Characterisation of cytokine expression in early synovitis and established rheumatoid arthritis

By

LORRAINE YEO

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ABSTRACT

In rheumatoid arthritis (RA), chronic inflammation and destruction of the joint is driven by local production of cytokines. My aims were to characterise cytokine mRNA expression in multiple synovial fluid cell populations in early and established RA and to study these, in addition to whole synovial tissue, in early synovitis patients in relation to disease outcome.

I established a novel method to determine cytokine mRNA expression in synovial fluid CD4 T cells, CD8 T cells, B cells, macrophages and neutrophils directly *ex vivo.* I made several novel observations, for example RA synovial fluid B cells expressed high levels of RANKL. As RANKL drives bone resorption, this suggests a potential role for B cells in bone erosion in RA. RANKL protein was expressed by B cells in synovial tissue, and mainly by memory B cells in synovial fluid. Furthermore, synovial RANKL levels were reduced after treatment with the B cell depleting therapy, rituximab.

Overall, both in whole synovial tissue and in sorted cells, the cytokine mRNA expression profile was very similar in early synovitis patients who subsequently developed RA or had resolving synovitis, and in patients with early or established RA. In comparison, cytokines and chemokines were upregulated in early and established arthritis patients compared to uninflamed controls. The finding that cytokine mRNA expression is largely similar in early synovitis patients who develop RA or have resolving disease, and in the early and established phases of RA, suggests that cytokine expression is reflective of general synovial inflammation, rather than being specific for early synovitis outcome or stage of RA.

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ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
Anti-CCP	Anti-cyclic citrullinated peptide
ACR	American College of Rheumatology
BSA	Bovine serum albumin
CRP	C-reactive protein
CIA	Collagen-induced arthritis
DAS	Disease Activity Score
DMARD	Disease-modifying anti-rheumatic drug
EULAR	European League Against Rheumatism
ESR	Erythrocyte sedimentation rate
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NK cell	Natural killer cell
NK T cell	Natural killer T cell
NLR	NOD-like receptor
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PAD	Peptidyl arginine deiminase
PBS	Phospho-buffered saline
RA	Rheumatoid arthritis
SF	Synovial fluid
SJC	Swollen joint count
ST	Synovial tissue
TCR	T cell receptor
TJC	Tender joint count
TLR	Toll like receptors
Treg	Regulatory T cell

1 INTRODUCTION

1.1 The role of the immune system

1.1.1 Introduction

The primary role of the immune system is to protect the body from danger. The immune system thus provides defence against pathogenic organisms and tissue damage, and eliminates altered-self cells. The first line of defence against a potentially harmful agent is provided by the physical and chemical barriers of the body. However, if these are breached, the body responds using specialised cells and effector molecules of the immune system in an attempt to eliminate pathogens, limit tissue damage, and stimulate tissue repair. A sign of host response to infection or injury is acute inflammation, which is characterised by the classical signs of heat, pain, redness and swelling.

The immune system can be divided into two arms: the innate and adaptive. Innate immunity provides an immediate line of defence against pathogens and is usually sufficient to eliminate potential pathogens encountered in peripheral tissue. While the innate immune system offers an immediate non-specific or broadly-specific response in order to deal with a wide range of pathogens, the adaptive immune response is highly specific for a given pathogen and provides immunological memory which allows an individual to mount a rapid and stronger response upon re-exposure. The key players of the innate immune system are macrophages, neutrophils, natural killer (NK) cells, mast cells, eosinophils and basophils, while lymphocytes are central to the adaptive immune system, and antigen presenting cells such as dendritic cells link innate and adaptive immunity.

1.1.2 The inflammatory response

Acute inflammation is a physiological response triggered by infection or tissue damage. The innate immune system can respond to various classes of microorganisms by recognising highly conserved molecular structures known as Pathogen Associated Molecular Patterns (PAMPs). The recognition of PAMPs is facilitated by Pattern Recognition Receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). PRRs are expressed on antigen presenting cells, and interaction of a PRR with its ligand results in the internalisation of the pathogen expressing the ligand through phagocytosis. Internalisation of the pathogen can be enhanced by the surface of the pathogen being coated by opsonins, which may be antibodies or proteins involved in the complement cascade. Activation of the complement cascade results in the recruitment of phagocytes to sites of infection, and can also kill pathogens directly. The immune system can also detect danger signals provided by endogenous molecules which are associated with tissue damage or stress, such as heat shock proteins, high mobility group box 1 and hyaluronic acid (Gallucci and Matzinger 2001).

An acute inflammatory response is characterised by the infiltration of leukocytes in which neutrophils dominate. Neutrophils are the most abundant leukocyte in the body and play a key role in the front-line defence against invading pathogens by utilising several mechanisms: (i) phagocytosis and destruction of microorganisms; (ii) release of cytoplasmic granules containing soluble anti-microbial products such as defensins, myeloperoxidase, elastase, proteinase-3 and cathepsin G and properdin; and (iii) release of neutrophil extracellular traps (NETs) comprising de-condensed chromatin and antimicrobial proteins which prevent spreading of microorganisms.

The initial influx of neutrophils is followed by monocytes which migrate from the bloodstream into the tissue and rapidly differentiate into tissue macrophages. Macrophages are polarised functionally in response to cytokines, being classified as M1 (classically activated) if induced by IFN-γ and TNF-α, or M2 (alternatively activated) if induced by IL-4 or IL-10 (Gordon 2003). Macrophages contribute to phagocytosis of the pathogens, and also produce cytokines which activate endothelial cells of local blood vessels, allowing circulating leukocytes to migrate into the inflamed tissue. In addition, macrophages produce lipid mediators of inflammation such as prostaglandins, leukotrienes and platelet-activating factor.

Normally the acute inflammatory response is followed by resolution of inflammation and tissue repair. Elimination or neutralisation of the pathogen halts further production of pro-inflammatory mediators such as eicosanoids, cytokines, and cell adhesion molecules, and leads to their catabolism. Neutrophils and eosinophils that have been recruited to the site of injury or infection are cleared by local death followed by phagocytosis by recruited macrophages. Macrophages then exit the site of inflammation via the lymphatics, while a small population may die locally by apoptosis.

Although traditionally viewed as a passive process, resolution of inflammation is an active process that requires activation of endogenous programs to maintain tissue homeostasis. These include negative feedback pathways which inhibit TLR and NLR signalling, and anti-inflammatory cytokine production by regulatory T cells. Stromal cells such as fibroblasts, endothelial cells and macrophages also contribute to resolution by reducing leukocyte infiltration by withdrawing survival signals, normalising chemokine gradients, and inducing resolution programs that result in apoptosis or exit from the inflamed tissue. Molecular

mechanisms involved in the resolution of inflammation include the lipid mediators, lipoxins, resolvins and protectins (Serhan *et al.* 2008). Resolution of the acute inflammatory response by such mechanisms normally allows the inflamed tissue to return to homeostasis. However, if these naturally occurring resolution programs become dysregulated in some way, it is possible that inflammation may become chronic. In certain conditions such as rheumatoid arthritis, psoriasis, multiple sclerosis, atherosclerosis and inflammatory bowel diseases, this chronic state of inflammation may last the lifetime of the individual.

1.1.3 Adaptive immunity

The functions of the adaptive immune system are primarily mediated by lymphocytes. Both T and B cell subsets have diverse repertoires of receptors on their surfaces that enable them to recognise specific motifs of any pathogen they may encounter. The diversity of lymphocyte receptors arises by somatic recombination of V(D)J segments during development (Tonegawa 1983). Lymphocytes bearing receptors with high affinity for self molecules are deleted, while those with receptors with high affinity for pathogen-associated molecules survive. Naïve lymphocytes which have not yet engaged their cognate antigen move continuously between the lymph nodes and the blood via the lymphatics to search for antigens.

Naïve T cells continuously search for antigens being presented on the surface of antigen presenting cells such as dendritic cells. CD8 T cells recognise endogenous antigen presented by class I major histocompatibility complex (MHC) antigens, and thus deal with the removal of virally infected or altered self cells. CD4 T cells recognise exogenous antigens that have been processed by professional antigen presenting cells such as monocytes, dendritic cells, macrophages and B cells, and are presented by MHC class II molecules, and thus deal with

bacterial, fungal and parasitic infection. Cross-presentation of antigens may also occur in which MHC class I molecules present antigen of exogenous origin, and MHC class II molecules present peptides derived from cytoplasmic or nuclear antigens. Phagocytosed or endocytosed antigens may leave the vacuole in which they have been engulfed, gain access to the cytosol and be presented by MHC class I molecules. Peptide exchange with MHC class I molecules recycling from the cell membrane may also result in peptides derived from exogenous antigens being presented by MHC class I molecules. Intracellular antigens may be presented by MHC class II molecules if cytosolic peptides are engulfed by autophagosomes and thereby gain entry into the class II processing and presentation pathway.

Recognition of presented antigen by the T cell receptor (TCR), and a simultaneous co-stimulatory signal, results in the proliferation and activation of T cells. Induction of IL-2 stimulates clonal expansion of the engaged T cell. Naïve T cells subsequently differentiate into effector T cells with specific functions according to the activating antigen and the local cytokine milieu, such as Th1, Th2, Th17, Th9, Th22, T follicular helper (Tfh), or inducible regulatory T cell (Treg) subsets. More recently, Th9 and Th22 T helper subsets have also been described (Dardalhon *et al.* 2008; Eyerich *et al.* 2009; Veldhoen *et al.* 2008). Transcription factors direct the development of T helper cell subsets with distinct cytokine-secreting potentials. Th1 cells, which are important in defence against intracellular pathogens, express the transcription factor T-bet, a member of the T-box family of transcription factors. Th2 cells provide defence against helminths, and express the master regulator for Th2 differentiation, GATA-3. Th17 cells contribute to host defence against extracellular bacteria and fungi; the retinoic acid-related orphan receptors, ROR-α and ROR-γt, are the key transcription factors in Th17 differentiation. Tfh cells help B cells in making antibody responses, and express the

master regulator Bcl-6. Inducible Tregs, which exert immunosuppressive functions, express the master regulator Foxp3. Th9 cells mediate defence against extracellular parasites; recent studies have suggested PU.1, IRF-4 and STAT-6 as transcription factors required for Th9 differentiation (Chang *et al*, 2010; Goswami *et a.* 2011.; Staudt *et al.* 2010). Th22 cells are also important in defence against extracellular parasites; the aryl hydrocarbon receptor (AHR) is the master regular of Th22 cell differentiation. Following activation and proliferation, T cells migrate to sites of inflammation. After the clearance of the pathogen, some antigen-specific T cells remain in the body as memory T cells which facilitate a more rapid response upon re-exposure than the original response.

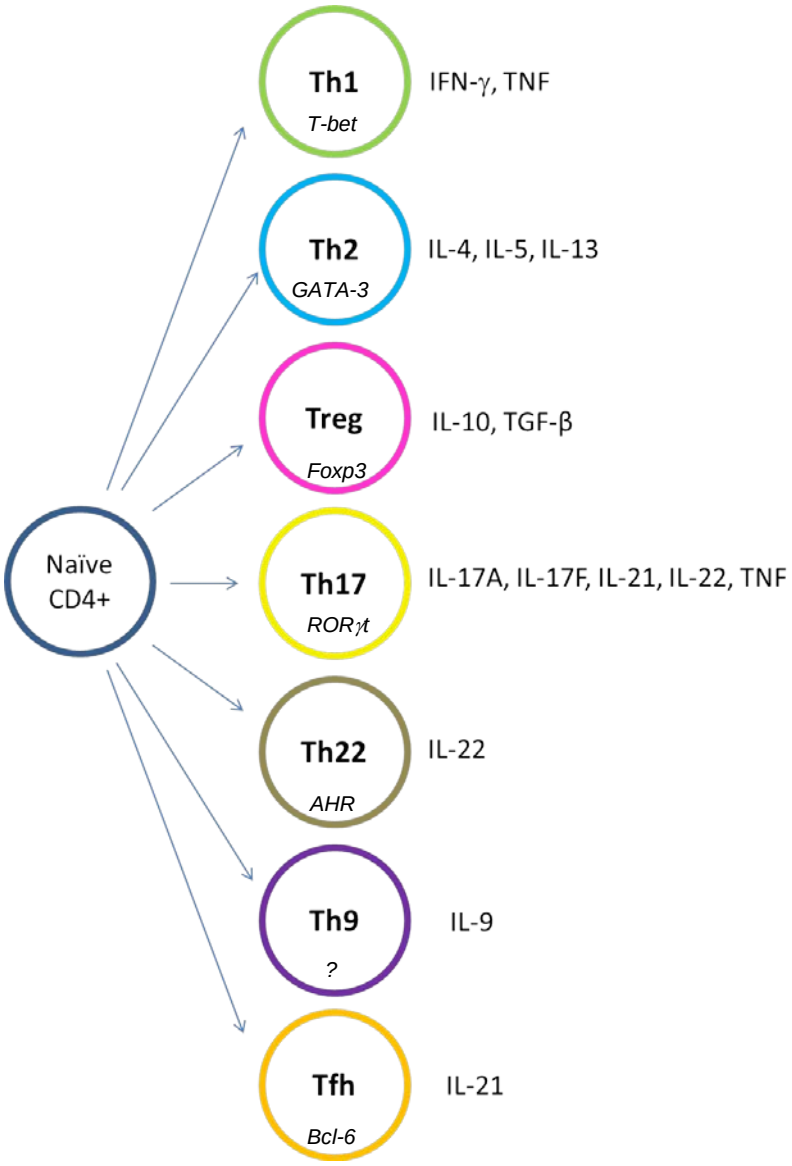


Figure 1.1 CD4 T helper cell subsets. Naïve CD4 T cells differentiate into different subsets of effector CD4 T cells which are specialised to deal with different types of pathogens. Effector T cells produce certain cytokines that produce distinct and appropriate effects on the target cell. Effector T cell subsets described at present include T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), T helper 9 (Th9), T helper 22 (Th22), T follicular helper (Tfh) and regulatory T cells (Treg). Th1 cells are important in defence against intracellular bacteria,

viruses and cancer, Th2 cells against helmiths and extracellular pathogens, Th17 cells against extracellular bacteria and fungi, and Th22 and Th9 cells against extracellular parasites. Tfh promote germinal centre responses, and Tregs maintain tolerance to commensals, innocuous antigens and self-antigens. The specific transcription factors required for the differentiation of distinct T helper cell subsets are shown in italics.

Naïve B cells become activated following an encounter with their cognate antigen, whereupon they may adopt one of two fates: (i) differentiation to short-lived plasma cells which produce antibody, or (ii) formation of germinal centres (Murphy et al, 2007). Within the germinal centre, B cell receptors undergo diversification by the process of somatic hypermutation. Lymphocytes bearing receptors with high affinity for antigen are selected for survival and further proliferation, whereas cells with receptors which have low affinity die. Selected B cells eventually differentiate into memory B cells or antibody-producing plasma cells. In addition to their important role in antibody production, B cells also act as antigen presenting cells. Antigen presentation by B cells and their recognition by T cells involves activation of co-stimulatory pathways and cytokine production, which triggers B cell proliferation and antibody secretion. During this process, cytokines are released by activated T helper cells which induce further T cell proliferation. In addition, B cells also produce polarising cytokines which influence the differentiation of T cells.

1.1.4 Autoimmunity

While the immune system is clearly essential for protecting the host from disease, it is also recognised that excessive activation of the immune system can be detrimental, and may lead to autoimmune diseases in which the immune system attacks an individual's own cells and tissues. The gene rearrangement that occurs during lymphocyte development in the central lymphoid organs is a random process, and inevitably leads to the formation of some lymphocytes with affinity for self antigens. Under normal circumstances, autoimmune attack is avoided by several mechanisms which maintain a state of self-tolerance including (i) removal of autoreactive lymphocytes during development in the thymus and bone marrow (central tolerance); (ii) tolerization of lymphocytes to antigens that are expressed at a high and constant concentration and thus correlate to self antigens; and (iii) peripheral tolerance

induced by encounter with self antigen in the absence of a co-stimulatory signal, which leads to a negative inactivating signal. However, all of these mechanisms are error-prone as none distinguishes self from non-self at the molecular level. A breakdown or failure of self-tolerance mechanisms can thus lead to development of an autoimmune disease and associated tissue damage.

1.2 Rheumatoid arthritis

1.2.1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic disease of unknown aetiology characterised by inflammation and destruction of diarthroidal joints. Patients typically develop a symmetrical synovitis involving the small joints of the hands and feet, which over time can lead to joint destruction and functional disability. RA is associated with substantial comorbidity due to the involvement of multiple organs and systems, and is estimated to reduce life expectancy by 3 to 18 years. RA affects approximately 1% of the population in the UK and the overall occurrence is approximately two times greater in women than men. The peak age of onset is between 40 and 50 years.

1.2.2 Classification

For the purpose of standardising epidemiologic studies and clinical trials, classification criteria for RA were developed by the American College of Rheumatology (ACR) in 1958 and were revised in 1987 (Arnett *et al.* 1988) (Table 1.1). In the list version of the 1987 ACR criteria, RA is classified based on the presence of four of seven criteria; these include five clinical variables and two investigation-based variables: radiographic evidence of erosions

and the presence of rheumatoid factor, an antibody directed against IgG. These criteria were developed based on data from patients with established disease, and it is widely recognised that some of these features are absent during early stages of the disease. In 2010, the ACR and the European League Against Rheumatism (EULAR) developed new classification criteria to facilitate identification of patients with RA with newly presenting inflammatory arthritis (Aletaha *et al.* 2010) (Table 1.2). In these criteria, classification as “definite RA” is based on the presence of synovitis, the number and site of involved joints, the presence of rheumatoid factor and anti-citrullinated protein antibodies (ACPA), elevated acute-phase response reactants, and symptom duration.

Criterion	Description
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic c**hanges**	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

* For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 or these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded.

Table 1.1 1987 American College of Rheumatology classification criteria.

Criterion	Score
A. Joint involvement	
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
B. Serology (at least 1 test result is needed for classification)	
Negative RF *and* **negative ACPA**	0
Low-positive RF *or* **low-positive ACPA**	2
High-positive RF *or* **high-positive ACPA**	3
C. Acute-**phase reactants (at least 1 test result is needed for classification)**	
Normal CRP *and* **normal ESR**	0
Abnormal CRP *or* **abnormal ESR**	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Table 1.2 2010 ACR/EULAR classification criteria. Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6/10 is needed for classification of a patient as having definite RA

1.2.3 Pathophysiology of RA

RA is regarded as a complex disease involving both genetic and environmental factors. Despite significant advances in our understanding of the pathogenesis of RA in recent years, so far the cause remains unknown. Current models imply an underlying immunologic abnormality, which may involve an autoimmune trigger that drives the chronic and destructive inflammatory response in the synovium.

The prevalence of RA in the general population is approximately 1%, but among siblings the prevalence increases to 2-4% (Seldin *et al.* 1999). In cross-sectional twin studies, concordance rates for RA were found to be between 12.3% and 15.4% for monozygotic twins, and 3.5% for dizygotic twins (MacGregor *et al.* 2000). From these studies it is estimated that the genetic contribution to RA is 50% to 60%. The strongest genetic association is the HLA-DRB1 locus which encodes the β-chain of MHC class II molecules. HLA-DRB1 alleles associated with increased risk of RA contain a common amino acid sequence (QKRAA, QRRAA, or RRRAA), known as the “shared epitope”, at positions 70–74 in the third hypervariable region of the DRβ1 chain, within the peptide-binding groove (Gregersen *et al.* 1987). The shared epitope hypothesis suggests that presentation of auto-antigenic peptides to T cells drives the disease process in RA. Another important genetic risk factor is PTPN22 which encodes the protein tyrosine phosphatase, Lyp. A single nucleotide polymorphism in this gene, which results in deregulated signalling downstream of the TCR, is associated with increased risk of a number of autoimmune diseases including RA (Begovich *et al.* 2004; Burkhardt *et al.* 2006; Hinks *et al.* 2005). Genome-wide association studies have identified several single nucleotide polymorphisms associated with RA, many of which are linked to T or B cell activation, or differentiation and cytokine signalling.

Anti-citrullinated protein/peptide antibodies (ACPA) have become associated with RA in recent years. ACPA have been found to be pathogenic in experimental murine models of arthritis (Lundberg et al, 2005; Kuhn et al., 2006), but it is not clear whether they have a directly pathogenic role in human RA. ACPA are present in approximately 70% of RA patients and are highly specific for RA (Nishimura *et al.* 2007; Schellekens *et al.* 2000). ACPA are most commonly measured as anti-cyclic citrullinated peptide (anti-CCP) auto-antibodies. They can be detected many years before clinical onset of disease and are predictive for the development of RA (Avouac *et al.* 2006; Bos *et al.* 2010; van der Helm-van Mil *et al.* 2007). ACPA recognise proteins or peptides in which the amino acid arginine is deiminated to citrulline through a post-translational modification process (Figure 1.1). Citrullination reduces the net charge of the protein by the loss of a positive charge per citrulline residue. As arginine residues often play a central role in the structural integrity of a protein, conversion into citrulline may result in an altered three-dimensional structure and altered protein function. Citrullination is mediated by peptidyl arginine deiminase (PAD) enzymes. PAD enzymes are normally present within cells and can be activated by high calcium levels when cells undergo apoptosis, for example during inflammation (Gyorgy *et al.* 2006). ACPA can recognise several citrullinated proteins such as vimentin, collagen, enolase and fibrinogen. ACPA have been shown to be enriched in RA synovial fluid (Snir *et al.* 2010) and citrullinated proteins have been detected in the RA synovium (De Rycke *et al.* 2005). In addition, antibodies against the citrullinating enzyme, peptidyl arginine deiminase type 4 (PAD4), have been found in the serum prior to clinical onset of RA (Kolfenbach *et al.* 2010).

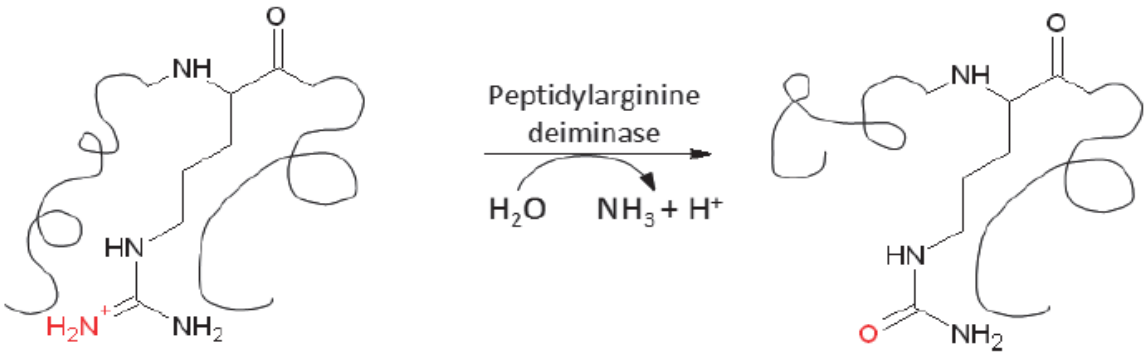


Figure 1.2 Conversion of peptidylarginine into peptidylcitrulline. This reaction is catalyzed by the family of peptidylarginine deiminase (PAD) enzymes. The positively charged arginine guanidino group is converted into the neutral citrulline ureido group in the presence of water, yielding ammonia and a hydrogen ion as by-products. The loss of a positive charge per citrulline residue may alter protein structure as shown schematically. Image taken from Wegner et al, Immunological Reviews 2010.

Increasing evidence indicates that ACPA-positive and ACPA–negative RA represent distinct disease entities. This notion is mainly based on differences in genetic and environmental risk factors between these two groups. The genetic associations of HLA-DRB1 and PTPN22 are confined to the ACPA-positive subset of disease, whereas other genes such as HLA-DRB1*03 are associated only with ACPA-negative disease (Padyukov *et al.* 2011; van der Helm-van Mil *et al.* 2006; Verpoort *et al.* 2005). Histological differences of the synovium are also apparent, with ACPA-positive RA being characterised by denser lymphocyte infiltrate and ACPA-negative RA showing more extensive fibrosis (van Oosterhout *et al.* 2008). Although ACPA-positive and ACPA-negative patients present with similar clinical features, they have different disease courses: ACPA-positive patients have a more severe disease course characterised by more radiological joint damage and worse disease activity scores (Forslind *et al.* 2004; Kastbom *et al.* 2004; Kroot *et al.* 2000; Ronnelid *et al.* 2005).

The best established environmental risk factor for RA is smoking. A genetic-environmental interaction between ACPA, shared-epitope alleles and smoking has been well described (Lundstrom *et al.* 2009). Recently, it has been shown that the combination of shared epitope alleles and smoking may also be present in autoantibody-negative disease (Bang *et al.* 2010; Mikuls *et al.* 2010). Another emerging environmental trigger for RA is the oral pathogen, *P. gingivalis* which is associated with periodontitis. *P. gingivalis* has the unique ability to citrullinate proteins using endogeneously produced PAD enzymes. Antibodies to *P. gingivalis* have been found to associate with ACPA in patients with RA and their relatives, suggesting a possible involvement in pathogenesis (Hitchon *et al.* 2010). Diet has also been associated with onset and persistence of RA. Dietary factors that may be relevant include vitamin C, β-cryptoxanthin, zeaxanthin and red meat (Pattison *et al.* 2005).

