution	**Points**
▪ 1 large joint	0
▪ 2-10 large joints	1
▪ 1-3 small joints (with or without involvement of large joints)	2
▪ 4-10 small joints (with or without involvement of large joints)	3
▪ > 10 joints (at least 1 small joint)	5

Serology	**Points**
▪ RF- and CCP-	0
▪ Low RF+ or CCP+	2
▪ High RF+ or CCP+	3

Acute Phase Reactants	**Points**
▪ Normal ESR or CRP	0
▪ Abnormal ESR or CRP	1

Appendix A 1.0: ACR/EULAR Classification Criteria for Rheumatoid Arthritis.

This criteria was used to define which one of the patients attending the Early Arthritis Clinic had a clear diagnosis of RA. RF: rheumatoid factor. CCP: anti-citrullinated citric peptide. ESR: erythrocyte sedimentation rate. CRP: C-reactive protein. Low: < 3 x upper limit of normal (ULN). High: > 3 x ULN

Appendix A 2.0:

ABCB1	*C6*	*CD164*	*CD96*	*CXCL11*
ABL1	*C7*	*CD19*	*CD97*	*CXCL12*
ADA	*C8A*	*CD1A*	*CD99*	*CXCL13*
AHR	*C8B*	*CD1D*	*CDH5*	*CXCL2*
AICDA	*C8G*	*CD2*	*CDKN1A*	*CYBB*
AIRE	*C9*	*CD209*	*CEACAM1*	*DEFB1*
APP	*CAMP*	*CD22*	*CEACAM6*	*DEFB103A*
ARG1	*CARD9*	*CD24*	*CEACAM8*	*DEFB103B*
ARG2	*CASP1*	*CD244*	*CEBPB*	*DEFB4A*
ARHGDIB	*CASP10*	*CD247*	*CFB*	*DPP4*
ATG10	*CASP2*	*CD27*	*CFD*	*DUSP4*
ATG12	*CASP3*	*CD274*	*CFH*	*EBI3*
ATG16L1	*CASP8*	*CD276*	*CFI*	*EDNRB*
ATG5	*CCBP2*	*CD28*	*CFP*	*EGR1*
ATG7	*CCL11*	*CD34*	*CHUK*	*EGR2*
ATM	*CCL13*	*CD36*	*CIITA*	*ENTPD1*
B2M	*CCL15*	*CD3D*	*CISH*	*EOMES*
B3GAT1	*CCL16*	*CD3E*	*CLEC4A*	*ETS1*
BATF	*CCL18*	*CD3EAP*	*CLEC4E*	*FADD*
BATF3	*CCL19*	*CD4*	*CLEC5A*	*FAS*
BAX	*CCL2*	*CD40*	*CLEC6A*	*FCAR*
BCAP31	*CCL20*	*CD40LG*	*CLEC7A*	*FCER1A*
BCL10	*CCL22*	*CD44*	*CLU*	*FCER1G*
BCL2	*CCL23*	*CD46*	*CMKLR1*	*FCGR1A/B*
BCL2L11	*CCL24*	*CD48*	*CR1*	*FCGR2A*
BCL3	*CCL26*	*CD5*	*CR2*	*FCGR2A/C*
BCL6	*CCL3*	*CD53*	*CRADD*	*FCGR2B*
BID	*CCL4*	*CD55*	*CSF1*	*FCGR3A/B*
BLNK	*CCL5*	*CD58*	*CSF1R*	*FCGRT*
BST1	*CCL7*	*CD59*	*CSF2*	*FKBP5*
BST2	*CCL8*	*CD6*	*CSF2RB*	*FN1*
BTK	*CCND3*	*CD7*	*CSF3R*	*FOXP3*
BTLA	*CCR1*	*CD70*	*CTLA4_all (common probe)*	*FYN*
C14orf166	*CCR10*	*CD74*	*CTLA4-TM (membrane-bound form)*	*GATA3*
C1QA	*CCR2*	*CD79A*	*sCTLA4 (soluble form)*	*GBP1*
C1QB	*CCR5*	*CD79B*	*CTNNB1*	*GBP5*
C1QBP	*CCR6*	*CD80*	*CTSC*	*GFI1*
C1R	*CCR7*	*CD81*	*CTSG*	*GNLY*
C1S	*CCR8*	*CD82*	*CTSS*	*GP1BB*
C2	*CCRL1*	*CD83*	*CUL9*	*GPI*
C3	*CCRL2*	*CD86*	*CX3CL1*	*GPR183*
C4A/B	*CD14*	*CD8A*	*CX3CR1*	*GZMA*
C4BPA	*CD160*	*CD8B*	*CXCL1*	*GZMB*
C5	*CD163*	*CD9*	*CXCL10*	*GZMK*