1.2.4 Phases of RA

The processes involved in the initiation and progression of RA remain poorly understood. The presence of rheumatoid factor and ACPA in the serum up to 14 years before the disease becomes clinically apparent indicates that immunological abnormalities precede the development of RA. Recently it has been reported that plasma levels of several cytokines and chemokines are increased prior to disease onset (Kokkonen *et al.* 2010). In addition, increased levels of C-reactive protein (CRP) can be detected in the peripheral blood together with rheumatoid factor and/or ACPA before clinical onset (Kolfenbach *et al.* 2009; Nielen *et al.* 2004). The observation that synovial inflammation is present in clinically unaffected knee joints from patients with established RA suggests that inflammatory changes may also occur in the pre-clinical phase (Kraan *et al.* 1998). In addition, several animal models of RA have

demonstrated inflammatory changes in the synovium in the latency phase of arthritis (Kraan *et al.* 1998).

It is not known if the initial immune response leading to the production of autoantibodies takes place in the synovium or at other sites in the body. One candidate site is the lung where cigarette smoke could trigger citrullination of specific peptides, which in combination with a loss of tolerance to these peptides could result in ACPA production. It has been proposed that a "second hit" is needed to induce citrullination of proteins in the synovium, such as minor trauma or a viral infection. Indeed, it has been reported that citrullination can occur in the synovium in any form of arthritis (Vossenaar *et al.* 2004). On the background of pre-existing immunity against citrullinated antigens, this could lead to epitope spreading and progression of synovitis, in accordance with the observation that ACPA immunoglobulin isotypes and epitopes recognised by ACPA evolve over time (Ioan-Facsinay *et al.* 2008; Verpoort *et al.* 2006).

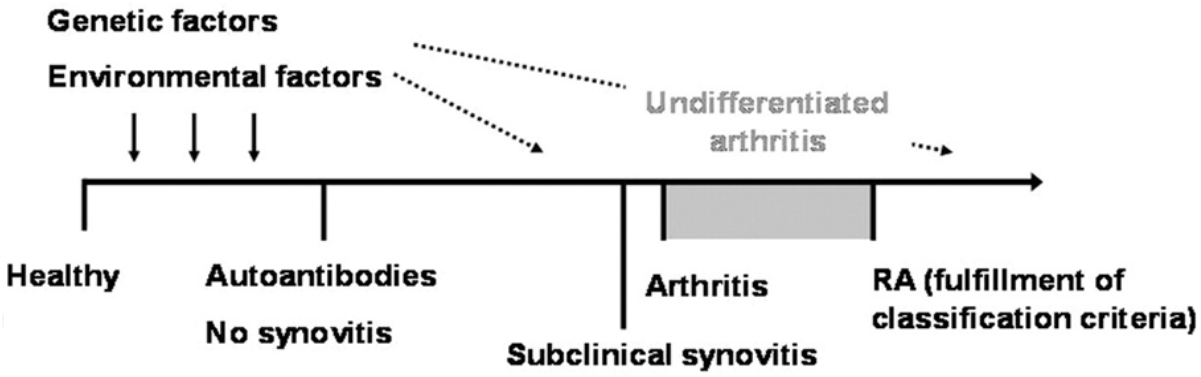


Figure 1.3 Timeline depicting the different stages of disease of autoantibody-positive rheumatoid arthritis. Autoantibody formation may precede the development of clinical signs and symptoms of RA by several years. Image taken from van de Sande M G H et al., Annnals of Rheumatic Disease 2011.

Clinically, RA appears to develop in several stages beginning with swelling and pain in a limited number of joints, followed by a subsequent increase in the number of affected joints and severity of symptoms, and finally resulting in destruction and deformity. In 1985 Konttinen and colleagues defined three phases of synovitis based on the predominant cell types found at each stage of disease. Phases described were an acute phase (< 3 months' duration in RA patients) in which monocytes and macrophages predominate with little T cell infiltration; a subacute phase (recent synovitis in patients with RA of longer than 3 years) in which primarily infiltrating T cells were found, and also B cells and plasma cells in addition to mononuclear cells seen in the acute phase; and a chronic phase (clinical synovitis and disease for longer than 3 years) which demonstrated a shift towards a predominance of T cell infiltration and dense plasma cell infiltrates. Other studies have reported that late stages of chronic RA are dominated by macrophages and synovial fibroblasts rather than T cells (Bromley and Woolley 1984; Kobayashi and Ziff 1975; Matsuno *et al.* 2002; Muirden 1982; Youssef *et al.* 1998), and NK cell numbers appears to decrease with disease duration (Tak *et al.* 1994).

Other studies which have examined synovial tissue from patients with early and long-standing RA reported no significant differences between the two groups in terms of macrophage infiltration, synovial thickening, or expression of cytokines, chemokines, granzymes, adhesion molecules and matrix metalloproteinases (MMPs) (Kraan *et al.* 1999; Schumacher and Kitridou 1972; Smeets *et al.* 1998a; Tak 2001). These findings suggest that different phases of RA cannot easily be distinguished on the basis of clinical disease duration, although there was a wide range in the definitions used for early disease (4 weeks to 12 months after symptom onset) and long-standing disease (>1 year to > 5 years in duration).

More recent evidence suggests that the pathology of very early RA differs from that of established RA. Early RA patients were shown to have a distinct and transient cytokine profile in first 12 weeks after symptom onset that was not seen in other early arthritides and not present in established RA. Synovial fluid from patients with early RA contained higher levels of IL-2, IL-4, IL-15 and GM-CSF compared to patients with established RA (Raza *et al.* 2005). Molecular profiling of synovial biopsies from patients with early and long-standing RA has also revealed distinct molecular signatures between the two groups, indicating that different biological processes operate at different stages of the disease (Lequerre *et al.* 2009).

In support of the concept of distinct pathological mechanisms operating in early and established RA is the observation that treatment initiated in the early stages of RA reduces disease activity to a greater extent than when started later in the disease course (Mottonen *et al.* 2002; Nell *et al.* 2005). Several trials have shown that institution of disease-modifying anti-rheumatic drug (DMARD) therapy is more beneficial in improving the outcome of RA when started early in the disease (Lard *et al.* 2001; Nell *et al.* 2005; van der Heide *et al.* 1996). Evidence suggests that there may a crucial “window of opportunity” within which disease course can be altered, and this may be as short as 12 weeks. Understanding this early phase of disease remains an important goal in order to facilitate early diagnosis of RA and to allow treatment to be targeted to the appropriate patient group.

1.3 Cells of the RA synovium

The synovium is a delicate membrane that encapsulates the joints, offering structural support and lubricating the surfaces, and providing nutrients to the cartilage. The synovium is composed of an intimal lining layer and a sublining layer. The intimal lining layer, which produces synovial fluid, is normally a single-cell layer composed of synovial fibroblasts and macrophages. The sublining layer is normally comprised of relatively acellular connective tissue and blood vessels. In RA, the synovium transforms into a hyperplastic, invasive tissue. The intimal lining layer expands to a depth of up to 10-20 cells due to an increase in synovial fibroblasts and macrophages, and the sublining layer becomes infiltrated by multiple cell types including T cells, B cells, plasma cells and macrophages, as well as a smaller number of mast cells, NK cells, neutrophils and dendritic cells. The organisation of the cellular infiltrate varies between patients, ranging from a diffuse infiltrate through to focal lymphoid aggregates and development of germinal centre-like structures. In addition, the sub-lining layer becomes oedematous and highly vascular. At the cartilage-bone interface, the inflamed synovium forms an aggressive pannus lesion formed of macrophages, osteoclasts and fibroblasts, which invades the adjacent cartilage and bone and promotes articular destruction by osteoclasts, chondrocytes and synovial fibroblasts. Neutrophils are also found at the cartilage-pannus junction, suggesting they may play a role in the marginal erosion of the joints (Bromley and Woolley 1984).

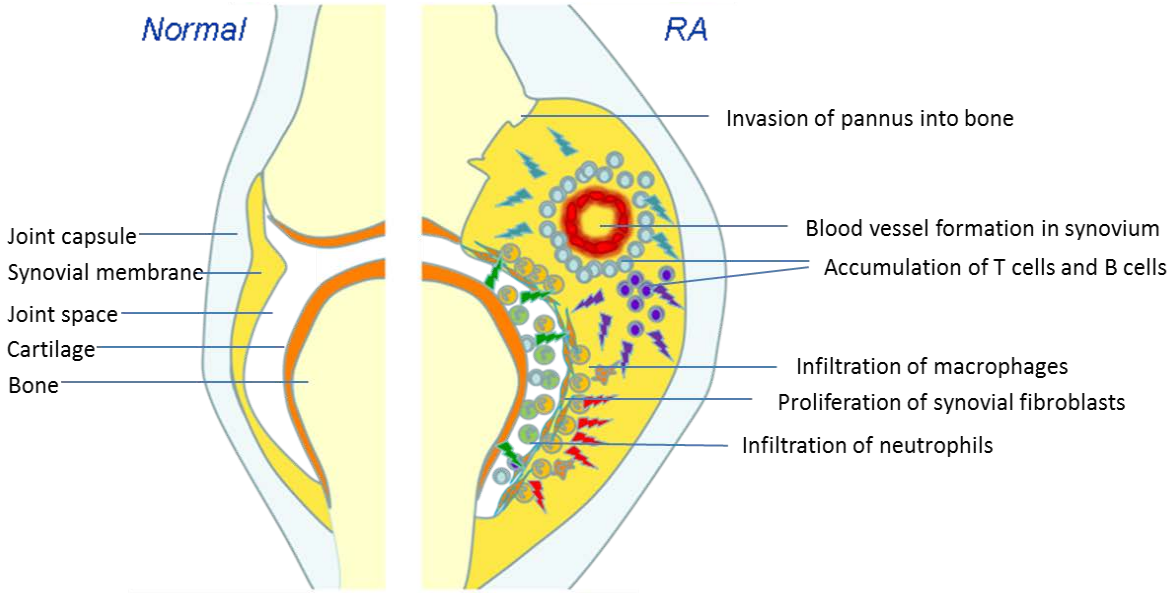


Figure 1.4 Changes in the synovium in RA. In the healthy joint there is a thin synovial membrane lining the joint. In RA the synovial lining becomes hyperplastic due to the proliferation of synovial fibroblasts, and the synovial membrane becomes infiltrated by inflammatory cells including macrophages, T cells, B cells, mast cells, NK cells and dendritic cells. Neutrophils accumulate in the synovial fluid. At the cartilage-bone interface, the inflamed synovium forms an aggressive pannus lesion which invades the articular cartilage and underlying bone.

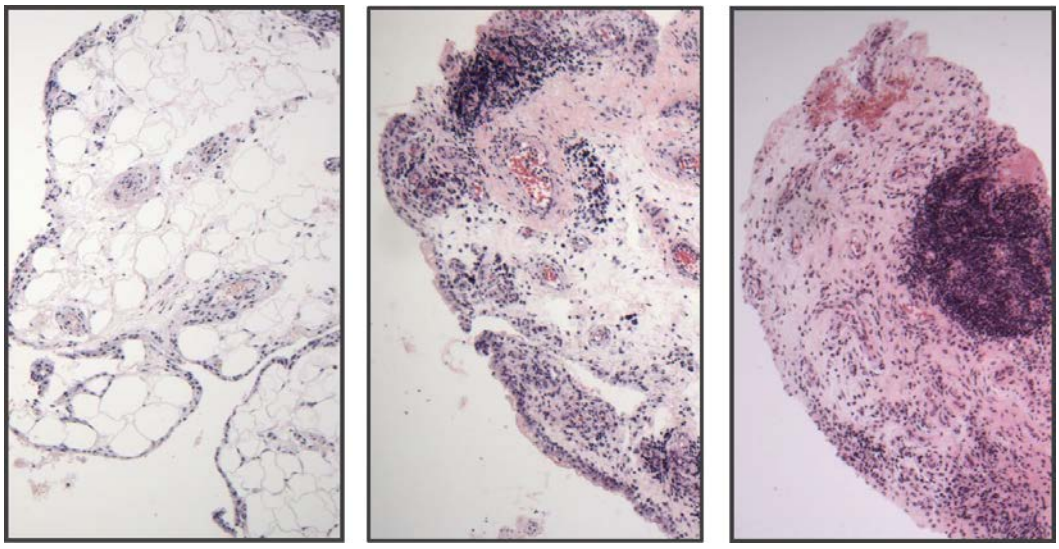


Figure 1.5 Patterns of leukocyte infiltration in the RA synovium. Cellular infiltration of the synovial tissue varies between patients, ranging from little infiltration (left), diffuse cellular infiltration (centre) to the formation focal lymphocytic aggregates (right). Images courtesy of D. Scheel-Toellner.

1.3.1 Macrophages

Macrophages are found in prominent numbers in the rheumatoid synovium and at the cartilage-pannus junction. They display an activated phenotype and are important contributors to inflammation and joint destruction in RA due to their production of pro-inflammatory mediators and tissue-degrading enzymes. The central role of macrophages in RA is supported by the fact that conventional therapies, including methotrexate and cytokine inhibitors, act to reduce levels of cytokines which are produced predominantly by macrophages (Lavagno *et al.* 2004). Furthermore, the number of synovial macrophage has been found to correlate with joint erosion in RA (Mulherin *et al.* 1996). Macrophage-like (type A) synoviocytes present in the intimal lining layer are thought to be derived from the CD14+ branch of the monocyte lineage, but they share features with dendritic cells, distinguishing them from typical monocytes. These cells likely have a phagocytic role in clearing debris from the synovial fluid, and animal models of arthritis suggest they have a role in propagating chronic synovitis (van Lent *et al.* 1998).

Macrophages are an important source of many pro-inflammatory cytokines which are considered to have a central role in RA, including TNF-α, IL-1 α and IL-6 (Chu *et al.* 1992; Chu *et al.* 1991). Other pro-inflammatory cytokines produced by synovial macrophages in RA include IL-15, IL-18 and IL-27 (McInnes *et al.* 1996). Synovial macrophages produce several chemokines which act as chemoattractants for leukocytes into the inflamed synovium and promote angiogenesis. Furthermore, synovial macrophages produce growth factors that promote cell survival in the rheumatoid synovium including GM-CSF, G-CSF, M-CSF, bFGF, VEGF and TGF-b. In addition, they produce MMPs which mediate tissue degradation and remodelling. Other roles for synovial macrophages in RA include Th1 polarisation via

production of IL-12, IL-15 and IL-18, and promoting B cell survival by their expression of BAFF, APRIL and LT-β.

1.3.2 Neutrophils

Neutrophils are often the most abundant cell type in RA synovial fluid, although relatively low numbers are found in the synovial tissue. While the primary role of neutrophils in RA pathogenesis remains unclear, they are known to release a wide range of inflammatory mediators which could support a range of pathological events. Neutrophils release reactive oxygen and nitrogen species, proteases, complement and pro-inflammatory cytokines including TNF-α, IL-1, IL-18, IL-15, IL-6 and BAFF which could promote synovial inflammation and joint destruction (Assi *et al.* 2007; Edwards and Hallett 1997). Activated neutrophils are found at the cartilage-pannus interface where they could have a direct role in promoting erosion (Bromley and Woolley 1984). Neutrophils may also be involved in bone remodelling through expression of RANKL, RANK and OPG (Poubelle *et al.* 2007). Although the normal lifespan of neutrophils is relatively short, neutrophils in patients with early RA have reduced rates of apoptosis compared to patients with other persistent forms of arthritis or self-limiting disease (Raza *et al.* 2006). Neutrophils have an essential role in the initiation and progression of joint inflammation in the mouse K/BxN spontaneous arthritis model, implicating an important role for this cell type in the pathogenesis of RA (Wipke and Allen 2001).

1.3.3 T cells

T cells are abundant in the rheumatoid synovium, found in close proximity to B cells, dendritic cells and synovial fibroblasts. Both CD4 T cells and CD8 T cells are found in the

RA synovium. CD4 T cells are often found clustered in perivascular cuffs, while CD8 T cells are found scattered throughout the synovium (Kurosaka and Ziff 1983). Synovial T cells have a highly differentiated $CD45RO^{+}CD45RB^{dull}$ phenotype and display markers of activation such as CD69, CD28 and CD40L, but are hyporesponsive to antigenic stimulation (Cope *et al.* 1994; Emery *et al.* 1985; Malone *et al.* 1984). Although cytokine secretion by synovial T cells appears to be suppressed, T cells can promote cytokine production by macrophages and synovial fibroblasts via CD40L-CD40 interactions (Aarvak and Natvig 2001; McInnes *et al.* 2000). Cytokine production and proliferation of T cells in RA may be impaired by chronic exposure to TNF-α in the RA synovium (Cope *et al.* 1997; Cope *et al.* 1994). Prolonged exposure of T cells to TNF-α downregulates expression of the TCRζ chain which is required for sensing and regulating lymphocyte responses (Isomaki *et al.* 2001). Furthermore, $TCR\zeta^{dim}$ lymphocytes accumulate in the RA synovium where they show limited proliferation and effector responses (Zhang *et al.* 2007).

T cells are implicated in the pathogenesis of RA due to the genetic association with MHC class II alleles, the abundance of T cells in the rheumatoid synovium, and the requirement for T cells in various animal models of arthritis (Rankin *et al.* 2008; Sakaguchi *et al.* 2003). Therapeutic targeting of T cells in RA using cyclosporin and antibodies specific for CD4 and CD52 have been disappointing (Keystone 2003). However, clinical benefit is seen with abatacept, a recombinant fusion protein consisting of the extracellular domain of CTLA4 and a fragment of the Fc portion of IgG that inhibits co-stimulatory signals required for T-cell activation, supporting a role for T cell co-stimulation and activation in RA (Wells *et al.* 2011).

Largely on the basis of animal models, RA has classically been considered a Th1 cell-mediated disease driven by a population of T cells producing inflammatory cytokines such as IFN-γ and TNF-α. However, in mice lacking the Th1-cell-associated genes *Ifng, Ifngr* or *IL12p35*, collagen-induced arthritis (CIA) is accelerated (Manoury-Schwartz *et al.* 1997; Murphy *et al.* 2003; Vermeire *et al.* 1997). In addition, IFN-γ is lacking or present at low levels in the RA synovium and is rarely detectable in the synovial fluid (Firestein and Zvaifler 1987), although some studies support the presence of Th1 cells in the synovium (Kanik *et al.* 1998; Ronnelid *et al.* 1998). The notion of RA being a Th1-mediated disease was originally supported by the observation that IL-12, a major stimulator of Th1 development, enhanced murine CIA (Germann *et al.* 1995). However it was subsequently found that the autoimmune actions ascribed to IL-12 were in fact attributable to the newly discovered cytokine IL-23 which is composed of a unique p19 subunit and the p40 subunit component of IL-12. Both mice lacking IL-23p19 and mice lacking IL-12p40 were protected from CIA, whereas mice lacking IL-12p35 developed more severe diseases (Becher *et al.* 2002; Cua *et al.* 2003; Murphy *et al.* 2003). While IL-12 induces development of Th1 cells, IL-23 binds to memory T cells and induces the proliferation of Th17 cells.

A new model implicates Th17 cells as a crucial effector subset in RA. The Th17 cell subset plays a central role in the pathogenesis of several animal models of autoimmune diseases including RA. In the CIA model of arthritis, disease is suppressed in IL-17-deficient mice, whereas administration of anti-IL-17 antibodies significantly reduces disease severity (reviewed in (Lubberts *et al.* 2005)). Autoimmune arthritis in SKG mice, a strain of mice that spontaneously develops inflammatory arthritis due to a mutation in the T cell signalling molecule ZAP-70, is also highly dependent on IL-17 production by CD4 T cells (Hirota *et al.*

2007). In RA, IL-17 has been detected in synovial fluid and synovium (Fossiez *et al.* 1996; Hitchon *et al.* 2004; Kotake *et al.* 1999; Ziolkowska *et al.* 2000). IL-17 expression is associated with synovial inflammation and joint destruction mediated by inducing the production of pro-inflammatory cytokines and tissue-degrading proteins by stromal cells.

Tregs play an important role in the maintenance of immune tolerance. Human Tregs were originally identified by their expression of the IL-2 receptor alpha chain, CD25, and the transcription factor Foxp3. However, CD25 and Foxp3 are also upregulated on activated CD4 T cells which do not have a Treg function, and a CD25-negative subset of regulatory T cells has also been described. After *in vitro* activation, Tregs can suppress proliferation and cytokine production of CD4+CD25- T cells, and can also supress monocytes, macrophages, B cells and dendritic cells (Misra *et al.* 2004; Taams *et al.* 2005; Thornton and Shevach 1998; Zhao *et al.* 2006). Tregs exert their suppressive function via secretion of inhibitory cytokines and cytotoxic factors, or by modulating antigen presenting cells. Tregs are present in the rheumatoid synovium, but appear to have impaired regulatory function, being unable to suppress pro-inflammatory cytokine secretion by activated T cells and monocytes (Ehrenstein *et al.* 2004). Additionally, T responder cells in RA synovial fluid are less susceptible to suppression compared with circulating T cells which is consistent with the observation that strongly activated CD4 T cells are resistant to suppression by Tregs (Baecher-Allan *et al.* 2002; van Amelsfort *et al.* 2004). The defective regulatory capacity of RA Tregs is associated with their reduced expression of the suppressive molecule CTLA-4 (Flores-Borja *et al.* 2008). Their regulatory capacity also appears to be modulated by TNF-α, as TNF-α inhibition in RA patients promotes the emergence of functional $Foxp3^{+}CD4^{+}CD25^{+}CD62L^{-}$ Treg population (Nadkarni *et al.* 2007). This is consistent with a recent paper which showed that TNF-α

increases localisation of PKCθ to the immune synapse where it acts as a negative regulator of Treg function, supporting the notion that TNF-α has an inhibitory effect of Treg function in RA (Zanin-Zhorov *et al.* 2010).

1.3.4 B cells

The therapeutic success of B cell depletion in RA has revived interest in the role of this cell type in RA pathogenesis. In the RA synovium, B cells are found within lymphocyte aggregates which are often surrounded by large numbers of plasma cells. In a proportion of patients, ectopic germinal-centre like structures can be observed in the synovium, with follicles containing T and B cells arranged around a network of follicular dendritic cells (Magalhaes *et al.* 2002; Takemura *et al.* 2001a). Naïve and memory B cells infiltrate and accumulate in the synovial tissue in RA, and there appears to be continuous activation of selected B cell clones with a high migratory capacity (Scheel *et al.* 2011).

For many years, B cells have been regarded as important in RA due to their production of autoantibodies. Autoantibodies initiate immune complex formation within the joint, resulting in complement activation and recruitment of cells to the synovium. B cells have several other functions relevant to the pathogenesis of RA. B cells are able to process and present antigen to T cells, and they may represent the predominant population of antigen presenting cells in later phases of the immune response (MacLennan *et al.* 1997). Rheumatoid-factor-producing B cells have been shown to be particularly effective in binding immune complexes and presenting antigens from the immune complex to T cells, irrespective of the antigen within the antigen-antibody complex (Roosnek and Lanzavecchia 1991). B cells can also secrete several cytokines including IL-6, IL-10, IFN-γ, TGF-β and LT-α, indicating that they may have a

direct inflammatory role and support B cell development (Duddy *et al.* 2007; Pistoia 1997). Studies in mice indicate that IL-10-producing B cells are involved in the suppression of autoimmune and inflammatory disease, including RA (Mauri *et al.* 2003). However, these so-called “regulatory B cells” have not been described in the RA synovium. In addition, B cells can regulate lymphoid tissue organisation and neogenesis (Gonzalez *et al.* 1998; Tumanov *et al.* 2002), and the transfer of synovial tissue into immunodeficient mice demonstrated that B cells were required for germinal centre CD4 T-cell activation (Takemura *et al.* 2001b; Weyand and Goronzy 2003).

B cell depletion in RA patients using rituximab significantly reduces synovial inflammation. However, the return of B cells is accompanied by return of the disease, suggesting that transient B cell depletion by rituximab is unable to reset the immune system, possibly due to the persistence of memory B cells in the bone marrow or synovium (Nakou *et al.* 2009; Teng *et al.* 2009).

1.3.5 Synovial fibroblasts

Synovial fibroblasts are a dominant cell type in the hyperplastic rheumatoid synovium where they have an important role in amplifying the inflammatory cascade by production of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Hashizume *et al.* 2008). Synovial fibroblasts also drive joint destruction by production of MMPs and cathepsins, and promote osteoclastogenesis by expression of RANKL (Hashizume *et al.* 2008).

Compared to synovial fibroblasts from healthy joints, synovial fibroblasts found in the rheumatoid synovium have an altered phenotype in terms of altered morphological features, changes in long-term growth and apoptosis, and altered response to extracellular stimuli. RA synovial fibroblasts show long-term growth and reduced apoptosis which is associated with upregulation of early response genes and proto-oncogenes such as egr-1, c-fos, myc and ras, and a corresponding deficiency of tumour-suppressor genes such as p53, p53-upregulated modulator of apoptosis (PUMA), mapsin and phosphatase and tensin homolog (PTEN) (Cha *et al.* 2006; Matsumoto *et al.* 1996; Pap *et al.* 2000; Pap *et al.* 2004). Synovial fibroblasts also upregulate the anti-apoptotic molecules, FLICE inhibitor protein (FLIP) and sentrin (SUMO-1), as well as members of the Bcl family such as Bcl-2 and Mcl-1 (Franz *et al.* 2000; Kataoka 2005; Palao *et al.* 2004; Salmon *et al.* 1997). This imbalance of pro-apoptotic and anti-apoptotic pathways is thought to contribute to sustaining their long-term survival in the rheumatoid synovium.

Rheumatoid synovial fibroblasts also have invasive properties, facilitating cartilage erosion in RA. In addition to the activating effects of cytokines and growth factors, cytokine-independent pathways appear to be required for induction of an aggressive phenotype. Such triggers could include epigenetic modifications or the galectin-3 system. Galectin-3, which is elevated in tumours and metastasis, induces angiogenesis and inhibits apoptosis, and has been found at high levels in the serum and synovial fluid of RA patients (Ohshima *et al.* 2003). A recent study showed that when healthy human cartilage and cartilage-invading synovial fibroblasts were implanted subcutaneously at distinct sites in SCID mice, the healthy cartilage was also invaded by synovial fibroblasts which appeared to have migrated via the vasculature (Lefevre *et al.* 2009). This has led to the suggestion that the migratory properties of synovial fibroblasts could participate in the spread of RA to previously unaffected joints.

1.4 Cytokines in RA

Cytokines are a heterogeneous group of low molecular weight proteins which act as intercellular mediators. They have pleiotropic effects which include regulation of cell growth, development, differentiation and activation, and are thus involved in almost every biological process. Most cytokines are soluble, although some remain cell-bound, and others exist in both soluble and cell-bound forms. They usually act at short range and are able to produce immediate responses in target cells by activating direct signalling pathways that rapidly effect changes in gene transcription.

Cytokines act within a complex network to regulate the processes involved in chronic inflammation and tissue destruction in RA. In view of the complex cross-talk that exists between immune cell pathways, it is important to try to understand the roles of cytokines as components of larger networks rather than isolated pathways. Novel cytokine activities are being described on an ongoing basis, but it is difficult to understand cytokine interactions and define regulatory hierarchies using existing model systems such as animal models of disease in which genes for individual cytokines are genetically manipulated. While therapeutic targeting of cytokines has been largely successful to date, further targets still need to be identified because of partial and non-responders, the loss of effect observed in most therapeutic regimes, and an increasing drive to achieve remission.

Bioinformatic analysis of the cytokine network as a whole has demonstrated how immune cells function via cytokines in innately integrated and hierarchical collectives. Frankenstein and colleagues (Frankenstein *et al.* 2006) identified a five-tiered system structure of the cytokine connectivities between immune cell types in which the macrophage and B cell form

a pair in the first tier, being reciprocally connected by cytokines to all other cells of the immune system; Th1 and Th2 T cells are paired in a second tier, being mutually connected to other cells, but unique in providing one-way cytokine signals to a variety of cell types; the cytotoxic lymphocyte is in its own tier, being the only cell type that receives multiple one-way signals; neutrophils and NK cells form another tier, receiving more limited one-way signals; while the remaining immune cells comprise the final tier, receiving and sending one-way signals. The analysis also revealed a high degree of cytokine connectivity between immune cells – macrophages, B cells, dendritic cells and NK cells – and non-immune cells – fibroblasts, endothelial cells, epithelial cells, platelets, osteoblasts, osteoclasts, chondrocytes and smooth muscle cells. The involvement of these cell types in wound healing, bone repair and angiogenesis suggests that cytokines have an important role in regulating the physiology of the body as well as its defence. This analysis is interesting in the context of the rheumatoid synovium in which abnormal cytokine networks between immune cells and non-immune cells are known to exist.

Cytokines can be grouped into families using several strategies. Grouping of cytokines into families may be based on structural homology, shared functional properties, or the type of receptor that they engage. Cytokine receptors themselves are grouped by structure into several families. A brief overview of the major cytokine families is given in this chapter.

1.4.1 IL-1 family

The IL-1 family is composed of 11 structurally related cytokines. The first members to be identified were IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-18. Seven additional members have been identified on the basis of sequence homology, three-dimensional

structure, gene location and receptor binding. A new system of terminology has been proposed in which IL-1 α, IL-1β, IL-1Ra and IL-18 become IL-1F1, IL-1F2, IL-1F3 and IL-1F4 respectively, and newly identified cytokines are termed IL-1F5 through IL-1F11, the latter representing IL-33. Although all of the IL-1 family cytokines are extracellular, only IL-1Ra is secreted by the endoplasmic reticulum and Golgi apparatus. The mechanism of secretion of the other IL-1 family members remain unknown. However, IL-1β and IL-18 are known to require processing by a protein assembly complex known as the inflammasome to generate the biologically active forms and to be secreted. IL-1β and IL-18 have amino-terminal pro-domains that require cleavage by the inflammasome. IL-1α also has a pro-domain, but it is not required to be cleaved for its biological activity. IL-1F5, IL-1F6, IL-1F8, IL-1F9 and IL-33 are functional as full-length molecules and do not require processing by the inflammasome. IL-1 family members signal through a group of closely related receptors.

The term IL-1 is often used to refer to IL-1α and IL-1β, as both proteins signal through the same receptor complex and have identical biological activities *in vitro*. However, IL-1β is secreted and circulates systematically, whereas IL-1α is generally associated with plasma membrane and acts locally. Their actions can be blocked by IL-1Ra, and by the decoy receptor, IL-1RII. IL-1α and IL-1β are mainly produced by stimulated monocytes and macrophages, and to a lesser extent by neutrophils, epithelial and endothelial cells, lymphocytes and fibroblasts (Dinarello 1994). IL-1 exerts a broad range of pro-inflammatory effects, inducing mononuclear and stromal cells to produce pro-inflammatory mediators, degradative enzymes, adhesion molecules, cytokines and chemokines. IL-1 also promotes articular destruction by stimulating the maturation of osteoclasts and activating chondrocytes. IL-1 is also reported to induce Th17 differentiation and IL-17 production in mice and humans

(Acosta-Rodriguez *et al.* 2007; Chung *et al.* 2009). IL-1α, IL-1β and IL-1Ra are abundant in the synovial membrane (Dayer 2003). Targeting IL-1 and the IL-1 receptor in rodent models of arthritis effectively reduces inflammation and cartilage and bone damage (Joosten *et al.* 1999a; van den Berg *et al.* 1994). Mice that are deficient in the IL-1Ra develop spontaneous arthritis, partly mediated by enhanced Th17-dependent inflammation (Nakae *et al.* 2003a). Evidence thus suggests that blockade of IL-1 in RA would be beneficial, especially in reducing bone destruction. Several IL-1-targeting agents have been developed for use in RA, but these have shown modest or negative results. The recombinant IL-1R antagonist, anakinra, and a fully human monoclonal anti-IL-1RI showed modest benefit, whereas other approaches to target IL-1 such as rilonacept (IL-1 trap fusion protein), canakinumab (anti-IL-1β antibody) and soluble IL-1RI showed no effect.

IL-18 is produced by a range of cells including macrophages, neutrophils, dendritic cells, osteoblasts and synovial fibroblasts. IL-18 activity is regulated by binding of the soluble IL-18-binding protein (IL-18BP) which prevents IL-18 from interacting with its receptor. IL-18 has diverse functions which include promoting Th1 or Th2 differentiation, depending on other cytokines present, and increasing NK cell cytotoxicity and IFN-γ production. IL-18 is implicated in the pathogenesis of RA and is found at elevated levels in the serum of RA patients and in the RA synovium (Gracie *et al.* 1999). Blockade of IL-18 using neutralising antibody or soluble IL-18 receptor is effective in rodent models of RA, and there is reduced incidence and severity of CIA in mice lacking IL-18 (Plater-Zyberk *et al.* 2001; Wei *et al.* 2001).

IL-33 is the most recently described member of the IL-1 family. It was originally identified as a nuclear protein prominent in high endothelial venules, and its cytokine function was described later. IL-33 is predominantly expressed in the nucleus of cells with a barrier function such as endothelial and epithelial cells. IL-33 can activate T cells and basophils, promote degranulation and pro-inflammatory cytokine production by mast cells and eosinphils, and induce Th2 cell and neutrophil migration (Allakhverdi *et al.* 2007; Alves-Filho *et al.* 2010; Komai-Koma *et al.* 2011; Kurowska-Stolarska *et al.* 2008; Schmitz *et al.* 2005; Smithgall *et al.* 2008). Expression of IL-33 is detected in RA synovial membrane and is produced predominantly by synovial fibroblasts (Xu *et al.* 2008). In murine models of arthritis, administration of IL-33 was found to exacerbate disease, whereas disease was reduced in mice lacking the IL-33 receptor and in mice administered sST2, a natural antagonist of IL-33 (Xu *et al.* 2008). The effects of IL-33 appear to be mediated by the activation of mast cells (Xu *et al.* 2008).

1.4.2 Cytokines binding a γ_c-containing receptor

Several cytokines are grouped on the basis of their usage of heterodimeric cytokine receptors which contain a common gamma chain receptor subunit as a signal-transducing chain, together with a cytokine-specific chain. These cytokines have the common structural feature of a four-helix bundle motif. Cytokines signalling through γ_c-containing receptors include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. IL-13 is closely related to IL-4, and the alpha chain and gamma chain components of the IL-4 receptor both play a role in the IL-13 receptor complex.

IL-2 was the first cytokine shown to bind a receptor containing the common gamma chain. IL-2 is produced by activated T cells and drives their proliferation and differentiation following

antigen stimulation. IL-2 also stimulates NK cells to proliferate and induces cytolytic activity, and stimulates B cells to divide and produce antibody. IL-2 has an important role in controlling immune responses through the development and maintenance of Tregs and also perhaps by suppression of Th17 differentiation and promotion of Fas-mediated cell death. Despite displaying a highly activated phenotype, RA synovial T cells express relatively low levels of IL-2, no spontaneous IL-2 production has been observed in freshly isolated RA T cells, and peripheral blood cells show reduced IL-2 production in RA compared to healthy controls (Combe *et al.* 1985; Firestein *et al.* 1988; Kitas *et al.* 1988; Smeets *et al.* 1998a).

IL-4 is a Th2-cell derived cytokine whose role in RA is not well understood. IL-4 exerts an anti-inflammatory effect by inhibiting the production of pro-inflammatory cytokines by macrophages and monocytes, such as IL-1, TNF-α, IL-6 and IL-12. IL-4 can also induce the production of several cytokine inhibitors such as IL-1Ra, IL-1RII and TNF receptors. IL-4 is the predominant cytokine capable of inducing differentiation of naive T cells into Th2 cells. *In vitro*, IL-4 inhibits MMP and induces tissue inhibitor of metalloproteinase (TIMP) production, and can inhibit osteoclast activity and survival, suggesting a protective role in bone resorption. Animal models of arthritis have suggested both pathogenic and protective roles for IL-4. IL-4 is required for the development of CIA (Ortmann and Shevach 2001). However, in mice with established CIA, treatment with IL-4 suppresses disease and bone erosion is reduced (Joosten *et al.* 1999b; Lubberts *et al.* 2000; Saidenberg-Kermanac'h *et al.* 2004). In addition, CIA is attenuated in mice injected with dendritic cells genetically engineered to produce IL-4 (Morita *et al.* 2001), and in rat adjuvant-induced arthritis IL-4 adenoviral gene therapy reduces disease severity and articular damage (Woods *et al.* 2001). In patients with RA, a polymorphism in the IL-4 receptor that results in reduced responsiveness

to IL-4 is associated with rapidly erosive disease (Prots *et al.* 2006). IL-4 has not been detected in RA synovium but IL-4 has been detected in the synovial fluid of early RA patients (Raza *et al.* 2005).

IL-13 is closely related to IL-4, sharing overlapping functions and being encoded by genes on the same chromosome. The IL-4 receptor alpha chain plays an important role in IL-13 signalling, and the common gamma chain also plays a role although it does not bind IL-13. Like IL-4, IL-13 is a classical Th2 cytokine and is involved in allergic responses and IgE class switching. IL-13 has multiple effects on the differentiation and functions of monocytes and macrophages, and is able to suppress their cytotoxic function (McKenzie *et al.* 1993). In addition, IL-13 can suppress the production of pro-inflammatory cytokines. IL-13 has been detected in synovial fluid from RA patients and is expressed by synovial fluid macrophages (Isomaki *et al.* 1996b; Raza *et al.* 2005; Tokayer *et al.* 2002). Animal models of arthritis indicate a protective role for this cytokine in RA. CIA is attenuated in mice treated with vector cells engineered to secrete IL-13 (Bessis *et al.* 1996; Bessis *et al.* 1999). Similarly, IL-13 adenoviral gene therapy reduces inflammation, vascularisation and bony destruction in rat adjuvant-induced arthritis (Woods *et al.* 2002). Overexpression of IL-13 in immune-complex-mediated arthritis reduced chondrocyte death and MMP-mediated cartilage damage, although there was increased joint inflammation (Nabbe *et al.* 2005).

IL-7 is produced by stromal cells at lymphopoietic sites and has a role in T cell and B cell development and homeostasis (Fry and Mackall 2005; Link *et al.* 2007). IL-7 can also induce TNF-α production by monocytes in a T-cell dependent manner, modify chondrocyte function and promote RANKL-mediated osteoclastogenesis. IL-7 is detected in RA synovial fluid and tissue, and its expression in the synovium has been localised to macrophages, endothelial cells

and fibroblasts (Harada *et al.* 1999; Pickens *et al.* 2011; van Roon *et al.* 2007). Addition of IL-7 to synovial cultures has a pro-inflammatory effect (Harada *et al.* 1999; van Roon *et al.* 2005). In the CIA model of arthritis, blockade of IL-7R ameliorates joint inflammation by reducing T cell trafficking and macrophage production of pro-inflammatory mediators (Hartgring *et al.* 2010).

IL-15 is widely expressed in numerous tissues and cell types, including activated monocytes, mast cells, dendritic cells and fibroblasts (Grabstein *et al.* 1994; Waldmann and Tagaya 1999). It mediates its effects by binding to IL-15R which is found on T and B cells, NK cells, monocytes, macrophages, DCs and fibroblasts. IL-15 can also participate in trans-signalling in which IL-15-IL-15Rα complexes on one cell can bind to IL-15Rβγ chains on adjacent cells. IL-15 has broad pro-inflammatory effects including induction of Th1 and Th17 cell proliferation, and activation of neutrophil, macrophage and mast cell effector functions (Lodolce *et al.* 2002; Miranda-Carus *et al.* 2004; Verri *et al.* 2007; Waldmann and Tagaya 1999). IL-15 is present in the RA synovium where it is expressed by macrophages and synovial fibroblasts, and is spontaneously produced in RA synovial cultures and isolated synovial fibroblasts (McInnes *et al.,* 1996; Harada *et al.,* 1999). In the RA synovium, IL-15 drives the release of IFN-γ, IL-17, TNF-α and various chemokines, and also promotes the survival and migration of synovial T cells and synovial fibroblasts (McInnes *et al.* 1996; McInnes *et al.* 1997; Miranda-Carus *et al.* 2004). IL-15 blockade reduces severity of CIA (Ferrari-Lacraz *et al.* 2004; Ruchatz *et al.* 1998). IL-15 has been targeted in RA using an antibody specific for human IL-15, and has showed clinical responses during clinical trial (Baslund *et al.* 2005).

IL-21 is a potent inflammatory cytokine which plays an important role in B cell maturation and plasma cell development, and is also involved in T cell activation and differentiation, including the generation of Th17 cells (Nakae *et al.* 2003b). IL-21 induces NK-cell and NKT-cell maturation and activation, and promotes the generation of Tfh cells. It may also suppress the generation of Tregs. IL-21 is detected in the synovial fluid and synovial tissue in patients with RA (Andersson *et al.* 2008). Addition of IL-21 to synovial T cell cultures induces activation and pro-inflammatory cytokine production (Jungel *et al.* 2004; Li *et al.* 2006), whereas neutralisation of IL-21 in synovial cultures suppresses release of TNF-α, IL-1 and IL-6 (Andersson *et al.* 2008). Mice deficient in IL-21 receptor are resistant to development of spontaneous arthritis, and have fewer homeostatically proliferating and Tfh cells than control mice, but more Th17 cells (Jang *et al.* 2009). In CIA, blockade of IL-21 delays onset and progression of disease (Young *et al.* 2007). In addition, polymorphisms in IL-21 loci have been identified as a risk factor for RA (Daha *et al.* 2009).

1.4.3 Cytokines binding a gp130-containing receptor

Several cytokines signal through a receptor containing the signal-transducing protein glycoprotein 130 (gp130). These cytokines have a four alpha-helix structure. This family includes IL-6, IL-11, IL-27, IL-31, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, and osteopontin.

IL-6 is predominantly produced by mononuclear phagocytic cells, and is also produced by T cells, B cells, fibroblasts, endothelial cells, keratinocytes, hepatocytes and bone marrow cells. IL-6 was originally identified as a substance produced by T cells which induced B cells to produce antibodies (Hirano *et al.* 1986). IL-6 is now known to have a wide range of biological

effects including the activation of B cells and T cells, macrophages, osteoclasts, chondrocytes and endothelial cells. IL-6 also induces and the proliferation of keratinocytes and has effects on haematopoiesis. IL-6 shares several activities with IL-1, including the induction of pyrexia and production of acute phase proteins. IL-6 exerts its effects by binding to the IL-6 receptor (IL-6R) which then associates with gp130 (Taga *et al.* 1989). IL-6R exists as a membrane-bound receptor and also in a soluble form (Lust *et al.* 1992; Matthews *et al.* 2003). Soluble IL-6R and IL-6 are able to stimulate cells which express gp130 but not membrane-bound IL-6R in a process known as trans-signalling (Mackiewicz *et al.* 1992). IL-6 is elevated in RA synovial fluid and synovial tissue (Houssiau *et al.* 1988), and IL-6 in synovium correlates with disease activity (Cohick *et al.* 1994; Tak *et al.* 1997). In CIA, deletion of the IL-6 gene is protective, and administration of antibodies specific for IL-6 ameliorates disease (Alonzi *et al.* 1998). Using an experimental arthritis model in IL-6-deficient mice, administration of a soluble IL-6R/IL-6 fusion protein restored disease activity, while blockade of soluble IL-6R by soluble gp130 ameliorated disease (Nowell *et al.* 2003). Tocilizumab is a monoclonal antibody targeted against the IL-6 receptor which reduces inflammation and erosions in RA, and has been approved for clinical use.

IL-11 regulates the growth and development of haematopoietic stem cells and polarises T cell differentiation towards a Th2 phenotype (Curti *et al.* 2001). It mediates anti-inflammatory effects by suppressing production of pro-inflammatory cytokines and nitric oxide production (Trepicchio *et al.* 1996). IL-11 is detected in the serum, synovial fluid and synovium of patients with RA (Hermann *et al.* 1998). IL-11 has immunoregulatory effects on RA synovial tissue cultures, inhibiting spontaneous production of TNF-α and MMPs (Hermann *et al.* 1998). In animal models of collagen-induced and adjuvant-induced arthritis, treatment with recombinant human IL-11 (rhIL-11) reduced the level of synovitis and the histologic lesion

scores in the joints, suggesting that IL-11 could have therapeutic activity in RA (Walmsley *et al.* 1998). However, in a phase-I/II randomised trial of recombinant human IL-11, no therapeutic benefit was found (Moreland *et al.* 2001).

1.4.4 IL-12 family

The IL-12 family comprises a family of structurally related cytokines which are composed of two covalently-linked subunits. IL-12 was the first cytokine to be identified as a heterodimeric cytokine, and is composed of the IL-12p40 and IL-12p35 subunits. The IL-23p19 subunit was later identified, which dimerises with IL-12p40 to form IL-23. IL-12 and IL-23 signal through receptors which share a common component, IL-12Rβ1. IL-27 is a cytokine formed from IL-27p28 and the Epstein-Barr virus (EBV)-induced molecule (EBI3). The receptor for IL-27 comprises WSX1 and gp130. The newest family member is IL-35, which is composed of EBI3 and IL-12 p35.

The two subunits of IL-12, p35 and p40, are both required for the biological activity of IL-12. The p40 subunit can be secreted in the absence of p35, but p35 cannot be secreted without co-expression of p40. p35 is found at low levels in many cell types, while expression of the p40 subunit is usually much higher and restricted to cells that produce the biologically active dimer. IL-12 is produced by dendritic cells and monocytes and macrophages in response to bacterial products and immune signals. IL-12 induces the development of Th1 cells. Some studies have demonstrated a correlation between elevated IL-12 in the serum and synovial fluid with disease activity in RA (Kim *et al.* 2000), whereas others have not found IL-12 to be increased in RA.

IL-23, composed of a unique p19 subunit and an IL-12p40 subunit, also requires expression of both subunits for the biologically active cytokine. *In vitro* experiments have shown that activated monocytes and dendritic cells are capable of inducing both subunits required for functionally active IL-23, but activated synovial fibroblasts only express the p19 subunit. IL-23 is produced predominantly by activated dendritic cells and macrophages and can be triggered by TLR agonists and CD40/CD40L interaction with T cells. IL-23 binds to its receptor on memory T cells, NK cells, macrophages and dendritic cells. IL-23 appears to be required for the induction and maintenance of Th17 cells (Veldhoen *et al.* 2006). IL-23 also upregulates RANK on myeloid precursors and induces RANKL on T cells indicating a role in osteoclastogenesis and bone erosion. In RA synovial tissue, high levels of IL-23p19 expression are reported, but only low levels of bioactive IL-23 (Brentano *et al.* 2009; Kim *et al.* 2007; Liu *et al.* 2007). In a murine model of CIA, administration of anti-IL-23 produced less severe disease (Yago *et al.* 2007), and in a spontaneous arthritis murine model administration of IL-23 increased disease severity (Ju *et al.* 2008). Furthermore, IL-23p19-deficient mice are protected against arthritis (Murphy *et al.* 2003), indicating a pathogenic role for IL-23 in disease. Ustekinumab, a human monoclonal antibody against IL-12p40 that targets IL-12 and IL-23 is in phase III clinical trials.

Another member of the IL-12 family is IL-27, composed of a specific IL-27p28 subunit and the EBI3 subunit. IL-27 is secreted by activated monocytes and macrophages and dendritic cells and binds to the IL-27R expressed on naïve CD4 T cells, NK cells, mast cells, macrophages and neutrophils. IL-27 induces the proliferation of naïve T cells, promoting Th1 differentiation (Lucas *et al.* 2003; Pflanz *et al.* 2002) and suppressing Th17 development (Amadi-Obi *et al.* 2007; Artis *et al.* 2004; Batten *et al.* 2006; Villarino *et al.* 2003). In addition, animal models of infection have demonstrated anti-inflammatory effects of IL-27, as

observed by the increased T cell and NK cell responses in the absence of the IL-27 receptor component WSX1, and increased mast cell and macrophage responses in the absence of the IL-27 receptor (Holscher *et al.* 2005; Pearl *et al.* 2004; Villarino *et al.* 2003). IL-27 has been detected in the synovial tissue of patients with RA (Niedbala *et al.* 2008). In murine models of arthritis, IL-27 has been found to have a protective or pathogenic role, depending on the model used. IL-27 has a pathogenic effect in adjuvant-induced arthritis and proteoglycan-induced arthritis, which are considered to be Th1-mediated (Cao *et al.* 2008; Goldberg *et al.* 2004). In contrast, IL-27 has a protective role in CIA, which is thought to be Th17-mediated (Niedbala *et al.* 2008).

1.4.5 IL-10 family

The IL-10 family comprises a group of cytokines which are structurally related to IL-10. Bioinformatics was used to identify molecules with a similar structure to IL-10, which contains six alpha helices. This led to the isolation and naming of new family members IL-19, IL-20, IL-22, IL-24 and IL-26.

IL-10 is an immunoregulatory cytokine that blocks production of pro-inflammatory cytokines and inhibits the antigen presenting capacity of macrophages and dendritic cells. IL-10 also has a weak stimulatory effect on B cells. IL-10 is produced by macrophages, T and B cells, dendritic cells, mast cells, NK cells and neutrophils. IL-10 is produced by macrophages and T cells in the RA synovium (Katsikis *et al.* 1994), and in RA synovial tissue membrane cultures is able to regulate endogenous pro-inflammatory cytokine production (Cohen *et al.* 1995; Katsikis *et al.* 1994). IL-10 levels have been found to be increased in patients with RA (Alanara *et al.* 2009; Cush *et al.* 1995; Isomaki *et al.* 1996a). High IL-10 producer genotypes

are increased in patients with a higher rate of joint destruction (Lard *et al.* 2003), and are associated with more severe radiographic damage in seronegative patients (Marinou *et al.* 2007). In animal models of arthritis, CIA is exacerbated in IL-10-deficient mice, whereas treatment of established CIA with IL-10 suppresses disease (Ortmann and Shevach 2001; Walmsley *et al.* 1996), indicating a protective role for IL-10 in the disease. However, clinical studies performed with human recombinant IL-10 in patients with rheumatoid arthritis (RA) have shown little efficacy (Maini *et al.*, 1997).

IL-20 is expressed by monocytes, epithelial cells, and endothelial cells and induces production of inflammatory mediators. IL-20 has been shown to induce cytokine secretion by synovial fibroblasts, and promote neutrophil chemotaxis, synovial fibroblast migration and endothelial cell proliferation. IL-20 and its three receptors are expressed in synovial membranes and synovial fibroblasts in synovial tissue of RA patients and CIA rats, and levels of IL-20 are elevated in RA synovial fluid compared to healthy controls (Hsu *et al.* 2006). In a rodent model of CIA, administration of soluble IL-20 receptor type 1 significantly reduced disease (Hsu *et al.* 2006), supporting a pro-inflammatory role for IL-20 in RA.