Appendix A 2.0 *Continued:*

HAMP	*IKZF3*	*IL4*	*KIR3DL3*	*MAP4K4*
HAVCR2	*IL10*	*IL4R*	*KIT*	*MAPK1*
HFE	*IL10RA*	*IL5*	*KLRAP1*	*MAPK11*
HLA-A	*IL11RA*	*IL6*	*KLRB1*	*MAPK14*
HLA-B	*IL12A*	*IL6R*	*KLRC1*	*MAPKAPK2*
HLA-C	*IL12B*	*IL6ST*	*KLRC2*	*MARCO*
HLA-DMA	*IL12RB1*	*IL7*	*KLRC3*	*MASP1*
HLA-DMB	*IL13*	*IL7R*	*KLRC4*	*MASP2*
HLA-DOB	*IL13RA1*	*IL8*	*KLRD1*	*MBL2*
HLA-DPA1	*IL15*	*IL9*	*KLRF1*	*MBP*
HLA-DPB1	*IL16*	*ILF3*	*KLRF2*	*MCL1*
HLA-DQA1	*IL17A*	*IRAK1*	*KLRG1*	*MIF*
HLA-DQB1	*IL17B*	*IRAK2*	*KLRG2*	*MME*
HLA-DRA	*IL17F*	*IRAK3*	*KLRK1*	*MR1*
HLA-DRB1	*IL18*	*IRAK4*	*LAG3*	*MRC1*
HLA-DRB3	*IL18R1*	*IRF1*	*LAIR1*	*MS4A1*
HRAS	*IL18RAP*	*IRF3*	*LAMP3*	*MSR1*
ICAM1	*IL19*	*IRF4*	*LCK*	*MUC1*
ICAM2	*IL1A*	*IRF5*	*LCP2*	*MX1*
ICAM3	*IL1B*	*IRF7*	*LEF1*	*MYD88*
ICAM4	*IL1R1*	*IRF8*	*LGALS3*	*NCAM1*
ICAM5	*IL1R2*	*IRGM*	*LIF*	*NCF4*
ICOS	*IL1RAP*	*ITGA2B*	*LILRA1*	*NCR1*
ICOSLG	*IL1RL1*	*ITGA4*	*LILRA2*	*NFATC1*
IDO1	*IL1RL2*	*ITGA5*	*LILRA3*	*NFATC2*
IFI16	*IL1RN*	*ITGA6*	*LILRA4*	*NFATC3*
IFI35	*IL2*	*ITGAE*	*LILRA5*	*NFIL3*
IFIH1	*IL20*	*ITGAL*	*LILRA6*	*NFKB1*
IFIT2	*IL21*	*ITGAM*	*LILRB1*	*NFKB2*
IFITM1	*IL21R*	*ITGAX*	*LILRB2*	*NFKBIA*
IFNA1/13	*IL22*	*ITGB1*	*LILRB3*	*NFKBIZ*
IFNA2	*IL22RA2*	*ITGB2*	*LILRB4*	*NLRP3*
IFNAR1	*IL23A*	*ITLN1*	*LILRB5*	*NOD1*
IFNAR2	*IL23R*	*ITLN2*	*LITAF*	*NOD2*
IFNB1	*IL26*	*JAK1*	*LTA*	*NOS2*
IFNG	*IL27*	*JAK2*	*LTB4R*	*NOTCH1*
IFNGR1	*IL28A*	*JAK3*	*LTB4R2*	*NOTCH2*
IGF2R	*IL28A/B*	*KCNJ2*	*LTBR*	*NT5E*
IKBKAP	*IL29*	*KIR_Activating_ Subgroup_1*	*LTF*	*PAX5*
IKBKB	*IL2RA*	*KIR_Activating_ Subgroup_2*	*LY96*	*PDCD1*
IKBKE	*IL2RB*	*KIR_Inhibiting_ Subgroup_1*	*MAF*	*PDCD1LG2*
IKBKG	*IL2RG*	*KIR_Inhibiting_ Subgroup_2*	*MALT1*	*PDCD2*
IKZF1	*IL3*	*KIR3DL1*	*MAP4K1*	*PDGFB*
IKZF2	*IL32*	*KIR3DL2*	*MAP4K2*	*PDGFRB*