IL-22 is known to be produced by Th17 and Th22 cells (Duhen *et al.* 2009; Liang *et al.* 2006; Trifari *et al.* 2009). IL-22 has been detected in RA synovial tissue macrophages and fibroblasts and synovial fluid mononuclear cells (Ikeuchi *et al.* 2005). Addition of recombinant IL-22 to synovial fibroblasts was found to enhance proliferation and production of CCL2. In the CIA model of arthritis, mice deficient in IL-22 have reduced incidence of arthritis and pannus formation, and IL-22 was found to promote osteoclastogenesis *in vitro*, indicating a pro-inflammatory role for IL-22 in RA (Geboes *et al.* 2009).

1.4.6 IL-17 family

The IL-17 family is a group of cytokines which shares little homology with other cytokines. Six family members have been identified, designated IL-17A to IL-17F. The first described cytokine known as IL-17 has been termed IL-17A. The five related cytokines which have since been identified share 20% to 50% sequence homology with IL-17A. The members of the IL-17 family bind to a unique class of cytokine receptors.

IL-17 plays a critical role in the defence against extracellular pathogens. IL-17 is able to induce production of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, nitric oxide species and MMPs by fibroblasts, macrophages and endothelial cells (Fossiez *et al.* 1996; Parsonage *et al.* 2008). IL-17 also induces several chemokines that attract T cells and macrophages to sites of inflammation. IL-17 promotes osteoclastogenesis by upregulating RANKL on osteoblasts (Fossiez *et al.* 1996; Parsonage *et al.* 2008; Sato *et al.* 2006).

Evidence from animal and human studies suggests that IL-17 may be of importance in the pathogenesis of RA. In CIA, the incidence and severity of disease is markedly reduced in IL-17-deficient mice (Nakae *et al.* 2003a), while administration of IL-17 promotes osteoclastic bone destruction (Lubberts *et al.* 2003). IL-17 is present in RA synovial fluid and Th17 cells have been detected in synovial tissue (Fossiez *et al.* 1996; Hitchon *et al.* 2004; Kotake *et al.* 1999; Ziolkowska *et al.* 2000). In RA synovial fibroblasts, IL-17 stimulated the production of IL-6, CXCL8, leukaemia inhibitory factor and prostaglandin E2, and could synergise with IL-1 and TNF-α to induce production of cytokines and MMPs (Miossec 2003). IL-17 stimulated dendritic cell migration and recruitment of T cells by inducing production of CCL20 by RA synoviocytes (Chabaud *et al.* 2001). In a recent study which assessed the cytokine profile and

frequency of IL-17-producing CD4 T cells in RA synovial fluid, synovial fluid mononuclear cells were found to have reduced levels of IL-22 (a Th17- and Th1-derived cytokine) and did not express IL-23R which is important for Th17 expansion and survival (Church *et al.* 2010). Compared to the peripheral blood and synovium, there was a modest enrichment of IL-17-producing CD4 T cells in the synovial fluid but the overall proportion of this population was low, suggesting that there may be an alternative source of IL-17 in the rheumatoid joint. Indeed, expression of IL-17 has been reported by mast cells in the RA synovium (Hueber *et al.*). IL-17 has been investigated as a potential therapeutic target in RA. In two phase I clinical trials, a monoclonal anti-IL-17 was reported to show benefit in RA compared to placebo (Genovese *et al.* 2010; Hueber *et al.* 2010).

1.4.7 TNF family

Several cytokines have been identified as part of the TNF family on the basis of structural and functional homology. TNF family cytokines are trimeric proteins which are membrane-bound, with the exception of TL1A which is synthesized as a secreted soluble protein. Some of the TNF family members may be cleaved to generate soluble forms. The founding members of the TNF family, TNF-α and LT-α were initially identified as proteins that possess tumour cytotoxicity; however, it is now recognised that TNF family members have a variety of cellular functions including regulating cell differentiation, effector function, survival and apoptosis. TNF family members are important in regulating key biological processes including inflammation, lymphoid organogenesis, bone homeostasis, and developmental processes. TNF family cytokines bind to membrane-bound receptors of the TNF receptor family. Members of this receptor family are typically transmembrane proteins, although some TNF family receptors exist as soluble proteins. The members of the TNF family are LT-α

(TNFSF1), TNF-α (TNFSF2), LT-β (TNFSF3), OX40L (TNFSF4), CD40L (TNFSF5), FASL (TNFSF6), CD70 (TNFSF7), CD30L (TNFSF8), 4-1BB-L (TNFSF9), TRAIL (TNFSF10), RANKL (TNFSF11), TWEAK (TNFSF12), APRIL (TNFSF13), BAFF (TNFSF13B), LIGHT (TNFSF14), TL1A (TNFSF15) and GITRL (TNFSF18).

TNF-α is considered a classical pro-inflammatory cytokine and plays an important role in host defence. TNF-α is mainly produced by activated macrophages but can also be released by other leukocytes including T cells, NK cells and neutrophils. During infection, production of TNF-α by macrophages triggers local containment of infection by acting on blood vessels to increase blood flow and permeability, and to increase the ability of leukocytes and platelets to adhere to the endothelial cells. The local release of TNF-α thus allows an influx of cells and proteins into the infected tissue where they participate in host defence. TNF-α is recognised as a pivotal pro-inflammatory cytokine that mediates inflammation and joint destruction in RA, and therapeutic targeting of this molecule has proved to be highly successful (Feldmann *et al.* 1994). Blockade of cytokines produced spontaneously by RA synovial cultures showed that blockade of TNF-α downregulated IL-1, and also GM-CSF, IL-6 and IL-8 (Butler *et al.* 1995; Haworth *et al.* 1991). Furthermore, samples from RA patients in clinical trials showed that following anti-TNF therapy there was a dramatic and rapid reduction in levels of serum IL-6 and many other cytokines in the blood and synovium (Charles *et al.* 1999). These findings support the concept of a TNF-α-dependent cytokine cascade operating in RA. In RA, TNF-α induces leukocyte and endothelial cell activation, leading to the production of other pro-inflammatory cytokines such as IL-1 and IL-6, and leukocyte accumulation in the inflamed synovium. Furthermore, TNF-α stimulates MMP release by fibroblasts leading to cartilage destruction, and also has a role in promoting angiogenesis. TNF-blocking agents

currently approved for clinical use are infliximab (a chimeric antibody specific for human TNF-α), etanercept (a fusion protein comprised of human soluble TNF receptor linked to the Fc component of human IgG1), adalimumab (a fully humanised antibody specific for human TNF-α), certolizumab pegol (a humanized antigen-binding fragment (Fab') of a monoclonal antibody that has been conjugated to polyethylene glycol), and golimumab (a fully humanised antibody specific for human TNF-α), all of which act by partly neutralising circulating and synovial TNF-α. TNF blockade is effective in reducing leukocyte trafficking to the joints, reducing fatigue and improving mood, and reducing pain. Importantly, anti-TNF therapy has been shown to reduce joint inflammation and slow radiographic progression of joint damage (Maini and Taylor 2000). Therapeutic blockade of TNF-α produces a clinical response in approximately 70% of patients with established RA, and is able to induce remission in a proportion of these patients (Quinn *et al.* 2005).

RANKL is a TNF family member which has an important role in bone homeostasis. RANKL is expressed at high levels in skeletal tissues and in primary and secondary lymphoid tissues. RANKL is produced either as a transmembrane protein or in a soluble form which can be derived from proteolytic cleavage or alternative splicing. Binding of surface-bound or soluble RANKL to its membrane-bound receptor, RANK, is critical for the terminal differentiation of osteoclasts from myeloid lineage precursors. Osteoclasts degrade bone via expression of effector molecules such as cathepsins, MMPs and local production of hydrogen ions. RANKL signalling can be regulated by osteoprotegerin (OPG), a soluble decoy receptor which is produced by bone-forming osteoblasts.

High levels of RANKL expression have been described in the RA synovium (Crotti *et al.* 2002; Gravallese *et al.* 2000; Takayanagi *et al.* 2000), and RANKL is expressed predominantly at the pannus-cartilage junction at sites of bone erosion (Pettit *et al.* 2006). Levels of soluble RANKL have also been reported to be elevated in the synovial fluid of RA patients (Skoumal *et al.* 2005; Ziolkowska *et al.* 2002). Cytokines present in the RA synovium such as TNF-α, IL-1β, IL-6 and IL-17 are capable of inducing RANKL expression by synovial fibroblasts, osteoblasts and bone marrow stromal cells (Gravallese *et al.* 2000; Hashizume *et al.* 2008; Kotake *et al.* 1999; Romas *et al.* 2002). In addition, RANKL is expressed by synovial T cells (Crotti *et al.* 2002). Anti-RANKL therapies have been shown to inhibit bone loss in post-menopausal women (Bone *et al.* 2008; Cohen *et al.* 2008; Kearns *et al.* 2008; McClung *et al.* 2006), and the fully human monoclonal antibody specific for RANKL, denosumab, is effective in suppressing erosive progression in RA (Cohen *et al.* 2008).

The observation that RANKL-deficient mice have a defect in the development of lymph nodes indicates that RANKL also has an important role in the immune system (Kong *et al.* 1999b; Theill *et al.* 2002). An immunoregulatory role for RANKL has been suggested by the observation that RANKL is involved in the generation of Tregs in a model of diabetes (Green *et al.* 2002).

BAFF and APRIL are TNF family members which regulate B cell maturation, differentiation and activation. BAFF is expressed by various cell types including monocytes, dendritic cells and bone marrow stromal cells. Several studies have suggested a role for BAFF in RA. BAFF is detected in the RA synovium in macrophages and synovial fibroblasts (Ohata *et al.* 2005).

In animal models of arthritis, silencing of BAFF ameliorates disease and suppresses Th17 cell generation (Lai Kwan Lam *et al.* 2008). In the human-synovium-SCID mouse model, disruption of BAFF-BAFF-receptor interaction with the TACI-Ig Fc fusion protein inhibits antibody production and cytokine production by B cells (Seyler *et al.* 2005). A fully humanised anti-BAFF antibody, belimumab, has demonstrated modest efficacy in clinical trials in RA.

The B cell survival factor, APRIL, is produced predominantly by monocytes and macrophages. APRIL has also been implicated in RA, and is found to be highly expressed by dendritic cells in RA synovial membranes containing ectopic germinal centres (Seyler *et al.* 2005). APRIL levels are elevated in RA synovial fluid and correlate with plasma cells in the synovium (Dong *et al.* 2009). APRIL-deficient mice have a reduced incidence of CIA with reduced antibody and IL-17 production (Xiao *et al.* 2008), whereas APRIL overexpression correlates with increased autoantibody production and induces pro-inflammatory production by RA synovial fibroblasts (Nagatani 2007). Heterodimeric complexes of BAFF and APRIL are found at elevated levels in the serum in RA, and correlate with synovial lymphoid architecture and disease severity (Seyler *et al.* 2005; Vallerskog *et al.* 2006). Atacicept, a fusion protein which prevents binding of both BAFF and APRIL to B cells, has been trialled in a phase II study in RA but did not produce significant improvement (van Vollenhoven *et al.* 2011).

LT-α and LT-β are TNF family members which are involved in the development of secondary lymphoid organs and germinal centre organisation. LT-α may be secreted as a homotrimer, or form a membrane-bound heteromeric complex with LT-β. LT-α is secreted by Th1 cells,

whereas LT-β does not appear to function by itself. Cell-surface lymphotoxin heteromers are expressed by activated lymphocytes and a subset of resting B cells. The receptor LTβR is expressed mainly by non-haematopoietic and myeloid lineage cells. LT-α activates macrophages, inhibits B cells and is directly cytotoxic for some cells. The membrane-bound form of lymphotoxin can promote production of cytokines, chemokines and MMPs by synovial fibroblasts, including IL-1, IL-6, GM-CSF, CCL2, CCL5, MMP1 and MMP3 (Braun *et al.* 2004). The lymphotoxin pathway has been implicated in the development of tertiary lymphoid structures in chronically inflamed tissue associated with autoimmune diseases, including RA. LT-α and LT-β are found in the RA synovial tissues containing B cell follicles, and B cell expression of LT-β is associated with germinal centre formation (Takemura *et al.* 2001a). LT-β expression in the RA synovium has also been found to correlate with the degree of inflammation (O'Rourke *et al.* 2008). Blockade of LTβR is beneficial in preventing disease in animal models of arthritis (Gommerman 2003, Han 2005), and treatment of mice with an LT-α-specific monoclonal antibody ameliorates disease (Chiang 2009). LTβR-Ig has been trialled in patients with RA, but failed to meet clinical end points in a Phase IIb clinical trial.

1.4.8 Interferons

Interferons are widely expressed cytokines with potent anti-viral and growth-inhibitory effects. There are three major classes of IFNs, designated type I, type II and type III. Within the family of type I IFNs, there are several members, all of which have considerable structural homology. The subtypes that exist in humans are IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω. IFN-α can be further subdivided into 13 different subtypes. The most highly expressed members of the type I IFN family are IFN-α and IFN-β. All type I IFNs bind a common cell-surface receptor known as the type I IFN receptor. There is only one member of the type II

IFN family, IFN-γ. IFN-γ does not have marked structural homology with type I IFNs, and binds a different receptor, the type II IFN receptor. IFN-γ was originally classified in the IFN family because of its ability to "interfere" with viral infections, consistent with the original definition of interferons. The type III IFNs consist of IFN-λ1, IFN-λ2 and IFN-λ3, which are also called IL-29, IL-28A, and IL-28B, respectively. Type III IFNs exhibit several features common with type I IFNs including activation of the same signalling pathways and anti-viral, anti-proliferative and anti-tumour activities. They act through a receptor composed of an IFN-γ-specific chain and an IL-10R2 chain.

IFN-α and IFN-β are produced by many cell types in response to infection by viruses, and induce the transcription of several proteins that help to inhibit viral replication. IFN-α and IFN-β also activate NK cells which can kill virus-infected cells. IFN-α and IFN-β activate the adaptive immune response by inducing the expression of co-stimulatory molecules on macrophages and dendritic cells to enable them to act as antigen presenting cells. IFN-α and IFN-β also stimulate increased expression of MHC class I molecules on all cell types, thus promoting the killing of virus-infected cells by CD8 T cells. Almost all cell types can produce IFN-α and IFN-β if required, although plasmacytoid dendritic cells are capable of expressing particularly high levels.

Evidence suggests that type I IFNs play a crucial role in RA. Gene expression profiling studies of RA patients have identified a type I IFN signature in a subset of patients (Thurlings *et al.* 2010; van der Pouw Kraan *et al.* 2007; Vosslamber *et al.* 2011). Circulating lymphocytes from patients with RA have been found to exhibit significantly reduced IFN-α

production after stimulation with virus compared to healthy controls (Seitz *et al.* 1987), and RA synoviocytes exposed to IFN-α exhibit increased expression of the anti-inflammatory molecules IL-1Ra and soluble TNF receptor (Wong *et al.* 2003). IFN-α therapy has been associated with *de novo* development of RA in the treatment of malignant melanoma and chronic HCV infection (Ionescu *et al.* 2008; Passos de Souza *et al.* 2001), but there have also been reports of the effectiveness of IFN-α in treating RA (Niewold and Gibofsky 2006; Shiozawa *et al.* 1992).

There has been much interest in the role of IFN-β in RA. IFN-β is involved in suppression of the pro-inflammatory cytokines IL-1β and TNF-α, induction of the anti-inflammatory cytokines IL-1Ra, IL-10 and TGF-β (Coclet-Ninin *et al.* 1997; Palmer *et al.* 2004; van Holten *et al.* 2002; Yong *et al.* 1998), suppression of MHC Class II expression (Yong *et al.* 1998), inhibition of T cell activation (Noronha *et al.* 1993), and reduction of adhesion molecule expression (Calabresi *et al.* 1997). IFN-β has also been identified as a stromal cell-derived factor that is able to rescue T cells from apoptosis (Pilling *et al.* 1999). Studies performed in animal models of arthritis suggest a protective role for IFN-β in RA. In the collagen-induced and adjuvant-induced models of arthritis, administration of IFN-β resulted in reduced disease activity and suppressed cartilage and bone destruction, whereas IFN-β deficiency worsened disease (Adriaansen *et al.* 2006; Treschow *et al.* 2005; van Holten *et al.* 2004). Systemic administration of IFN-β has been trialled for the treatment of RA but did not show clinical benefit in a phase II study (van Holten *et al.* 2005).

IFN-γ is the main cytokine released by CD8 effector T cells, which can block viral replication by inducing MHC class I and class II expression in somatic cells, and activating NK cells. IFN-γ is also produced by Th1 cells, and has potent effects in activating macrophages. IFN-γ is detected at relatively low levels in RA synovial T cells (Firestein and Zvaifler 1987; Smeets *et al.* 1998a). In animal models of arthritis, IFN-γ has been reported to have both protective and pathogenic effects, which may be explained by its ability to both induce inflammation and also inhibit Th17 differentiation. In the CIA model of arthritis, disease is accelerated in IFN-γ-deficient mice, indicating a protective role. (Cooper *et al.* 1988; Manoury-Schwartz *et al.* 1997; Mauritz *et al.* 1988; Sarkar *et al.* 2009; Vermeire *et al.* 1997). In contrast, proteoglycan-induced arthritis is dependent on IFN-γ, suggesting a pathogenic role (Doodes *et al.* 2008; Finnegan *et al.* 1999).

1.4.9 Growth factors

Growth factors are diverse group of proteins capable of stimulating cell growth, proliferation and differentiation. Growth factors can be grouped by structure or function into several families.

Colony-stimulating factors were originally identified as growth and differentiation factors for haematopoietic precursor cells. Granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF) were initially defined by their abilities to generate colonies of mature myeloid cells from bone-marrow precursor cells - granulocytic and macrophage colonies in the case of GM-CSF, macrophage colonies in the case of M-CSF, and granulocytic colonies in the case of G-CSF.

M-CSF (CSF1) promotes the survival, proliferation and differentiation of monocytes/macrophages, and is also required for osteoclast differentiation. M-CSF can be expressed by fibroblasts, macrophages, bone marrow stromal cells and endothelial cells. M-CSF is detected in RA synovial fluid, and is produced at high levels by synovial fibroblasts (Kawaji *et al.* 1995; Seitz *et al.* 1994; Williamson *et al.* 1989). M-CSF is also produced by endothelial cells from RA synovium and has been shown to stimulate osteoclastogenesis (Nakano *et al.* 2007). Administration of M-CSF exacerbates disease in murine models of arthritis (Abd *et al.* 1991; Bischof *et al.* 2000; Campbell *et al.* 2000), supporting a role in promoting disease and joint destruction.

GM-CSF (CSF2) stimulates the growth of cells of the myelomonocytic lineage. GM-CSF is produced by a range of cell types including T cells, B cells, macrophages, mast cells, endothelial cells, fibroblasts and adipocytes. GM-CSF is detected in RA synovial fluid and is spontaneously produced by RA synovial cells (Bell *et al.* 1995; Haworth *et al.* 1991). Administration of GM-CSF exacerbates murine CIA, indicating a pathogenic effect (Bischof *et al.* 2000). A phase I study using a human monoclonal antibody targeting the GM-CSF receptor-alpha in RA has shown preliminary evidence of pharmacodynamic activity (Burmester *et al.* 2011).

G-CSF (CSF3) stimulates the development and differentiation of neutrophils. G-CSF is mainly produced by monocytes and macrophages, and can also be secreted by other cell types including fibroblasts, endothelial cells and bone marrow stromal cells. G-CSF is used to treat neutropenia and mobilise hematopoietic stem cells for transplantation. G-CSF is present in RA synovial fluid (Nakamura *et al.* 2000; Williamson *et al.* 1989). IL-1 and TNF-α induce the

production of G-CSF by human synovial fibroblasts and chondrocytes *in vitro* (Campbell *et al.* 1991; Leizer *et al.* 1990). Administration of G-CSF exacerbates murine CIA (Campbell *et al.* 2000), and a passive transfer model of CIA in rats (`Miyahara *et al.* 1993) whereas G-CSF blockade in murine CIA reduces disease activity (Lawlor *et al.* 2004). Administration of r-Hu-methionyl G-CSF to patients with RA in a trial for using haematopoietic stem cell mobilisation produced flare of RA in a proportion of patients (Snowden *et al.* 1998). Evidence thus suggests a pro-inflammatory role for G-CSF in RA.

Macrophage migration inhibitor factor (MIF) regulates the activation of macrophages and lymphocytes. MIF is expressed by several cell types including T cells and macrophages. MIF has a pro-inflammatory effect mediated by its ability to induce macrophages to release a range of cytokines including TNF-α, IL-1, IL-6 and IL-8, and stimulate synovial fibroblast to produce phospholipase $A_{2,}$ cyclooxygenase-2, prostaglandin E2 and MMPs. Elevated levels of MIF have been reported in RA synovial fluid (Onodera *et al.* 1999), and MIF expression in the synovium has been localised to macrophages, endothelial cells, synovial fibroblasts and T cells (Leech *et al.* 1999; Onodera *et al.* 1999). MIF induces RA synovial fibroblasts to produce pro-inflammatory mediators and MMPs including IL-1, IL-6, COX2 and PGE2 (Onodera 2000, Santos 2004). In murine models of arthritis, antagonism of MIF delayed onset and decreased frequency of CIA, and inhibited adjuvant-induced arthritis; conversely, adjuvant-induced arthritis severity was decreased in MIF-deficient mice (Leech *et al.* 2003; Mikulowska *et al.* 1997; Santos *et al.* 2001; Santos *et al.* 2008). Synovial MIF expression and polymorphisms in the MIF promoter have been reported to correlate with disease activity and increased risk of erosions in RA (Morand 2002, Baugh 2002, Radstake 2005).

Epidermal growth factor (EGF) is a pleiotropic cytokine with roles in cell proliferation, apoptosis, angiogenesis, invasion, and metastasis. EGF is a mitogen for fibroblasts, epithelial cells and endothelial cells. EGF stimulates expression of inflammatory mediators such as COX2 and PGE2, and is found at high levels in the synovial fluid of RA patients (Maruotti *et al.* 2006; Satoh *et al.* 2001). EGF has also been found to be constitutively produced by RA synovial fibroblasts (Bucala *et al.* 1991).

The transforming growth factor-β (TGF-β) family includes a large number of structurally and functionally related growth factors which regulate many cellular functions including cell growth, adhesion, migration, differentiation and apoptosis. The first member of the family, TGF-β, was identified by its ability to induce or 'transform' the growth of cultured fibroblasts. Among the TGF-βs, six distinct isoforms with a variable degree of homology have been identified. Three closely related isoforms, TGF-β1, TGF-β2, and TGF-β3 are expressed in mammals. TGF-βs are secreted in an inactive form and are activated by proteolytic processing which releases them from a multiprotein complex. Recent studies have demonstrated a critical role for TGF-β in the regulation of immune responses due to its role in differentiation of Th17 cells and regulatory T cells. TGF-β also has an important role in cartilage matrix metabolism. Serum levels of TGF-β1 have been found to correlate with joint damage in RA (Mieliauskaite *et al.* 2009).

The family of fibroblast growth factors (FGFs) are involved in many aspects of development, including the regulation of cell proliferation and differentiation, survival and migration. FGFs regulate developmental pathways from the early embryo stage through to angiogenesis and

wound repair in the adult. The mammalian FGF family comprises 18 structurally-related members which signal through four highly conserved receptors. One member of the FGF family, FGF-2 was originally identified based on its stimulatory activity on fibroblast proliferation, and has subsequently been found to be involved in numerous cellular functions in various cell types, including angiogenesis, cell proliferation and differentiation, wound healing and tissue remodelling. The relevance of these processes in the pathogenesis of RA has promoted interest in the role of FGF-2 in RA. FGF-2 is detected in RA synovial tissue and synovial fluid, and expression in the synovium has been localised to mast cells and vascular cells (Qu *et al.* 1995).

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that induces proliferation and migration of endothelial cells to form new blood vessels. VEGF also increases vascular permeability and directly increases production of the pro-inflammatory cytokines TNF-α and IL-6. VEGF is found to be elevated in the serum and synovium of patients with RA and raised serum levels have been associated with disease activity (Lee *et al.* 2001; Sone *et al.* 2001). Expression of VEGF in the RA synovium is associated with distinct vascular morphological patterns including the formation of new blood vessels (Fearon 2003). The hypoxic environment of the RA joint results in increased VEGF gene transcription through the binding of hypoxia-inducible transcription factors such as HIF-1 which is upregulated in the RA synovium (Hollander 2001). In the CIA model of arthritis, administration of an antibody specific for VEGF prevents disease (Choi *et al.* 2009b).

1.4.10 Chemokines

Chemokines, or “chemotactic cytokines”, are small peptide molecules (8-12 kDa) which have the ability to regulate cell migration and are involved in multiple processes including lymphoid organ development, lymphocyte trafficking, angiogenesis and inflammation (Rot and von Andrian 2004). To date, approximately 50 chemokines have been discovered with shared sequence homology (Borish and Steinke 2003). Chemokines mediate their biological effects by binding to their own family of chemokine receptors which are G Protein Coupled Receptors (Murphy *et al.* 2000). Chemokines are often described as “promiscuous” due to their ability to bind to multiple receptors.

Chemokines were originally named for their presumed function, but due to the large number of chemokines discovered, a systematic nomenclature system has been established. Chemokines are classified according to the spacing and positioning of the first two N-terminal cysteine residues in specific motif, and can be thus divided into four families (CC, CXC, XC and CX3C). Chemokines in the CC family have the first two N-terminal cysteine residues adjacent to each other. The CC family is the largest chemokine family, consisting of 28 members, CCL1 through CCL28. The majority of CC chemokines have important roles in recruiting cells to sites of inflammation, including CCL2, CCL3, CCL5, CCL7, CCL8 and CCL13.

The levels of several chemokines are increased in the synovium of RA patients. Chemokines are implicated in RA pathology primarily due to their ability to recruit and retain leukocytes in the joint. In addition, chemokines can stimulate the release of inflammatory cytokines and proteases by other cell types. For example, CCL2, CCL5 and CXCL12 induce synovial

fibroblasts to increase production of IL-6 and IL-8 (Nanki *et al.* 2001). CCL2 and CCL5 are also known to increase gelatinase and collagenase activities, and stimulate MMP3 and proteoglycan release by chondrocytes (Borzi *et al.* 2000; Garcia-Vicuna *et al.* 2004; Yuan *et al.* 2001). Chemokines can also enhance cell proliferation, thus contributing to synovial hyperplasia. CCL2 and CXCL12 are able to enhance proliferation of synovial fibroblasts (Garcia-Vicuna *et al.* 2004; Iwamoto *et al.* 2007). Several chemokines such as CXCL8 and CXCL1 have angiogenic properties.

1.4.11 Adipokines

Adipokines are a family of soluble mediators with metabolic activities which are now known to also have inflammatory properties. They are produced primarily by adipose tissue, but are also expressed by chondrocytes, synoviocytes and immune cells. Emerging evidence suggests that adipokines may promote and perpetuate inflammatory responses in RA. There are several adipokines, which include leptin, adiponectin, resistin, visfatin, ghrelin, chemerin, lipocalin 2, serum amyloid 3, vaspin and omentin. The roles of some of these family members and their potential involvement in RA, are discussed here.

The first adipokine to be cloned was leptin, a protein belonging to the class I cytokine superfamily. Leptin decreases food intake and increases energy consumption, and also acts as a pro-inflammatory cytokine which induces T cell activation and promotes Th1 differentiation. Leptin also has anabolic effects, such as stimulating the expression of cartilage growth factors and increasing proteoglycan synthesis (Loeser 2003). Adipocyte-derived leptin is associated with maintaining a balance between Th1 cells and Tregs (Matarese *et al.* 2010).

Several studies have reported a strong correlation between the risk of an aggressive disease course of RA and leptin levels (Lee *et al.* 2007; Targonska-Stepniak *et al.* 2010; Targonska-Stepniak *et al.* 2008). Leptin levels are elevated in patients with RA, and serum levels and synovial fluid:serum ratio of serum are correlated with disease duration and activity (Olama *et al.* 2010). However, it has also been reported that leptin levels correlate inversely with radiographic joint damage, and conflicting results concerning the effect of leptin on joint damage have been reported in experimental models of arthritis (Rho *et al.* 2009). Leptin has a pro-inflammatory activity in chondrocytes (Otero *et al.* 2003; Otero *et al.* 2007; Otero *et al.* 2005) and synovial fibroblasts (Kitahara *et al.* 2009; Tong *et al.* 2008), and can also induce anergy in Tregs (Notley and Ehrenstein 2010).

Adiponectin has structural homology with collagens VIII and X and complement factor C1q. It increases fatty acid oxidation and reduces glucose synthesis in the liver. Adiponectin has been reported to have both pro-inflammatory and anti-inflammatory properties. High levels of adiponectin have been reported in RA which correlate with disease severity (Ebina *et al.* 2009). Adiponectin induces the production of pro-inflammatory cytokines and MMPs in synovial fibroblasts which may contribute to joint destruction in RA, and also recruits inflammatory cells to the synovium (Choi *et al.* 2009a; Frommer *et al.* 2010; Tang *et al.* 2007).

Resistin is a dimeric protein, so named for its apparent induction of insulin resistance in mice. It has been found in adipocytes and macrophages, and induces production of IL-6 and TNF-α (Bokarewa *et al.* 2005). Resistin levels are elevated in the serum of patients with RA, and levels have been found to correlate with inflammation and joint destruction (Forsblad d'Elia

et al. 2008). Resistin is also detected in the RA synovium, being expressed by macrophages, B cells and plasma cells (Senolt *et al.* 2007). Resistin levels in RA synovial fluid are higher than in osteoarthritis and correlate with CRP levels and Disease Activity Score for 28 joints (DAS28) (Gonzalez-Gay *et al.* 2008).

Visfatin is an insulin-mimetic adipokine which was originally described as a growth factor for B lymphocyte precursors (hence its alternative name, pre-B-colony enhancing factor). Visfatin has pro-inflammatory properties such as inducing the production of IL-1β, TNF-α and IL-6 by monocytes (Moschen *et al.* 2007). Levels of visfatin are elevated in the serum and synovial fluid of RA patients, and correlate with inflammation, clinical disease activity, rheumatoid factor levels and radiographic joint damage (Otero *et al.* 2006; Rho *et al.* 2009). Visfatin is expressed in RA synovial fibroblasts, and high levels are found in the synovial lining layer and at the site of cartilage destruction. It promotes the expression of pro-inflammatory cytokines and MMPs by RA synovial fibroblasts, and also induces pro-inflammatory cytokine production by monocytes (Brentano *et al.* 2007).

1.5 Objectives

Cytokines are directly implicated in many processes involved in RA pathogenesis. Multiple inflammatory cell populations infiltrating the RA synovium contribute to the local cytokine environment. There remains a need for systematic characterisation of cytokine expression by each of the major cell populations present in the rheumatoid joint. An important objective of this work was therefore to determine the cellular sources of cytokines in separated populations of the synovial fluid and peripheral blood of patients with RA.

When a patient first presents with an inflammatory arthritis, it is often difficult to distinguish patients destined to develop RA from those with a self-limiting synovitis. Differences between early synovitis patients with divergent outcomes may be reflected at the level of synovial cytokine gene expression, as indicated by a previous study which found a differences in cytokine protein expression patterns in the synovial fluid of patients with early synovitis (with a symptom duration up to 3 months) who subsequently developed RA, compared to early resolving synovitis patients and established RA patients (Raza et al., 2005). The present study set out to explore the mRNA expression of a wide range of cytokines, chemokines and growth factors in synovial tissue from early synovitis patients with divergent clinical outcomes (resolving synovitis and persistent RA) and patients with established RA. In addition, I sought to investigate mRNA cytokine expression by individual synovial fluid cell populations in early synovitis patients with diverging clinical outcomes and at different stages of RA. Finally, I aimed to follow up novel observations made in mRNA profiling studies by characterising expression on the protein level in synovial fluid and synovial tissue.

2 METHODS

2.1 Patient selection and sample collection

2.1.1 Samples from early arthritis and established RA patients

All studies involving patients and controls were approved by the Solihull Local Research Ethics Committee. All participating patients gave informed, written consent before samples were taken. Samples were obtained from patients seen by consultant rheumatologists, Dr Karim Raza or Dr Andrew Filer. Samples (blood, synovial fluid and synovial tissue) were obtained from patients presenting with early synovitis or with established RA. RA was diagnosed using the 1987 ACR criteria (Arnett *et al.* 1988). Patients with early synovitis were selected on the basis of having at least one swollen joint and symptoms (inflammatory joint pain and/or early morning stiffness and/or joint related soft tissue swelling) for ≤3 months. Early synovitis patients were followed for 18 months then diagnosed as having RA, non-RA persistent synovitis or self-limiting synovitis. RA was diagnosed using the 1987 ACR criteria; non-RA persistent synovitis was diagnosed if the patient showed clinical evidence of persistent joint swelling (or had been established on a DMARD) but did not satisfy the 1987 ACR criteria for RA; self-limiting synovitis was diagnosed if clinically apparent joint swelling was not clinically apparent at the 18-month assessment and the patient had not received glucocorticoid or DMARD treatment within the previous 3 months. At initial assessment, the disease activity score in 28 joints (DAS28) was calculated, and blood samples were analysed for CRP, erythrocyte sedimentation rate (ESR), rheumatoid factor and anti-CCP antibody.

For comparison, peripheral blood samples were obtained from age and sex-matched healthy control individuals, and synovial biopsies were obtained from patients undergoing surgery following joint trauma who had no previous history of inflammatory disease.

Synovial fluid was aspirated from joints under palpation or ultrasound guidance. Paired peripheral blood was collected in heparinised tubes. Synovial tissue biopsies were collected by needle arthroscopy under ultrasound guidance. To minimise sampling error, eight biopsy samples were obtained from the different sites in the joint. Specimens were directly embedded in TissueTek OCT and snap-frozen in liquid nitrogen. From each frozen tissue block, sections of 20 μm thickness were cut for real-time PCR analysis. Serial sections of 5μm thickness were cut for immunofluorescence staining. Sections were stored at -80°C.

2.1.2 Samples from rituximab-treated patients

Synovial tissue samples from patients treated with rituximab were obtained from the Academic Medical Center, Amsterdam, The Netherlands. The study was approved by the Medical Ethics Committee of the Academic Medical Centre/University of Amsterdam and all patients gave written informed consent before participation. All patients enrolled in the study had active RA. RA was diagnosed according to the 1987 ACR classification criteria for RA, and active disease was defined as having ≥4 tender joints and ≥4 swollen joints of 28 joints assessed, and at least one of the following: ESR ≥28 mm/h, serum CRP levels ≥15 mg/l or morning stiffness ≥45 minutes. All patients were treated with two intravenous infusions of 1,000 mg rituximab on days 1 and 15 after premedication with 2 mg clemastine fumarate intravenously and 1000 mg acetaminophen orally. The DAS28 was measured before and every month after treatment. Serum and synovial tissue were obtained at three time points:

before treatment, and 4 and 16 weeks after the first infusion of rituximab. Synovial biopsies were collected by needle arthroscopy from the same inflamed joint at each time point. To minimise sampling error, at least six biopsy samples were obtained from the different sites in the joint. Specimens were directly embedded in TissueTek OCT (Miles Diagnosis, USA) and snap-frozen in liquid nitrogen. From each frozen tissue block, serial sections of 5μm thickness were cut. Sections were stored at -80°C.

2.2 Isolation of cells from synovial fluid and peripheral blood

2.2.1 Isolation of mononuclear cells

Synovial fluid was incubated with 10μl/ml hyaluronidase (1000 U/ml, endotoxin tested, formulated for injection) at 37°C for 15 mins to reduce viscosity. Peripheral blood was collected in a heparinised tube. Blood was diluted with an equal volume of RPMI. Synovial fluid and peripheral blood were layered onto Ficoll-Paque in a 3:1 ratio and centrifuged at 300 g for 30 minutes at 22°C with no brake. The mononuclear cell population at the Ficoll-Paque interface was removed using a plastic Pasteur pipette and transferred to a fresh tube. Cells were suspended in 20 ml RPMI, centrifuged at 300 g for 8 mins, then the supernatant was removed and the cell pellet was resuspended by gently flicking the tube. Cells were washed twice more in 20 ml RPMI, then resuspended in freezing medium containing HIFCS supplemented with 10% dimethyl sulphoxide (DMSO) which was pre-warmed to 37°C. Cells were frozen at -80°C in a freezing container with isopropranol.

2.2.2 Isolation of neutrophils

Neutrophils were isolated using magnetic anti-CD15 microbeads (Miltenyi Biotech). Whole blood was incubated with 50 µl/ml anti-CD15 microbeads for 15 mins at 4˚C, then washed with 5 ml MACS buffer (phospho-buffered saline (PBS, Sigma) supplemented with 0.5 % bovine serum albumin (BSA, Sigma) and 20 mM ethylenediamine tetra-acetic acid (EDTA, Sigma) per ml of blood. Cells were centrifuged at 445 g for 10 mins with no brake. The supernatant was aspirated leaving 1-2 mm above the cell pellet. The cell pellet was resuspended in a total volume of 1 ml with MACS buffer. The labelled cells were then applied to a MACS column, which had been primed by adding 3 x 3 ml MACS buffer in the presence of a strong magnetic field. Unlabelled cells were washed through the column with 10 x 3 ml cold MACS buffer. The column was then removed from the magnetic field and the magnetically-labelled cells were eluted by adding 5 ml of Elution Buffer (Miltenyi Biotech) and immediately flushing cells through with a plunger. Collected cells were washed twice by resuspending in 20 ml PBS supplemented with 0.5% BSA and centrifuging at 300 g for 6 min. To determine the purity of the separated cell population, a sample of the cells was stained by resuspending the cell pellet in FITC-labelled anti-CD15 antibody diluted in freshly-prepared PBS supplemented with 0.5% BSA at 1 ml/10^8 cells. Cells were incubated on ice in the dark for 20 min. Cells were then washed twice by resuspending in 100 µl PBS supplemented with 0.5% BSA and centrifuging at 300 *g* for 4 minutes. A sample of the isolated cells was used to assess purity. Cells were labelled with mouse anti-CD15 FITC (BD Pharmingen, 1:40 dilution) or mouse IgM FITC (Immunotools) using the staining protocol described. The percentage of cells labelled with anti-CD15 was measured by flow cytometry.

2.2.3 FACS sorting of mononuclear cell populations

Staining was carried out in a flexible 96-well plate coated with heat-inactivated foetal calf serum (HIFCS, Sigma) on ice. Freshly-prepared PBS supplemented with 0.5% BSA was used as antibody diluent and wash solution. 100 µl wash solution was used for each wash and centrifugation was performed at 300 g for 4 minutes at 4°C. Wash solution was removed by inversion of the plate. Antibodies used to stain cells are shown in Table 2.1 Cells were incubated with antibody on ice for 20 min in the dark. Following staining, cells were washed and re-suspended in 500µl wash solution, filtered using 50µm filters and transferred to FACS tubes. Cells were sorted by FACS using a MoFlo ^{TM} high speed cell sorter (Dako, Ely, UK).

Cells stained with isotype control antibodies were used to determine the level of background non-specific binding. Compensation was performed using cells stained with each fluorochrome conjugated-antibody individually. Debris and dead cells were excluded from analysis by gating on forward and side scatter. Doublets were excluded by gating on the forward scatter versus pulse width.

The purity of each sorted cell population was determined by measuring the percentage of cells in the sample that were labelled with the antibody specific for the population by flow cytometry. For this purpose the forward scatter/side scatter region examined was enlarged to include all live cells.

Antibody	Dilution	Supplier
CD4 APC	1:40	eBioscience
CD8 PE Cy7	1:100	eBioscience
CD45RO FITC	1:40	Biostat
CD14 FITC	1:50	Immunotools
CD19 PE	1:40	Immunotools
IgG2a APC	*	Immunotools
IgG1 PECy7	*	Immunotools
IgG1 PE	*	Immunotools
IgG1 FITC	*	Immunotools

Table 2.1 Antibodies used for sorting mononuclear cell populations by FACS. All antibodies were mouse monoclonal antibodies raised against human antigens.

2.3 RNA extraction

2.3.1 RNA extraction from isolated cell populations

RNA was extracted from synovial fluid and peripheral blood mononuclear cell populations and neutrophils using the Nucleospin XS kit (Macherey-Nagel). In this method, cells are lysed by incubation in a solution containing high concentrations of chaotropic ions which immediately inactivates RNases which would otherwise degrade RNA. Ethanol is added to create appropriate binding conditions, then RNA is adsorbed to a silica membrane. Contaminating DNA, which is also bound to the silica membrane, is removed by applying rDNAse solution to the membrane within a column. The membrane is then washed with two different buffers to remove salts, metabolites and macromolecular cellular components. Pure RNA is eluted under low ionic strength conditions with water. RNA extraction was performed according to manufacturer's instructions as follows. Cells were centrifuged at 4000 g for 5 min. The cell pellet was resuspended in 100 μl Lysis Buffer RA1 + 2 μl TCEP. Cells were

vortexed twice for 10 sec then frozen at -80˚C. Cells were defrosted at room temperature. 5 µl of 20 ng carrier RNA was added to lysate. 100 µl of 70% ethanol was added to the lysate and mixed well by pipetting. The sample was applied to a Nucleospin column placed in a collection tube and centrifuged for 30 sec at 11000 g to bind the sample to the column. The column was placed in a new collection tube. 100 µl of Membrane Desalting Buffer was added to the column and centrifuged for 30 sec at 11000 g. A DNase reaction mixture was prepared with 3 µl rDNAse and 27 µl DNAse reaction buffer, then 25 µl of the mixture was added to the column and incubated for 15 min at room temperature. 100 µl of Buffer RA2 was added to the column and incubated for 2 min at room temperature, then centrifuged for 30 sec at 11000 g. The column was placed in a new collection tube. The column was washed by adding 400 µl of Buffer RA3 then centrifuging for 30 sec at 11000 g. A further 200 µl of Buffer RA3 was added, then the sample was centrifuged for 2 min at 11000 g. Finally, RNA was eluted by adding 10 µl of RNase-free water to the column and centrifuging for 30 sec at 11000 g.

2.3.2 RNA extraction from synovial tissue

RNA was extracted from 20 µm synovial tissue sections using an RNeasy kit (Qiagen). In this method, cells are lysed in a buffer containing guanidine isothiocyanate which immediately inactivates RNases. Ethanol is added to provide appropriate binding conditions, then the sample is applied to a column where it binds to a silica membrane. Contaminants are washed away using two wash buffers. Finally RNA is eluted in water. RNA extraction was performed according to manufacturer's instructions as follows. Cells were lysed by adding 350 µl Buffer RLT, vortexing for 1 min, then freezing at -80˚C. Cells were defrosted at room temperature and 350 µl of 70% ethanol was added. The sample was applied to a column and centrifuged for 15 sec at 13000 g to bind the sample to the column. The flow-through was discarded. 700

μl of Buffer RW1 was added to the column, the sample was centrifuged for 15 sec at 13000 g, and the flow-through discarded. 500 μl of Buffer RPE was added to the column and the sample was centrifuged for 15 sec at 13000 g. The flow-through was discarded. A further 500 μl of Buffer RPE was added and the sample was centrifuged for 2 mins at 13000 g. The column was transferred to a new collection tube. Finally, 30 μl RNase-free water was added to the column, and the sample was centrifuged for 1 min at 13000 g to elute the RNA.

RNA was also isolated from a second synovial tissue sample from each patient using a procedure which included DNA digestion. Contaminating DNA was removed using a DNase kit (Qiagen) in conjunction with an RNeasy RNA extraction kit (Qiagen), according to the manufacturer's instructions. Cells were lysed by adding 350 μl Buffer RLT, vortexed for 1 min, then frozen at -80˚C. Cells were defrosted at room temperature and 350 μl 70% ethanol was added. The sample was applied to a column and centrifuged for 15 sec at 13000 g to bind the sample to the column. The flow-through was discarded. 350 μl of RW1 was added to the column, the column was centrifuged for 15 sec at 13000 g, and flow-through was discarded. 10 μl DNAse I stock solution was added to 70 μl of Buffer RDD and mixed by gentle inversion. The DNase I mix was added to the membrane and incubated at room temperature for 15 min. 350 μl of Buffer RW1 was added, the sample was centrifuged for 15 sec and the flow-through discarded. 500 μl of Buffer RPE was added to the column and the sample was centrifuged for 15 sec at 13000 g. The flow-through was discarded. A further 500 μl of Buffer RPE was added and the sample was centrifuged for 2 mins at 13000 g. The column was transferred to a new collection tube. Finally, 30 μl RNase-free water was added to the column, and the sample was centrifuged for 1 min at 13000 g to elute the RNA.

2.4 Microfluidic cards

2.4.1 Microfluidic card design

Microfluidic cards containing 384 real-time PCR assays were custom ordered from Applied Biosystems. Each assay contained gene-specific primers, probes and a reaction buffer for reverse transcription and real-time PCR. RNA samples from cell populations isolated from synovial fluid and peripheral blood were analysed using a microfluidic card containing 48 real-time PCR assays per sample (listed in Appendix 8.1).

Synovial tissue samples were analysed using the following microfluidic cards:

i) Two sets of microfluidic cards, each containing 96 real-time PCR assays per sample (listed in Appendix 8.2), were used to analyse RNA from synovial tissue extracted without DNase treatment. One-third of the total RNA isolated from each synovial tissue section was used per 96 genes analysed.

ii) A microfluidic card containing 48 assays which detect genomic DNA and assays for genes that were weakly expressed (listed in Appendix 8.3) was used to analyse RNA from synovial tissue was treated with DNase. The total RNA isolated from each synovial tissue section was used per 48 genes analysed

The microfluidic card designed to include the assays listed in 8.3 was designed in order to address two issues:

i) For some genes, the real-time PCR assays contained primers which were designed within a single exon and thus by definition would detect genomic DNA. Some real-time PCR assays were described as assays that may detect genomic DNA. Therefore

RNA which was treated with DNase to remove contaminating genomic DNA was used to detect expression of these genes.

ii) Several cytokines were expressed at low levels in synovial tissue. Therefore RNA obtained from a whole synovial tissue section rather than one-third of the section was used to detect expression of these genes.

2.4.2 Reverse transcription and real-time PCR

Reverse transcription and real-time PCR was performed using a Quantitect Probe RT-PCR kit (Qiagen). A reaction mix was made with 90 µl of RNA, 50 µl of Quantitect-RT Master Mix and 1 µl of QuantiTect Reverse Transcriptase Mix. 95 µl of the sample was loaded into each port of the microfluidic card. The microfluidic card was centrifuged twice at 300 g for 1 min in a vertical centrifuge, then the plate was sealed and the loading ports were cut off. The card was placed in an Applied Biosystems 7900 PCR machine. The cycling program used for reverse transcription and real-time PCR was 50˚C for 30 min, 94.5˚C for 15 sec then 40 cycles of 96˚C for 30 sec and 59.7˚C for 1 min.

Gene expression was analysed by setting the baseline and threshold values on the amplification plot for each gene. The threshold cycle (Ct) value was then used to calculate the fold change in expression of each gene relative to the housekeeping gene by using the $2^{-\Delta\Delta Ct}$ method, and the relative quantification was plotted. In the synovial fluid and peripheral blood study, expression was normalised to 18S. In the synovial tissue study, expression was normalised to GAPDH. For real-time PCR, a program of 40 cycles was used. Genes which had a Ct value > 40 were below the level of detection of the assay and were and assigned an RQ value of zero.

2.5 Flow cytometry

Cells were stained as described earlier for FACS in 2.2.4. Antibodies used are shown in Table 2.2. Flow cytometry was performed using a Dako Cyan ADP High Performance flow cytometer (Dako, Ely, UK). Flow cytometry data were analysed using SUMMIT software (Dako).

Antibody	Dilution	Supplier
CD19 Pacific Blue	1:100	Biolegend
CD27 APC	1:100	BD Pharmingen
IgD FITC	1:100	BD Pharmingen
RANKL PE	1:500	eBioscience
IgG1 Pacific Blue	*	Biolegend
IgG1 APC	*	Immunotools
IgG2a FITC	*	Immunotools
IgG2b PE	*	eBioscience

Table 2.2 Antibodies used for flow cytometry. All antibodies were mouse monoclonal antibodies raised against human antigens.

2.6 Confocal microscopy

Antibodies were incubated at room temperature in the dark on a shaking tablet. Sections were firstly adsorbed with PBS supplemented with 10% fetal calf serum for 10 mins. Primary antibodies were incubated overnight at 4˚C in PBS supplemented with 10% fetal calf serum, then washed for 5 mins in PBS. Secondary antibodies were incubated for 45 mins respectively

at room temperature. Sections were immersed in Hoechst 33258 (Sigma) for 2 mins for nuclear staining, then mounted and kept in the dark at -20˚C.

Antibodies used are shown in Table 2.3. CD19 was developed with goat Alexa 488 anti-FITC, CD22 was developed with goat-anti mouse FITC and goat Alexa 488 anti-FITC, and RANKL was developed with donkey anti-rabbit rhodamine. A Zeiss confocal LSM 510 microscope (Zeiss) was used to visualise staining of synovial tissue stained for RANKL and CD19. Images were captured and processed using Zeiss LSM Image Examiner software (Zeiss). Synovial tissue sections from the rituximab study were visualised using a Zen confocal microscope (Zeiss). Images were captured and analysed using Zen software (Zeiss).