Appendix A 2.0 *Continued:*

PECAM1	*SELL*		*TLR3*	*ZEB1*
PIGR	*SELPLG*		*TLR4*	
PLA2G2A	*SERPING1*		*TLR5*	
PLA2G2E	*SH2D1A*		*TLR7*	
PLAU	*SIGIRR*		*TLR8*	
PLAUR	*SKI*		*TLR9*	
PML	*SLAMF1*		*TMEM173*	
POU2F2	*SLAMF6*		*TNF*	
PPARG	*SLAMF7*		*TNFAIP3*	
PPBP	*SLC2A1*		*TNFAIP6*	
PRDM1	*SMAD3*		*TNFRSF10C*	
PRF1	*SMAD5*		*TNFRSF11A*	
PRKCD	*SOCS1*		*TNFRSF13B*	
PSMB10	*SOCS3*		*TNFRSF13C*	
PSMB5	*SPP1*		*TNFRSF14*	
PSMB7	*SRC*		*TNFRSF17*	
PSMB8	*STAT1*		*TNFRSF1B*	
PSMB9	*STAT2*		*TNFRSF4*	
PSMC2	*STAT3*		*TNFRSF8*	
PSMD7	*STAT4*		*TNFRSF9*	
PTAFR	*STAT5A*		*TNFSF10*	
PTGER4	*STAT5B*		*TNFSF11*	
PTGS2	*STAT6*		*TNFSF12*	
PTK2	*SYK*		*TNFSF13B*	
PTPN2	*TAGAP*		*TNFSF15*	
PTPN22	*TAL1*		*TNFSF4*	
PTPN6	*TAP1*		*TNFSF8*	
PTPRC_all (common probe)	*TAP2*		*TOLLIP*	
CD45R0	*TAPBP*		*TP53*	
CD45RA	*TBK1*		*TRAF1*	
CD45RB	*TBX21*		*TRAF2*	
PYCARD	*TCF4*		*TRAF3*	
RAF1	*TCF7*		*TRAF4*	
RAG1	*TFRC*		*TRAF5*	
RAG2	*TGFB1*		*TRAF6*	
RARRES3	*TGFBI*		*TYK2*	
RELA	*TGFBR1*		*UBE2L3*	
RELB	*TGFBR2*		*VCAM1*	
RORC	*THY1*		*VTN*	
RUNX1	*TICAM1*		*XBP1*	
S100A8	*TIGIT*		*XCL1*	
S100A9	*TIRAP*		*XCR1*	
S1PR1	*TLR1*		*ZAP70*	
SELE	*TLR2*		*ZBTB16*	

Appendix A 2.0: Full list of genes included on NanoString nCounter Human Immunology V2 Panel.

Customised probe	Sequence
AluYa5	GGAAGAAGGGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCA GCACTTTGGGAGGCCGAGGCGGGCGGATCACGAGGTCAGGAG ATCGAGACCATCCCGGCTAAAACGGTGAAACCCCGTCTCTACT AAAAATACAAAAAATTAGCCGGGCGTAGTGGCGGGCGCCTGTA GTCCCAGCTACTTGGGAGGCTGAGGCAGGAGAATGGCGTGAAC CCGGGAGGCGGAGCTTGCAGTGAGCCGAGATCCCGCCACTGCA CTCCAGCCTGGGCGACAGAGCGAGACTCCGTC
AluYb9	GTAGGCAATGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCA GCACTTTGGGAGGCCGAGGCGGGTGGATCATGAGGTCAGGAG ATCGAGACCATCCTGGCTAACAAGGTGAAACCCCGTCTCTACT AAAAATACAAAAAATTAGCCGGGCGCGGTGGCGGGCGCCTGT AGTCCCAGCTACTGGGGAGGCTGAGGCAGGAGAATGGCGTGA ACCCGGGAAGCGGAGCTTGCAGTGAGCCGAGATTGCGCCACTG CAGTCCGCAGTCCGG CCTGGGCGACAGAGCGAGACTCCGTC
LTR5 5’ end	AAGGGGGAAATGTGGGGAAAAGCAAGAGAGATCAGATTGTTA CTGTGTCTGTGTAGAAAGAAGTAGACATAGGAGACTCCATTTT GTTATGTACTAAGAAAAATTCTTCTGCCTTGAGATTCTGTTAAT CTATGACCTTACCCCCAACCCCGTGCTCTCTGAAACATGTGCTG TGTCCACTCAGAGTTGAATGGATTAAGGGCGGTGCAGGATGTG CTTTGTTAAACAGATGCTTGAAGGCAGCATGCTCCTTAAGAGTC ATCACCACTCCCTAATCTCAAGTACCCAGGGACACAAAAA
LTR5 3’end	GGTGGGACCTGCGGGCAGCAATACTGCTTTGTAAAGCACTGAG ATGTTTATGTGTATGCATATCTAAAAGCACAGCACTTAATCCTT TACATTGTCTATGATGCAAAGACCTTTGTTCACATGTTTGTCTG CTGACCCTCTCCCCACAATTGTCTTGTGACCCTGACACATCCCC CTCTTCGAGAAACACCCACAGATGATCAATAAATACTAAGGGA ACTCAGAGGCTGGCGGGATCCTCCATATGCTGAACGCTGGTTC CCCGGGTCCCCTTCTTTCTTTCTCTATACTTTGTCTCTGTGTCTTT TTCTTTTCCAAATCTCTCGTCCCACCTTACGAGAAACACCCACA GGTGTG
L1 5’UTR	GGGGGAGGAGCCAAGATGGCCGAATAGGAACAGCTCCGGTCT ACAGCTCCCAGCGTGAGCGACGCAGAAGACGGTGATTTCTGCA TTTCCATCTGAGGTACCGGGTTCATCTCACTAGGGAGTGCCAGA CAGTGGGCGCAGGCCAGTGTGTGTGCGCACCGTGCGCGAGCCG AAGCAGGGCGAGGCATTGCCTCACCTGGGAAGCGCAAGGGGT CAGGGAGTTCCCTTTCCGAGTCAAAGAAAGGGGTGACGGACGC ACCTGGAAAATC
L1 ORF2	GTAGGGACATGGATGAAATTGGAAACCATCATTCTCAGTAAAC TATCGCAAGAACAAAAAACCAAACACCGCATATTCTCACTCAT AGGTGGGAATTGAACAATGAGATCACATGGACACAGGAAGGG GAATATCACACTCTGGGGACTGTGGTGGGGTCGGGGGAGGGGG GAGGGATAGCATTGGGAGATATACCTAATGCTAGATGACACAT TAGTGGGTGCAGCGCACCAGCATGGCACATGTATACATATGTA ACTAACCTGCACAATGTGCACATGTACCCTAAAACTTAGAGTA TAA