Antibody	**Dilution**	**Supplier**
Primary antibodies		
Rabbit anti-human RANKL	1:50	AbCam
Mouse anti-human CD19 FITC	1:50	Dako
Mouse anti-human CD22	1:500	UoB
Mouse IgG1	*	Southern Biotech
Mouse IgG2b	*	Southern Biotech
Rabbit Ig	*	Sigma
Secondary/Tertiary antibodies		
Goat AlexaFluor 488-anti FITC	1:100	Invitrogen
Donkey anti-rabbit rhodamine	1:50	Jackson ImmunoResearch
Goat anti-mouse FITC	1:250	Jackson ImmunoResearch

Table 2.3 Antibodies used for confocal staining

3 CYTOKINE PRODUCTION BY SYNOVIAL FLUID CELL POPULATIONS

3.1 Introduction

Cytokines are recognised as important factors in the pathogenesis of RA. Many cytokines are present in the synovium, and murine and rodent models of arthritis have demonstrated the effectiveness of cytokine modulation in altering disease outcome. Furthermore, the use of anti-TNF therapy as a treatment for RA has provided proof of principle that cytokine targeting is an acceptable and effective therapeutic strategy. This highlights the importance of investigating the role of cytokines in RA in order to further our understanding of disease pathogenesis and identify new targets for therapy. In RA, the joint is infiltrated by multiple inflammatory cell populations including T cells, B cells, macrophages and neutrophils, all of which contribute to the local cytokine network. Elucidating the cytokine network in RA represents an important challenge for furthering our understanding of disease pathogenesis. The first step towards achieving this goal is to determine the cellular sources of the cytokines found in the rheumatoid joint.

Early studies of cytokine expression by T cells indicated that synovial T cells produce low levels of IL-2 and IFN-γ (Combe et al., 1985; Firestein et al., 1988; Smeets et al., 1998). More recently, IL-17-producing T cells have been reported in the RA synovium, where they are thought to promote inflammation by activating local stromal cells (Chabaud *et al.* 1999; Kotake *et al.* 1999). Furthermore, RA synovial T cells are reported to produce pro-inflammatory cytokines including TNF-α and IL-6 (Chu *et al.* 1991; Field *et al.* 1991). They are also known to produce BAFF and APRIL, LT-α/LT-β and RANKL (Crotti *et al.* 2002;

Takemura *et al.* 2001a). Until recently, cytokine production by B cells in RA has remained relatively unstudied. It is known that B cells are capable of producing several cytokines which are relevant to RA pathogenesis including IL-1, TNF-α, IL-15 and IL-6, indicating a direct pro-inflammatory role. Production of LT-β by synovial B cells is thought to be required for synovial lymphoid neogenesis (Weyand *et al.* 2005).

Macrophages are regarded as an important source of pro-inflammatory cytokines found in the RA synovium such as TNF-α, IL-1 and IL-18 (Chu *et al.* 1991; Tanaka *et al.* 2001; Wood *et al.* 1992). Through expression of IL-7, IFN-β, IL-23, BAFF and APRIL, they are thought to play a role in synovial T cell and B cell differentiation and survival (Gabay *et al.* 2009; Seyler *et al.* 2005; Stamp *et al.* 2009; van Holten *et al.* 2005; van Roon *et al.* 2007). Neutrophils represent the largest cell population in RA synovial fluid and are known to produce a range of cytokines including IL-1β, BAFF, APRIL and TGF-β (Assi *et al.* 2007; Beaulieu and McColl 1994; Gabay *et al.* 2009; Quayle *et al.* 1994). They are also sources of chemokines such as CCL3 and CXCL8 (Beaulieu and McColl 1994; Hatano *et al.* 1999).

Our present knowledge of cytokine sources within the RA joint is largely based on studies which have determined cytokine expression in a limited number of cell populations. Many *in vitro* and *in vivo* models have demonstrated the effect of cytokine modulation on various cell types, but not the cellular source of the cytokine itself. Thus for many cytokines it is assumed that the cellular sources reported in physiological conditions or *in vitro* studies are the same as in the RA joint. To date, no study has systematically determined cytokine production by each of the major synovial cell populations simultaneously. The aim of the present study was therefore to identify the predominant cellular sources of cytokines in RA by comparing

expression across the five cell populations in RA synovial fluid and peripheral blood. To achieve this we developed a new approach which has the advantage of characterising cytokine mRNA profiles in cells directly *ex vivo* without culture or prior stimulation in order to reflect the *in vivo* situation as closely as possible.

3.2 Method development and validation

We developed a novel technical approach to determine the mRNA expression of 41 cytokines in T cells, B cells, macrophages and neutrophils isolated from a single sample of synovial fluid or peripheral blood from RA patients. In each of the isolated cell populations, the cytokine mRNA expression profile was determined by real-time PCR using microfluidic cards. In a series of validation experiments we excluded bias due to sample preparation, normalisation and staining techniques. The results of these preliminary experiments confirmed the robustness of the experimental approach taken.

CD4 T cells, CD8 T cells, B cells and macrophages were sorted by FACS based on their expression of CD4/CD3/CD45R0, CD8/CD3/CD45R0, CD19 and CD14, respectively (Figure 3.1A and B). Neutrophils were isolated with CD15-coated magnetic beads (Figure 3.1C). Samples were reanalysed after sorting by flow cytometry and only samples with a purity of ≥95% were included in the study (Figure 3.1D).

In some instances, only a small volume of synovial fluid containing a low number of cells can be aspirated from the joint. Therefore it was important to establish the minimum number of cells needed for the detection of cytokine gene expression using microfluidic card real-time

PCR assays. mRNA expression from samples containing $1x10^3$, $1x10^4$ or $1x10^5$ cells were directly compared in three independent experiments (Figure 3.2A). A close correlation was observed between results yielded by detection of 43 cytokine genes in $1x10^4$ and $1x10^5$ cells, whereas comparison of the results from $1x10^3$ genes and $1x10^5$ cells showed a large degree of variation. This confirmed that using a minimum cell number of $1x10^4$ would be sufficient for the sensitivity of the real-time PCR microfluidic card assay.

We also sought to determine if an anti-CD3 antibody could be used as a specific marker to sort T cells without inducing cytokine gene expression under the experimental conditions used. The cytokine gene expression for mononuclear cells sorted on the basis of CD3 and CD4 expression was compared with T cells sorted on the basis of CD4 expression and lack of expression of the myeloid cell markers CD11c and CD11b (Figure 3.2B). Under controlled experimental conditions, keeping the cells below 4°C, T cells sorted using anti-CD3 antibody did not have an altered expression profile compared to T cells sorted using alternative markers, showing that under these conditions there is no induction of cytokine gene expression by the anti-CD3 antibody.

Due to the limited availability of the cell sorter, we determined whether mononuclear cells and neutrophils could be stored in freezing medium before sorting. As shown in Figure 3.2C, the cytokine mRNA expression was not altered by storing synovial fluid mononuclear cells in HIFCS supplemented with 10% DMSO at -80°C, compared to using freshly isolated cells. In the case of neutrophils, the majority of genes expressed by freshly isolated neutrophils were not detectable in neutrophils that had been stored in freezing medium (Figure 3.2C). Therefore throughout the study, mononuclear cells were stored in freezing medium prior to

sorting, whereas neutrophils were isolated immediately after samples were received. mRNA from peripheral blood mononuclear cells stimulated with magnetic beads coated with anti-CD3 and anti-CD28 antibody was used as a positive control to test the real-time PCR assays. With the exception of G-CSF, CCL11 and CXCL12, expression of all cytokine genes was detected (Figure 3.2D).

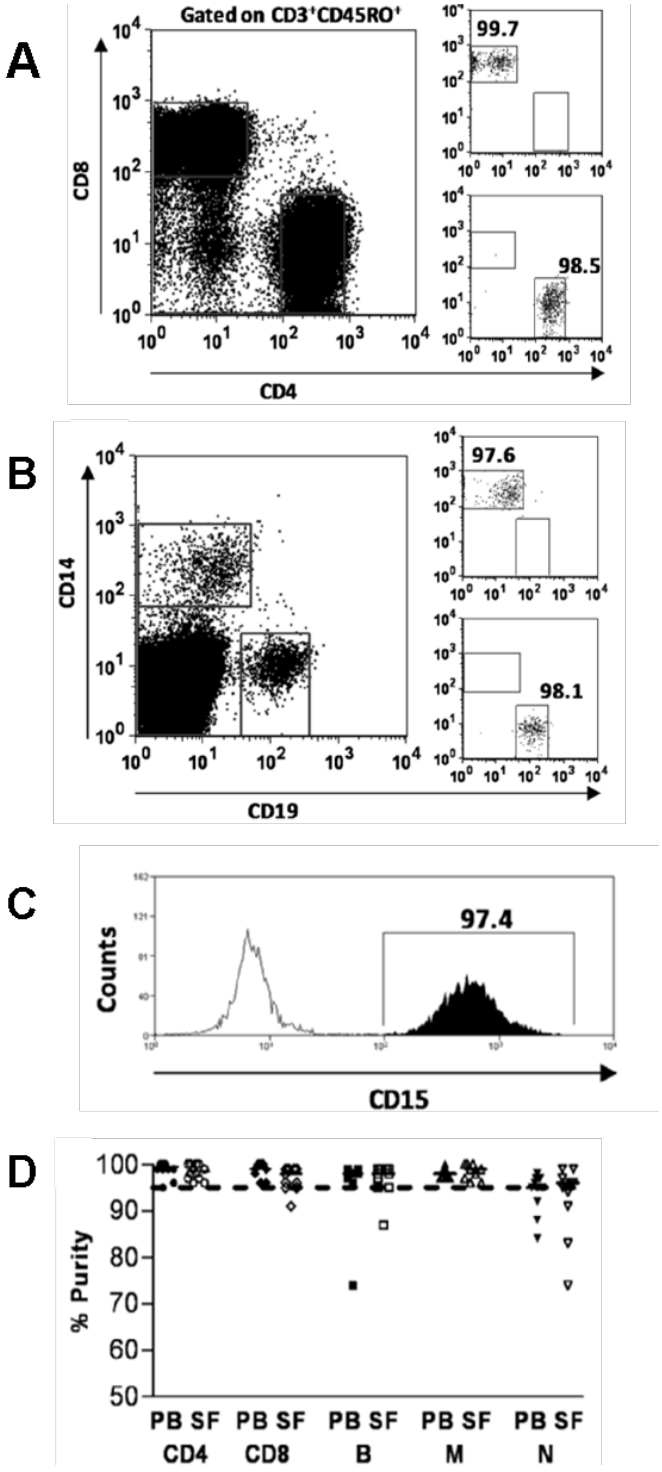
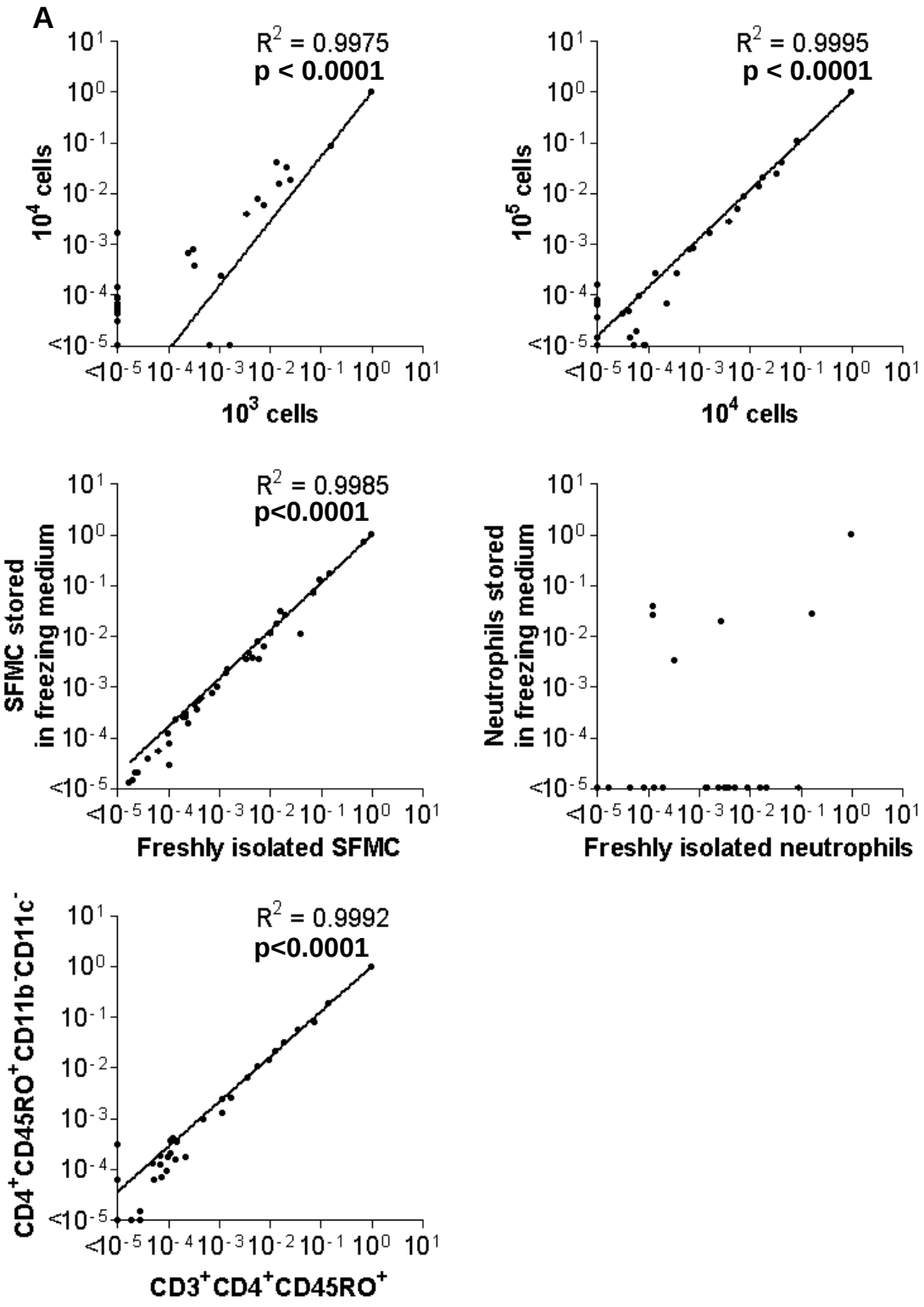


Figure 3.1 Isolation of mononuclear cell populations from synovial fluid and peripheral blood. (A, B) CD4 and CD8 T cells, B cells and monocytes were sorted based on their expression of CD4/CD3/CD45RO, CD4/CD3/CD45RO, CD19 and CD14 respectively. Purity of each sorted cell sample is indicated. (C) Neutrophils were isolated using CD15-coated magnetic beads. Percentages indicate purity of isolated populations as determined by flow cytometry. (D) Purity of all isolated population after isolation. Samples with more than 5% contaminating cells were rejected.


A
R2 = 0.9975
p < 0.0001
10^4 cells
10^3 cells
R2 = 0.9995
p < 0.0001
10^5 cells
10^4 cells
R2 = 0.9985
p<0.0001
SFMC stored in freezing medium
Freshly isolated SFMC
Neutrophils stored in freezing medium
Freshly isolated neutrophils
R2 = 0.9992
p<0.0001
CD4+CD45RO+CD11b-CD11c-
CD3+CD4+CD45RO+

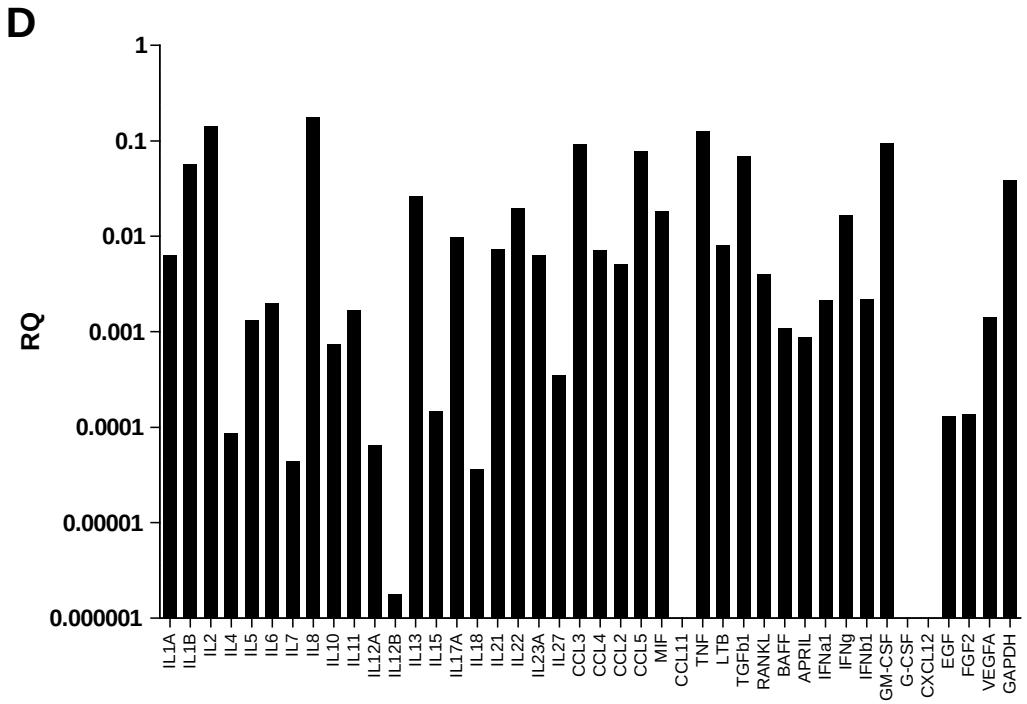


Figure 3.2 Method validation. (A) Comparison of cytokine mRNA expression from samples containing 10^3, 10^4 and 10^5 cells in order to establish the cell number needed for detection of cytokine expression. (B) Comparison of cytokine mRNA expression of cells stored in freezing medium at -80°C and freshly isolated cells. Gene expression is shown for synovial fluid mononuclear cells (left) and neutrophils (right). (C) Comparison of cytokine mRNA expression of mononuclear cells sorted on the basis of CD3 and CD4 expression compared to T cells sorted on the basis of CD4 expression and lack of expression of myeloid cell markers CD11c and CD11b. (D) Cytokine expression in PBMC stimulated with anti-CD3/anti-CD28-coated beads used as a positive control for the real-time PCR assays.

3.3 Cytokine expression in synovial fluid cell populations

Having established and validated the methods to be used in the study, we set out to determine cellular cytokine sources in RA synovial fluid and peripheral blood. Synovial fluid and peripheral blood were obtained from 12 patients with established RA. Clinical details of patients are shown in Appendix Table 8.4. CD4 T cells, CD8 T cells, B cells, macrophages and neutrophils were isolated from synovial fluid and peripheral blood as described previously. In each population mRNA expression of the following cytokines was determined by real-time PCR: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-11, IL-12p35, IL-12p40, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL23p19, IL-27, TNF-α, LT-β, RANKL, APRIL, BAFF, TGF-b1, CCL2, CCL3, CCL4, CCL5, CCL11, CXCL8, CXCL12, MIF, IFN-γ, IFN-α1, IFN-β1, CSF-2, CSF-3, EGF, FGF2 and VEGF-α. Levels of expression were quantified relative to 18S. mRNA expression was not detected for IL-4, IL-5, IL-13, IL-22, CCL11, CXCL12, CSF-3 or EGF. The complete set of data showing cytokine expression by all five cell populations in peripheral blood and synovial fluid of RA patients is shown in Appendix 8.3.5.

Cytokines expressed predominantly by synovial fluid lymphocytes are shown in Figure 3.3. In synovial fluid T cells we detected the expression of IFN-γ and IL-2 mRNA. In the CD4 T cell population we observed mRNA expression of the Th17 cell-related cytokines, IL-17 and IL-21. The CD4 T cell population also expressed high levels of mRNA for the neutrophil and macrophage survival factor, GM-CSF. CD4 and CD8 T cell populations both expressed mRNA for IL-11, IL-12p35, IL-23p19 and CCL5. Interestingly, T cells did not express IL-12p40 mRNA which is required for production of functional IL-12 and IL-23. Synovial B cells expressed mRNA for IL-12p40 in addition to IL-12p35. T cell and B cell populations

expressed comparable levels of mRNA for the T cell and B cell development and survival factors IL-7 and LT-β. Intriguingly, we observed that RANKL mRNA was expressed predominantly by B cells, while lower levels were expressed by CD4 T cells.

Cytokines expressed predominantly by the synovial fluid myeloid cell populations investigated are shown in Figure 3.4. Macrophages were found to express the highest levels of mRNA for the Th1-differentiation factors IL-18 and IL-27, and were also found to be the predominant mRNA source of APRIL, CCL2 and VEGF-a. Synovial fluid neutrophils were found to have a cytokine expression pattern very similar to macrophages. Both populations expressed comparably high mRNA levels of the pro-inflammatory cytokine IL-1β, and the chemokines CCL3 and CXCL8. Neutrophils expressed the highest mRNA levels of BAFF, which was also expressed by macrophages. In addition, neutrophils expressed the highest mRNA levels of IFN-α1 and IFN-β1.

Several cytokines were highly expressed both in lymphoid and myeloid populations, as shown in Figure 3.5. IL-1α mRNA was expressed at similar levels by macrophages, neutrophils and B cells in synovial fluid. B cells and macrophages expressed the highest levels of IL-6 mRNA, while T cell and macrophage populations expressed high levels of IL-10 mRNA. T cells, B cells and neutrophils expressed comparable levels of IL-15 mRNA. All lymphocyte and myeloid cell populations investigated expressed high mRNA levels of TNF-α, TGF-β1, MIF and CCL4.

Comparison of cytokine profiles in synovial fluid and peripheral blood populations showed that a wide range of cytokines was expressed at a higher level in synovial fluid (Figure 3.6 and Appendix 8.3.5). In the T cell, B cell and macrophage populations the majority of cytokines detected were more highly expressed in the synovial fluid. Interestingly, this was not the case for neutrophils. In addition, IFN-γ expression was found to be up-regulated in synovial fluid T cells whereas IL-2 expression was down-regulated in synovial fluid T cells.

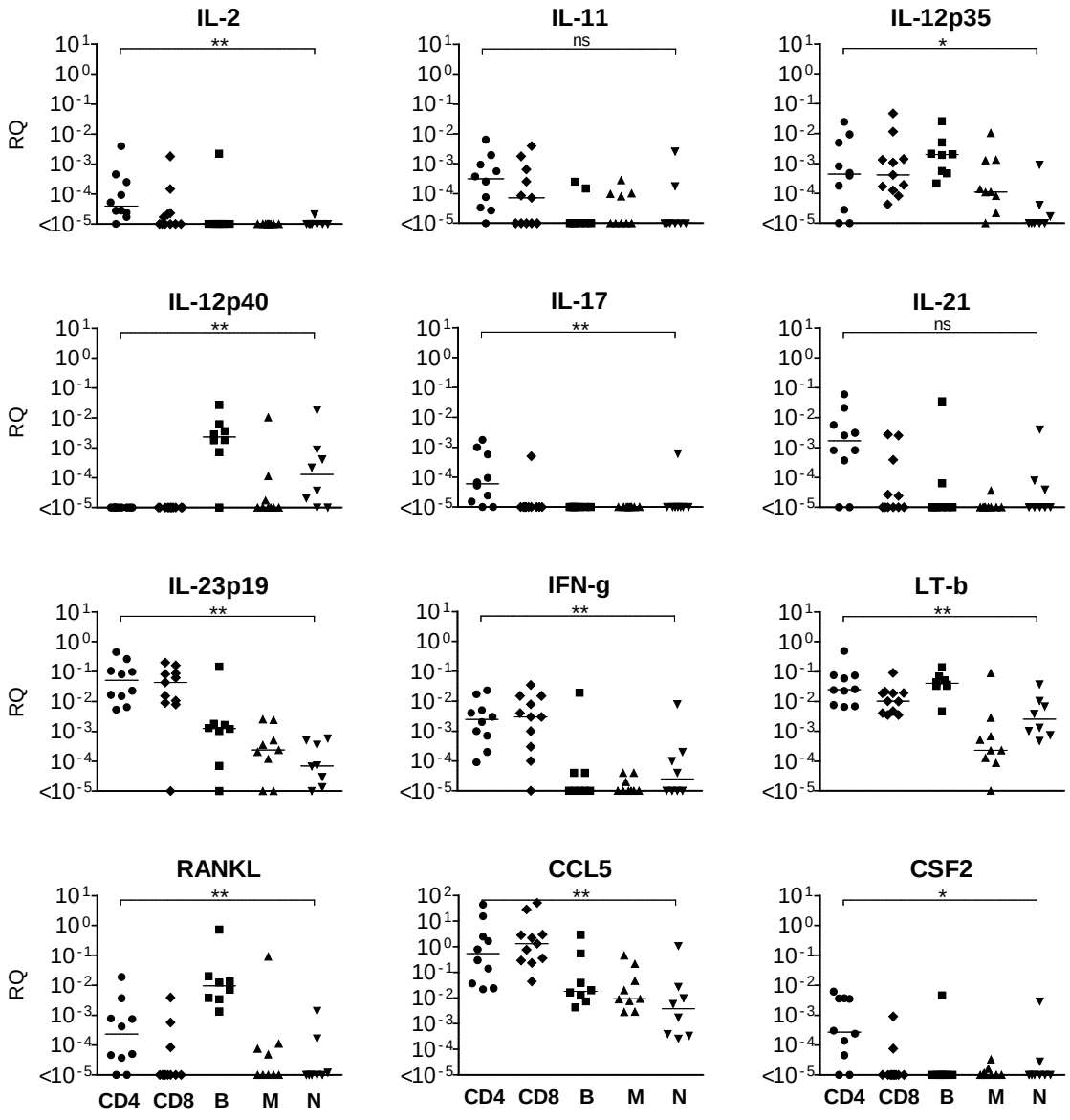


Figure 3.3 Cytokines expressed predominantly by synovial fluid lymphoid cells. mRNA expression of cytokines expressed predominantly by CD4 T cells, CD8 T cells or B cells. Cytokine expression is shown for CD4 T cells (CD4), CD8 T cells (CD8), B cells (B), macrophages (M) and neutrophils (N). Kruskal-Wallis test; *p<0.01, **p<0.001, ***p<0.0001.