Appendix A 3.0: Sequences of customised probes used in NanoString panel plus nCounter to identify retroelement activity.

Appendix B: Presentations and publications pertaining to this thesis

Published Abstracts

1. Cooles FAH, Anderson AE, Lendrem DW, Norris J, Pratt AG, Hilkens C and Isaacs JD. Peripheral blood plasmacytoid dendritic cells in early RA [abstract]. *Ann Rheum Dis* 2017 [in press]
2. Cooles FAH, Anderson AE, Lendrem DW, Norris J, Pratt AG, Hilkens C and Isaacs JD. The interferon gene signature is increased in early RA and predicts a poor response to initial therapies[abstract]. *Arthritis Rheumatol*. 2016 [in press]
3. Cooles FAH, Anderson AE, Hilkens C, Isaacs JD. The prevalence of a raised interferon gene signature is increased in early RA and is associated with worse disease activity. *Ann Rheum Dis* 2016; **75**:A6 doi:10.1136/annrheumdis-2016-209124.1
4. Cooles FAH, Anderson AE, Hilkens C, Isaacs JD. The Interferon Gene Signature in Early Rheumatoid Arthritis Demonstrates No Significant Association with Disease Activity [abstract]. *Arthritis Rheumatol*. 2015; 67 (suppl 10).

Presentations

Oral – regional

1. Annual Northern and Yorkshire Rheumatology Meeting: *The interferon response gene MxA demonstrates significant association with clinical parameters in early RA*. York, UK 30/09/2015

Poster – international

1. 37th EWRR workshop, Athens, Greece. 2nd-4th March, 2017. *Peripheral blood plasmacytoid dendritic cells in early RA*
2. American College Rheumatology/ARHP congress, Washington, USA, 12-16th November 2016. *The interferon gene signature is increased in early RA and predicts a poor response to initial therapies*
3. 36th EWRR workshop, York, UK. 25-27th February, 2016. *The prevalence of a raised interferon gene signature is increased in early RA and is associated with worse disease activity.*
4. American College Rheumatology/ARHP Congress, San Francisco, USA 6-11th November 2015. *The interferon gene signature in early rheumatoid arthritis demonstrates no significant association with clinical parameters*.

Poster – regional and local

1. RACE meeting (ARUK rheumatoid arthritis pathogenesis centre of excellence), University of Glasgow, Glasgow. 3-4th October 2016. *The interferon gene signature is increased in early RA and predicts a poor response to initial therapies*
2. Newcastle University Directors Day. 16th June 2016. *The interferon gene signature is increased in early RA and predicts a poor response to initial therapies*
3. Newcastle Academic Health Partners Research Day. Newcastle 15th June 2016. *The interferon gene signature is increased in early RA and predicts a poor response to initial therapies*
4. MRC Mission Training Day, Newcastle, UK. 12th March 2015. *Plasmacytoid dendritic cells in early rheumatoid arthritis.*

Prizes

- Best Poster Prize. *The interferon gene signature is increased in early RA and predicts a poor response to initial therapies*. ICM Directors Day. Newcastle University. June 2016
- Best Clinical Poster Prize. *Plasmacytoid dendritic cells in early rheumatoid arthritis*. MRC Mission Training Day, Newcastle, UK. March 2015