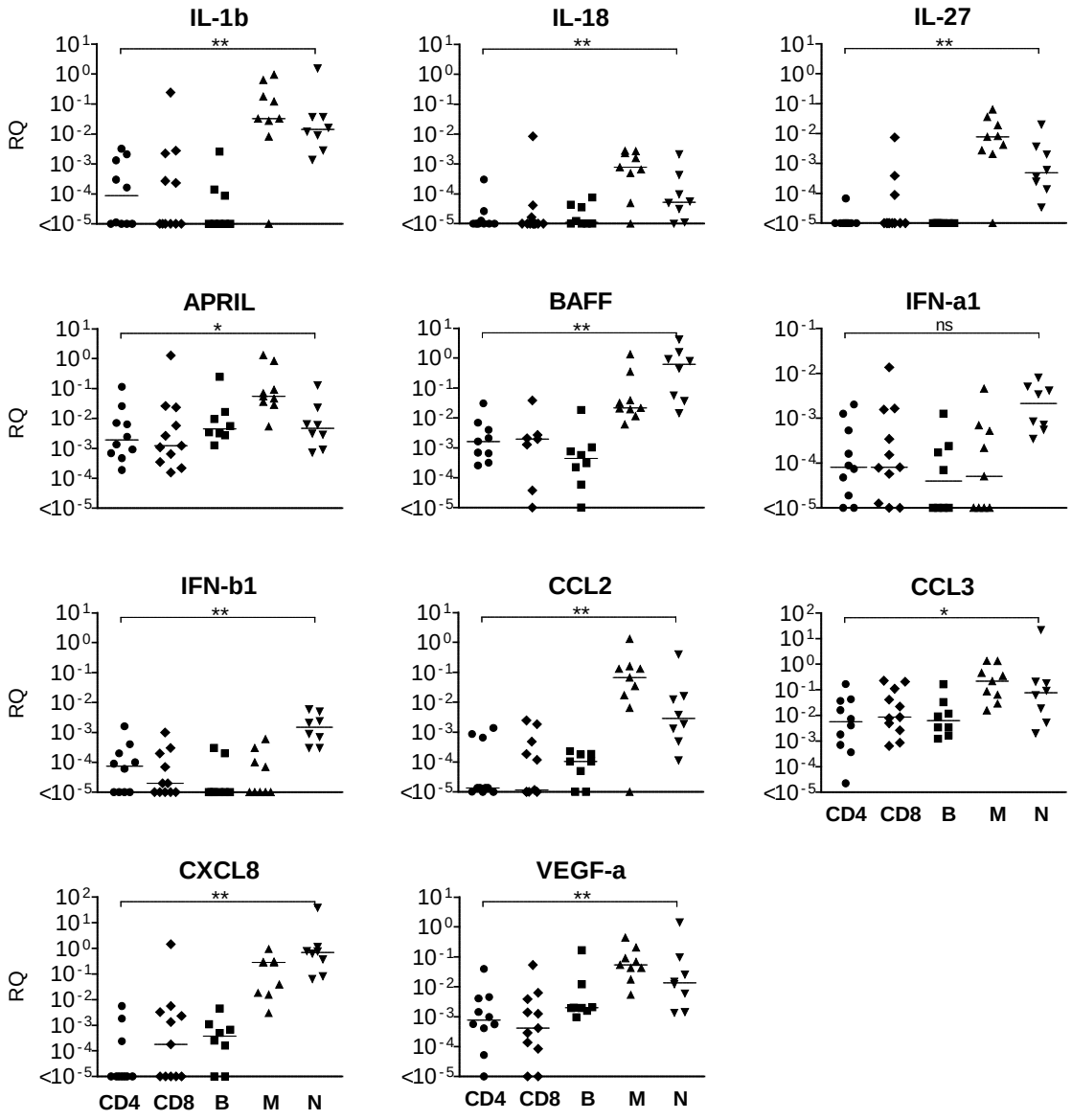


Figure 3.4 Cytokines expressed predominantly by synovial fluid myeloid cells. mRNA expression of cytokines expressed predominantly by macrophages or neutrophils. Cytokine expression is shown for CD4 T cells (CD4), CD8 T cells (CD8), B cells (B), macrophages (M) and neutrophils (N). Kruskal-Wallis test; *p<0.01, **p<0.001, ***p<0.0001.

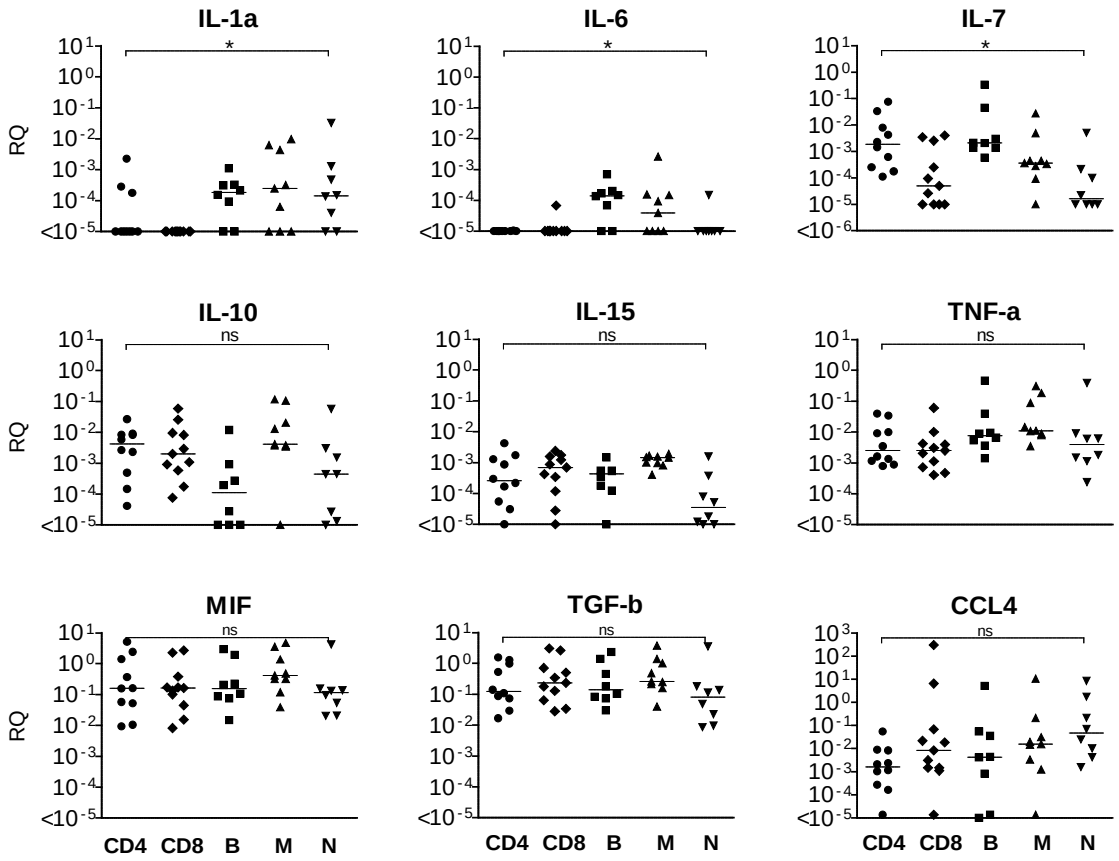
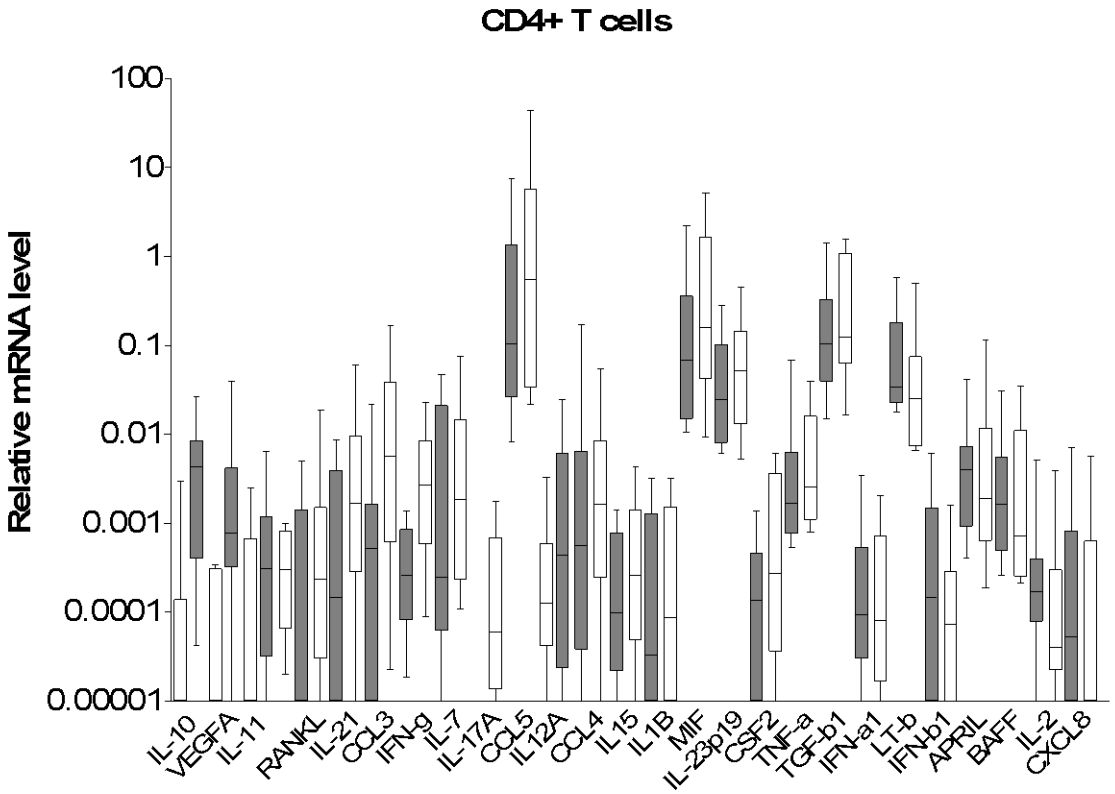
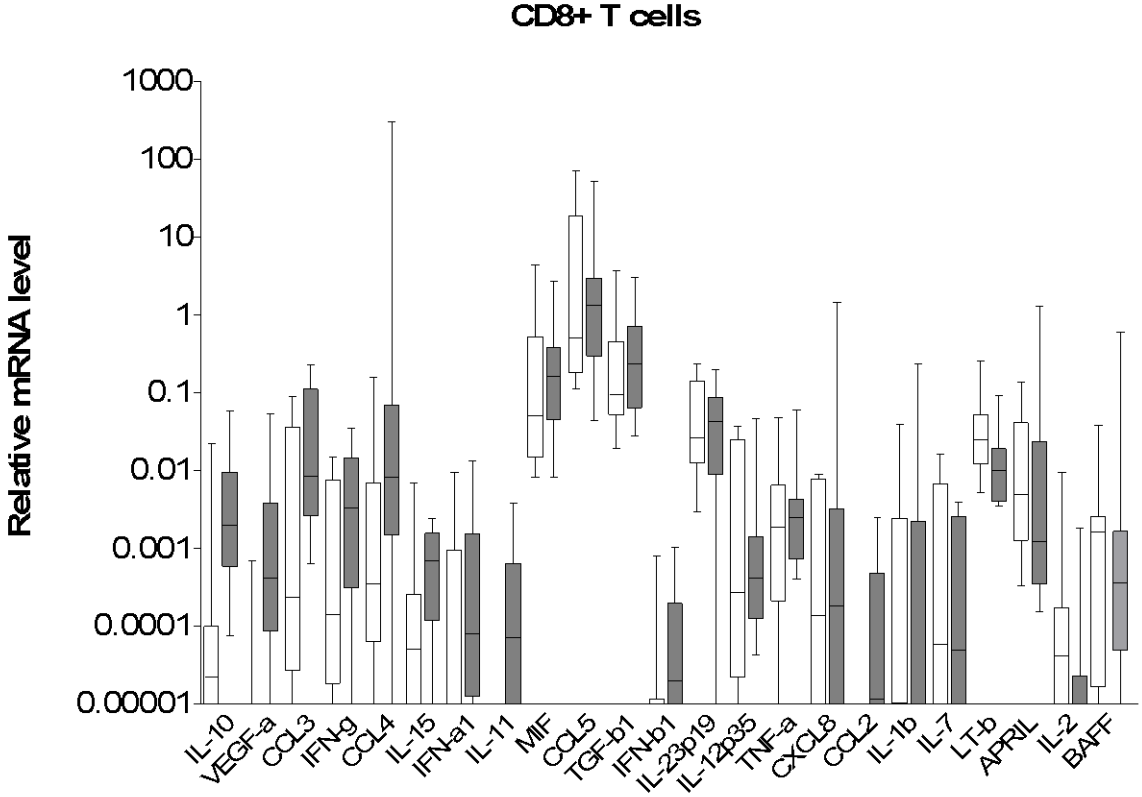
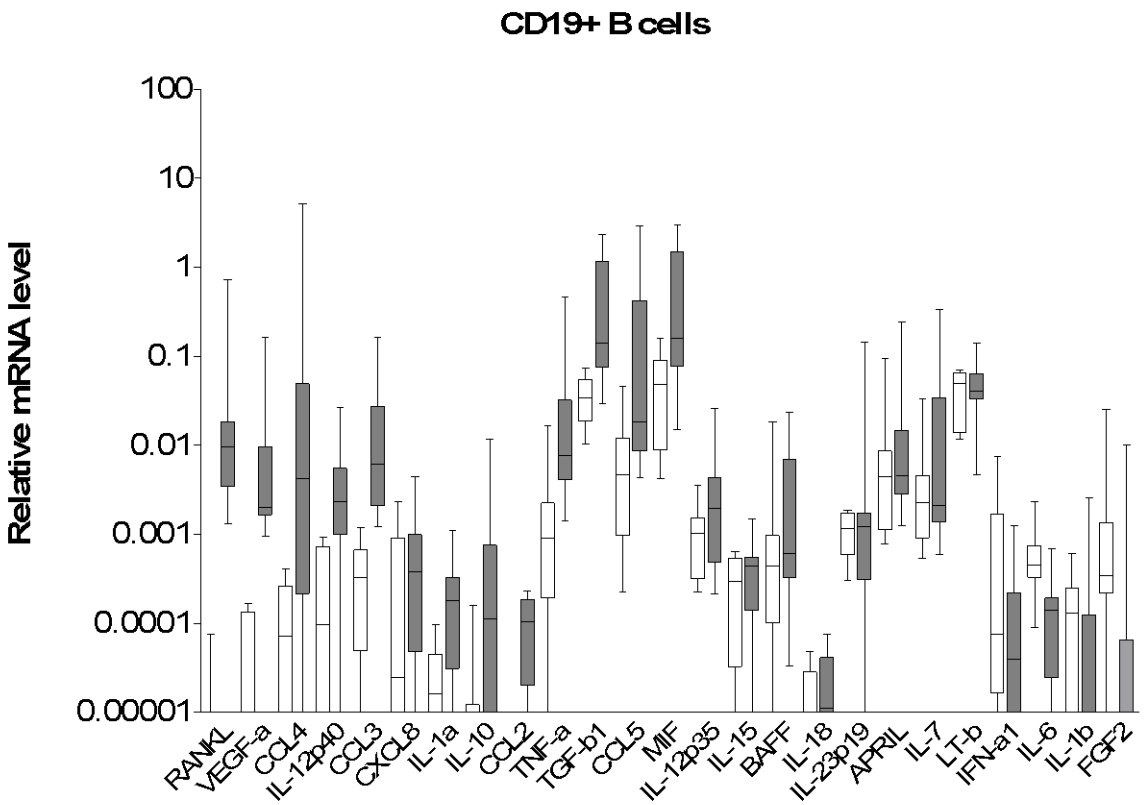


Figure 3.5 Cytokines expressed predominantly by both synovial fluid lymphoid and myeloid cell populations. mRNA expression of cytokines expressed by at least one of the lymphoid cell populations, CD4 T cells (CD4), CD8 T cells (CD8) or B cells (B), as well as either macrophages (M) or neutrophils (N). Kruskal-Wallis test; *p<0.01, **p<0.001.


CD4+ T cells
Relative mRNA level
100
10
1
0.1
0.01
0.001
0.0001
0.00001
IL-10
VEGFA
IL-11
RANKL
IL-21
CCL3
IFN-g
IL-7
IL-17A
CCL5
IL12A
CCL4
IL15
IL1B
MIF
IL-23p19
CSF2
TNF-a
TGF-b1
IFN-a1
LT-b
IFN-b1
APRIL
BAFF
IL-2
CXCL8


CD8+ T cells
Relative mRNA level
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
IL-10
VEGF-a
CCL3
IFN-g
CCL4
IL-15
IFN-a1
IL-11
MIF
CCL5
TGF-b1
IFN-b1
IL-23p19
IL-12p35
TNF-a
CXCL8
CCL2
IL-1b
IL-7
LT-b
APRIL
IL-2
BAFF


CD19+ B cells
Relative mRNA level
100
10
1
0.1
0.01
0.001
0.0001
0.00001
RANKL
VEGF-a
CCL4
IL-12p40
CCL3
CXCL8
IL-1a
IL-10
CCL2
TNF-a
TGF-b1
CCL5
MIF
IL-12p35
IL-15
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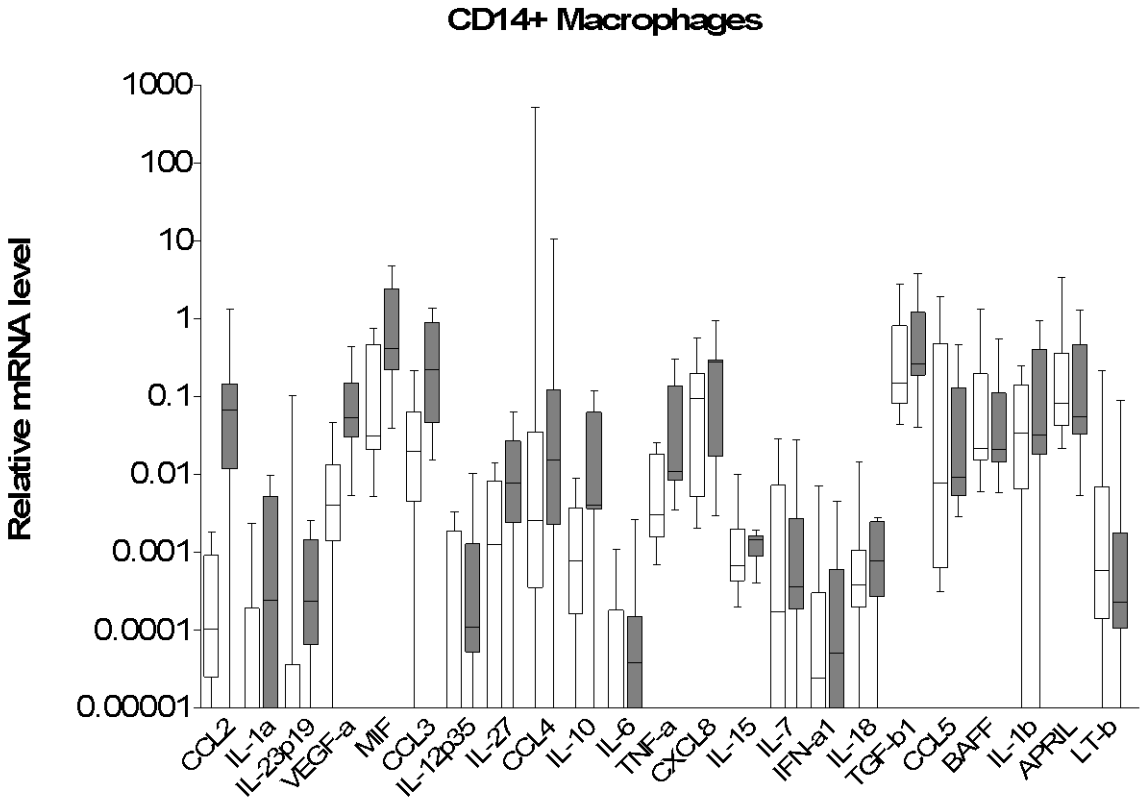
IL-23p19
APRIL
IL-7
LT-b
IFN-a1
IL-6
IL-1b
FGF2


CD14+ Macrophages
Relative mRNA level
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
CCL2
IL-1a
IL-23p19
VEGF-a
MIF
CCL3
IL-12p35
IL-27
CCL4
IL-10
IL-6
TNF-a
CXCL8
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CCL5
BAFF
IL-1b
APRIL
LT-b

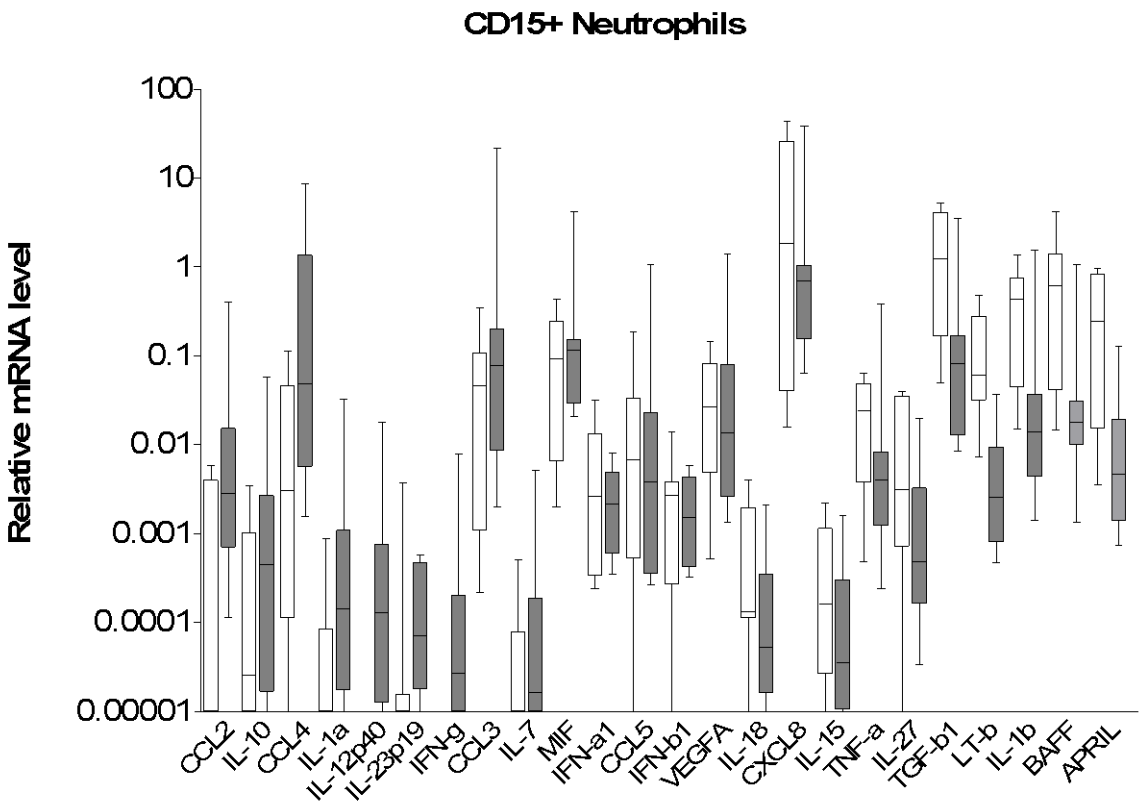


Figure 3.6 Comparison of cytokine mRNA expression in sorted populations from peripheral blood (white bars) and synovial fluid (grey bars) in established RA. For each population, mRNA expression is shown for the cytokines which had a median RQ value above the threshold value of 10^{-5}. Data are arranged hierarchically according to fold change in cytokine expression between synovial fluid and peripheral blood. Error bars indicate the range.

3.4 Discussion

In this study a novel strategy was used to compare cytokine expression in the five dominant immune cell populations in the synovial fluid and peripheral blood of RA patients without *ex vivo* stimulation. While many cytokines were found in the populations we would have predicted based on the existing literature, we also made several unexpected findings. Notably, we observed that synovial fluid B cells express high levels of pro-inflammatory cytokines. The B cell population was the only synovial fluid population that expressed both IL-12p35 and IL-12p40, the two subunits that are required for the production of functional IL-12. This observation is supported by previous reports of IL-12 production by B cells (Airoldi *et al.* 2002). Synovial fluid B cells also expressed IL-23p19, the subunit that heterodimerises with IL-12p40 to form IL-23, indicating that synovial fluid B cells also have the capacity to produce functional IL-23. We found that IL-23p19 was expressed predominantly by synovial fluid T cells, in agreement with the original publication that reported high levels of IL-23p19 in polarised Th1 cells (Oppmann 2000). However, our observation that synovial fluid CD4 and CD8 T cells do not express IL-12p40 mRNA raises the question of whether synovial fluid T cells express a yet undescribed alternative binding partner for IL-23p19. A discrepancy between the level of IL-23p19 mRNA expression and functional expression of IL-23 at the protein level has also been described in RA synovial tissue and fibroblast-like synoviocytes.

While B cells are regarded as an important cell type in RA, little is known about direct cytokine production by B cells in the RA synovium. An anti-inflammatory role has been proposed for a subset of B cells that can produce IL-10 (Iwata *et al.* 2011; Llorente *et al.* 1994; Mauri *et al.* 2003). These cells have been observed in peripheral blood of patients with autoimmune diseases including RA, but so far have not been reported in the rheumatoid joint.

Overall, the mRNA cytokine profile of synovial fluid B cells detected in this study supports a generally pro-inflammatory role for this cell type in the synovial fluid.

This is the first study to report that B cells contribute to RANKL production in the inflamed rheumatoid joint. Previous studies have reported the expression of RANKL by RA synovial fibroblasts, T cells, macrophages and chondrocytes (Crotti *et al.* 2002; Gravallese *et al.* 2000; Shigeyama *et al.* 2000; Skoumal *et al.* 2005). We also found RANKL was expressed by synovial fluid CD4 T cells, which is consistent with previous studies of expression by T cells in RA synovial tissue (Crotti *et al.* 2002). The study by Crotti et al also reported RANKL in approximately 40% of macrophages in RA synovial tissue, although it was noted that it was not possible to verify whether macrophages were the source of RANKL or bound RANKL that was produced by other cells. We did not consistently detect RANKL expression in synovial fluid macrophages, although a low level of expression was observed in a minority of samples. Consistent with our results, a previous study has reported that RANKL expression is not increased in RA synovial fluid neutrophils compared to peripheral blood neutrophils (Poubelle *et al.* 2007). RANKL is known to be required for osteoclast development and plays a key role in mediating bone erosion. Its clinical importance is underlined by the effectiveness of the monoclonal antibody against RANKL, denosumab, in limiting erosive progression in RA. Our observation of B cells as a novel source of RANKL in the rheumatoid joint was subsequently followed up on the protein level, as described in Chapter 5.

We detected IFN-γ expression in synovial fluid T cells and low IL-2 expression compared to peripheral blood, consistent with previous reports in synovial tissue and fluid (Abreu *et al.* 2009; Combe *et al.* 1985; Dolhain *et al.* 1996; Firestein *et al.* 1988; Raza *et al.* 2005; Steiner *et al.* 1999; Yamada *et al.* 2011). Detection of these cytokines in the expected populations

confirmed the robustness of our experimental strategy. We found synovial fluid CD4 T cells expressed mRNA for IL-17 supporting the notion of a Th17 population in the synovium as has been suggested by other studies (Chabaud *et al.* 1999; Kotake *et al.* 1999; Raza *et al.* 2005; Yamada *et al.* 2011; Ziolkowska *et al.* 2000). Interestingly a T cell subset producing both IL-17 and IFN-γ has recently been described in humans, which is elevated in the blood and inflamed tissues of patients with chronic inflammatory diseases (Annunziato *et al.* 2007; Kebir *et al.* 2009). In addition, a recent study has provided evidence for an IFN-γ+IL-17- subset of T cells which can arise under Th17-inducing conditions and has distinct properties from the Th1 lineage (Boniface *et al.* 2010). It is becoming increasingly apparent that the plasticity of T cells, and in particular the relationship between Th17 and Th1 subsets, needs to be taken into account when analysing IL-17 and IFN-γ-producing T cells in RA. Recent studies suggest that Th1 cells rather than Th17 cells are the predominant effector subset in the RA synovium (Yamada *et al* 2011.; Yamada *et al.* 2008). Further studies are required to elucidate the relative contribution and relevance of these T cell subsets in RA. In keeping with the notion that Th2 cells do not have a central role in established RA, we did not detect expression of Th2-associated cytokines IL-4, IL-5 and IL-13 in synovial fluid T cells. Other studies have reported that levels of these cytokines are not elevated in synovial fluid compared to peripheral blood (Berner *et al.* 2000; Isomaki *et al.* 1999).

Macrophages are considered an important source of synovial pro-inflammatory cytokines in RA. We found that synovial fluid macrophages expressed high levels of TNF-α, IL-6 and IL-1α, but other synovial fluid populations also expressed comparable levels of mRNA for these cytokines. In addition, several cytokines and chemokines that were highly expressed by macrophages were also expressed at comparable levels by neutrophils including IL-1β, CCL3

and CXCL8. The neutrophil population was found to express high levels of mRNA for IFN-α1 and IFN-β1, which has not been previously described in RA.

The results of this study have allowed us to map cytokine mRNA production in the five largest cell populations in established RA synovial fluid. It should be noted that there are many other synovial fluid cell populations which were not assessed including NK cells, NKT cells and mast cells which represent small populations in RA synovial fluid. Furthermore, cytokine production by stromal cells in the synovial tissue such as synovial fibroblasts, macrophage-like synoviocytes and endothelial cells were not investigated. For example, mast cells have been reported as a source of IL-17 in the RA synovium (Hueber *et al.* 2010). It would be interesting to further investigate cytokine expression by different subsets of the cell populations analysed here, for example in the case of T cells, B cells and macrophages. It should also be noted that the cytokine-expressing characteristics of cell populations present in the RA synovial fluid may not necessarily be representative of those found in the synovial tissue. Indeed, differential regulation of cytokines has been reported in these two compartments, for example, different components of the IL-6 receptor expressed are at different levels in RA synovial fluid and synovial tissue (Hidalgo *et al.* 2011).

In this study, gene expression was normalised to ribosomal 18S RNA. The use of any normalisation strategy across different cell populations is always a potential weakness, whether expression is normalised to metabolic enzymes such as GAPDH, structural proteins such as β-actin, or ribosomal RNA. Due to the nature of the strategy used to detect cytokine gene expression, it is intrinsically a screening assay. Eventually, the success of such a screening strategy is validated by the novel and important findings made, and whether these findings can be confirmed at the level of protein production.

Characterisation of cytokine expression by individual synovial populations is the first step towards understanding the complex cytokine network that operates in the joint in RA. One of the strengths of this study is that cytokine expression has been determined directly *ex vivo* in order to try to reflect the situation within the inflamed joint as closely as possible. Considerable effort was made to avoid stimulation of cells during the process of isolation. However, it is still conceivable that cytokine mRNA may be affected by the isolation procedure. It should be taken into account that mRNA expression as determined in this study is not necessarily reflective of protein production due to post-transcriptional regulation of cytokine expression. Interpretation of the results of this study also needs to take into account multiple factors that influence the biological activity of cytokines in the joint. The biological activity of a given cytokine depends on the simultaneous production of agonist and antagonistic cytokines, the presence of inhibitors, and the extent to which its receptor is expressed. Cytokine activity is also regulated by interactions between intracellular signals which may occur due to priming or reprogramming of signalling, or the uncoupling of ligand/receptor pairing from signal transduction. Importantly, some cytokines such as IL-10 can have pro-inflammatory or anti-inflammatory activities depending on the cytokine environment. In addition, mediators belonging to other families of molecules, such as hormones and adipokines, can also regulate cytokine activity. Understanding the complex crosstalk that exists in the cytokine network in the RA joint and the functional hierarchies therefore remains an important challenge.

In summary, our data have allowed us to determine cytokine mRNA expression by each of the five main cell populations present in the synovial fluid of patients with established RA (Figure 3.6). We have been able to compare relative expression of cytokines between cell types, and also determine cytokine expression of cell populations in synovial fluid compared

to peripheral blood. Several novel observations of cytokine gene expression have been made in the context of this study which will need to be followed up and validated on the protein level.

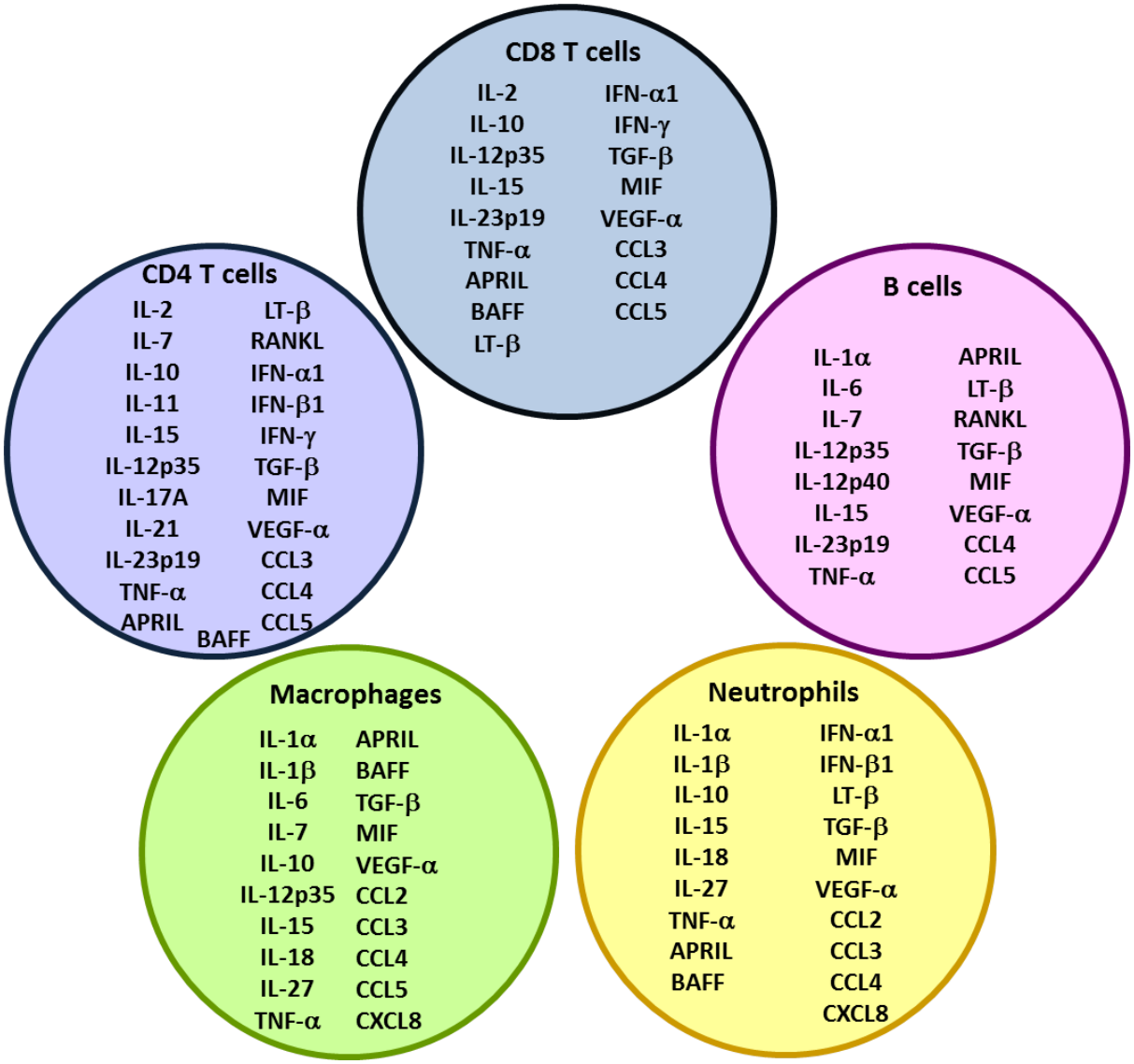


Figure 3.7 Summary of cytokines expressed by CD3+CD45RO+CD4+ T cells, CD3+CD45RO+CD8+ T cells, CD19+ B cells, CD14+ macrophages and CD15+ neutrophils in RA synovial fluid. For each population, cytokines are shown if their level of expression was at least 2 logs higher than in other cell populations.

4 CYTOKINE PROFILING IN EARLY AND ESTABLISHED ARTHRITIS

4.1 Introduction

When a patient presents with inflammatory arthritis, it is frequently unclear whether the synovitis will resolve or if the patient will develop a persistent inflammatory arthritis such as RA. In the Birmingham Early Arthritis cohort of patients with recent onset arthritis of up to 12 weeks' duration, approximately half of the patients had spontaneously resolving synovitis whereas the rest went on develop a persistent synovitis such as RA (Raza *et al.* 2005). In other early inflammatory arthritis cohorts, self-resolving synovitis was reported in 25% of patients (Harrison *et al.* 1996), 55% of patients (Tunn and Bacon 1993) and 60% of patients (Visser *et al.* 2002). It is important to differentiate individuals destined to develop RA from those whose disease will resolve in order to be able to inform patients of their prognosis and target treatment to the appropriate individuals. Furthermore, early recognition of RA is necessary to facilitate early therapeutic intervention, as initiation of DMARD therapy within the first few months of symptom onset is associated with better outcome than when treatment is commenced later in the course (Mottonen *et al.* 2002; Nell *et al.* 2004; van der Linden *et al.* 2010).

Clinical prediction models have been developed to determine outcomes of patients with early arthritis at presentation. A prediction rule for use in patients with undifferentiated arthritis, developed by van der Helm van Mil and colleagues, based on nine clinical variables (sex, age, localization of symptoms, morning stiffness, tender joint count, swollen joint count, CRP level, and positivity for rheumatoid factor and ACPA) was found to have a positive predictive

value of 84% and a negative predictive value of 91%, and has been validated in several cohorts (Kuriya *et al.* 2009; van der Helm-van Mil *et al.* 2008). However, the biological characteristics of synovial pathology have not yet been systematically assessed to see if they would contribute meaningfully to predictive algorithms.

Assessment of synovial histology using haematoxylin and eosin-based staining of tissue sections and electron microscopic analysis of synovial tissue from patients with synovitis of less than 2 months' duration found no clear differences between patients with self-limiting synovitis and those who subsequently developed RA (Schumacher and Kitridou 1972). In contrast, a study which compared the level of cytokines in the synovial fluid of early synovitis patients with diverging clinical outcomes showed that patients who developed RA could be distinguished from those who developed non-RA persistent disease or resolving arthritis based on their synovial fluid cytokine pattern (Raza *et al.* 2005). Synovial fluid from patients with early RA contained higher levels of the T cell, macrophage and stromal cell-related cytokines, IL-2, IL-4, IL-15 and GM-CSF.

Several studies have investigated whether differences can be found in synovial pathology during the early and established phases of RA. In studies comparing early RA (defined as a duration of <1 year since the diagnosis of RA) and established RA, immunohistological analysis of the synovium (Tak *et al.* 1997) and cytokine mRNA expression in synovial fluid mononuclear cells (Bucht *et al.* 1996) were found to be similar. However, higher levels of T cell, macrophage and stromal cell-related cytokines have been reported in the synovial fluid of patients with early RA compared to patients with established RA (Raza *et al.* 2005), and levels of BAFF and APRIL are higher in the serum in early compared to established RA

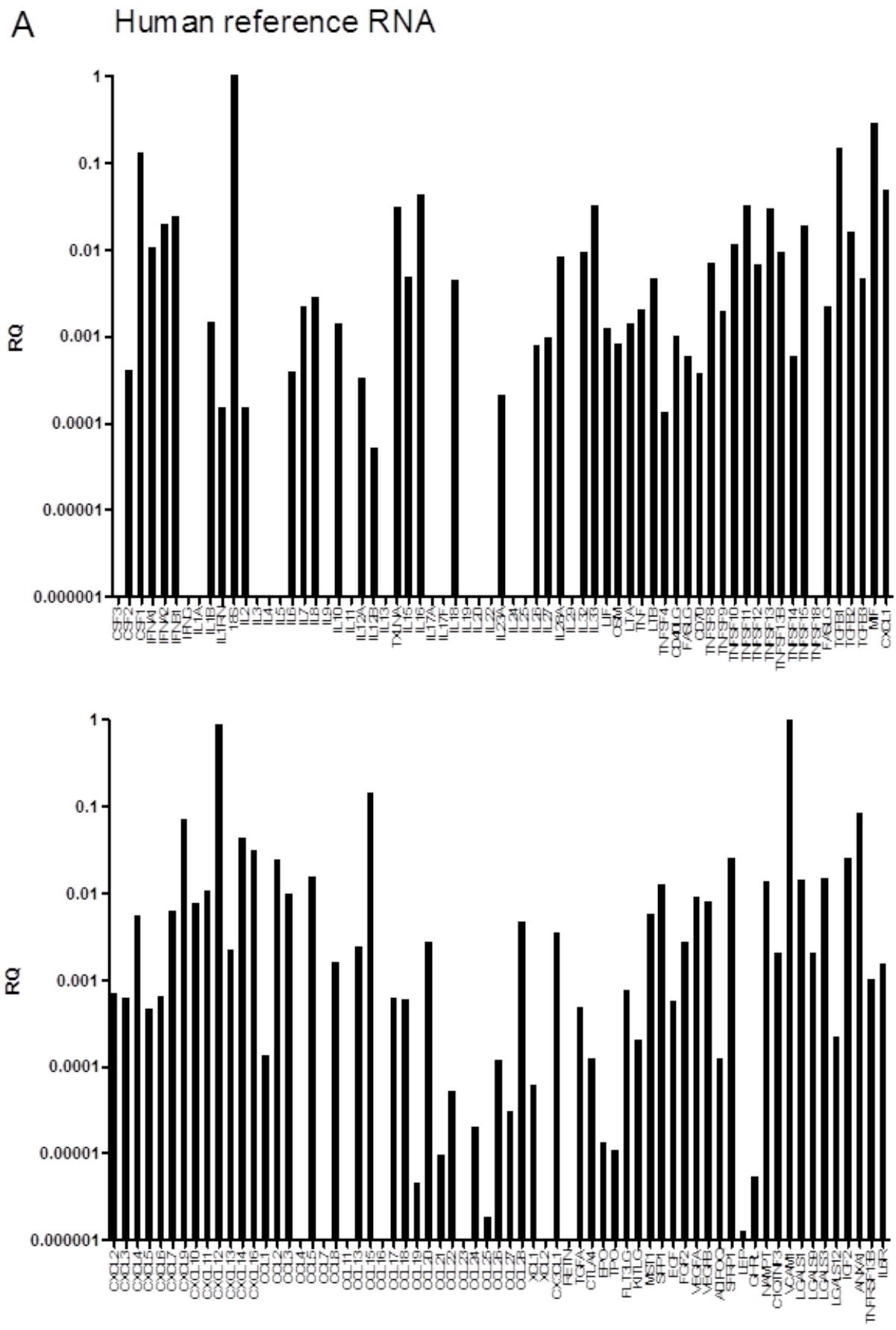
(Moura *et al.* 2010). In addition, in gene expression profiling studies of peripheral blood cells, early and long-standing RA have been found to have distinct molecular signatures associated with different biological processes (Lequerre *et al.* 2009; Olsen *et al.* 2004). This supports the notion that different molecular and cellular mechanisms operate in early versus established RA, and features of early synovitis could relate to disease outcome.

As cytokine production in the RA synovium can be considered to reflect the pathological processes operating in a given stage of disease, we hypothesised that cytokine expression in the synovial tissue and fluid would differ between patients in the early and established stages of RA. We also hypothesised that cytokine expression in the synovial tissue and fluid of early synovitis patients who developed RA would differ from patients with self-resolving disease. Genes selected for investigation were cytokines or cytokine-related molecules belonging to families which have been associated with RA; these included interleukins, TNF family members, chemokines, growth factors, adipokines and interferons. In this study we examined the cytokine gene expression profiles of patients with early synovitis within 12 weeks of symptom onset, which represents a phase of disease that has not previously been studied using this approach.

4.2 Synovial tissue cytokine expression in early and established arthritis

Synovial tissue biopsies were prospectively obtained from 18 early synovitis patients who developed RA, 13 early synovitis patients with self-limiting synovitis, 12 patients with established RA, and 12 control subjects. The control subjects were patients undergoing

arthroscopy with no macroscopic or radiological evidence of inflammatory pathology. These patients were undergoing arthroscopy for treatment of non-inflammatory conditions, such as ligament or meniscal repair. A microfluidic card was designed to analyse expression of a panel of genes which included 135 cytokines and related molecules (interleukins, TNF family members, chemokines, growth factors, interferons, adipokines and other cytokine-like factors; see Appendix Table 8.2) and housekeeping genes. mRNA expression was determined by real-time PCR, and the expression of each gene was calculated relative to the levels of GAPDH. Of the three housekeeping genes included (GAPDH, β2 microglobulin and 18S), GAPDH was selected because it showed the least variation in expression across samples. mRNA from PBMC stimulated with magnetic beads coated with anti-CD3 and anti-CD28 antibodies, and a commercially available blend of RNA from human cell lines representing different tissues (Stratagene) were used as positive controls to test whether the microfluidic card real-time PCR assays worked. With the exception G-CSF, CCL23, XCL2 and RETN, expression of all cytokine genes was detected (Figure 4.1A,B). As described in 2.4.1, gene expression was assessed using two approaches: (i) expression of all genes was analysed using RNA which was not treated with DNase, and (ii) expression of genes with real-time PCR assays containing non-intron spanning primers or genes expressed at very low levels was analysed using RNA treated with DNase, and using six times more RNA per assay than originally used. Variability between the real-time PCR assays on separate microfluidic cards was assessed by running a sample of reference RNA or RNA extracted from a synovial tissue biopsy section on two different cards (Figure 4.1C). Comparison of mRNA expression between the two cards showed that results were very similar, emphasising the reproducibility of this approach.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Human reference RNA
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IFNW1
IFNA2
IFNB1
IFNG
IL1A
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IL12A
IL12B
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TxLNA
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IL17A
IL17F
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IL31
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LIF
OSM
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TNF
LTB
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FASLG
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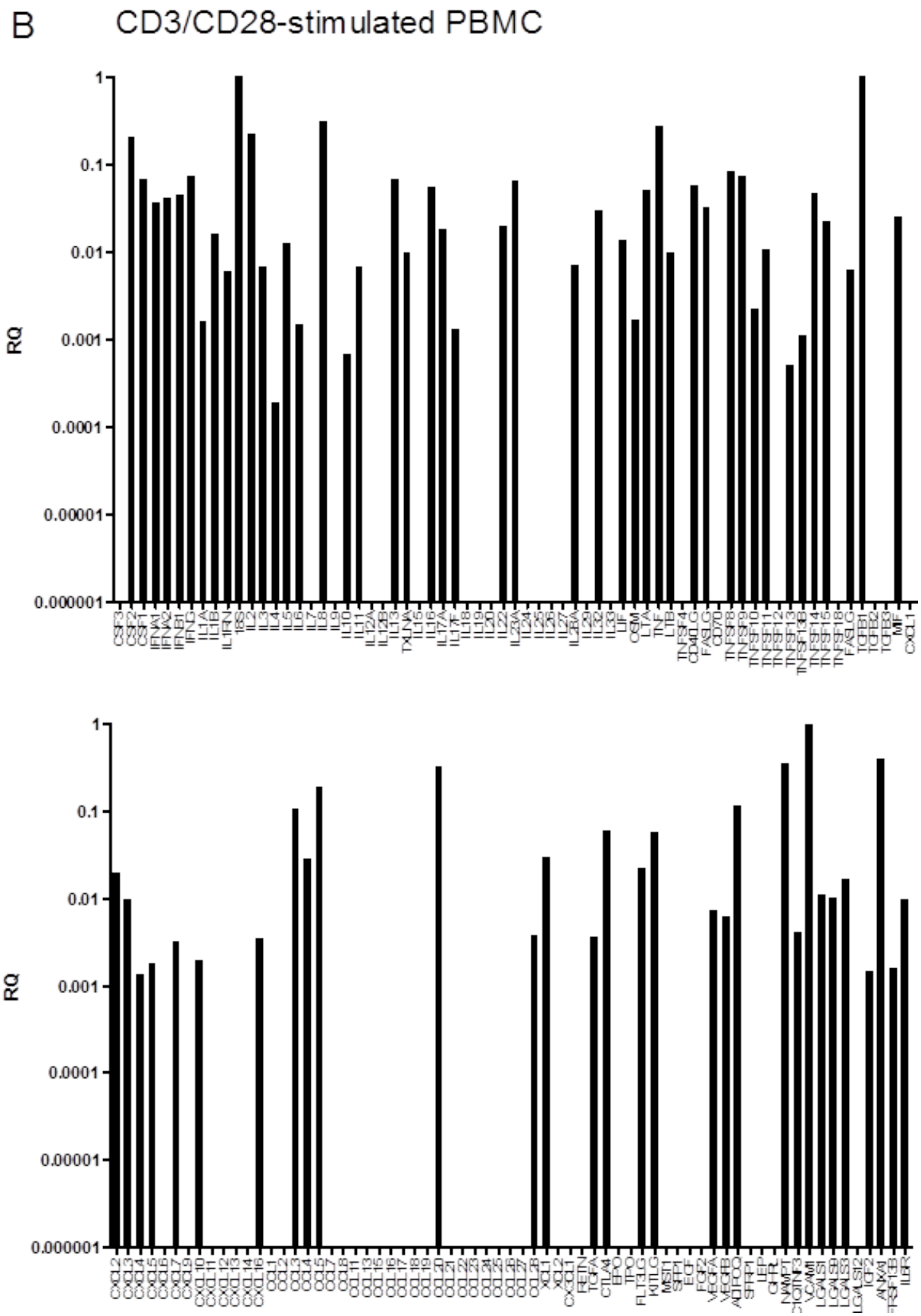
B
CD3/CD28-stimulated PBMC
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IL12A
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TXLNA
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IL17F
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IL28A
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TNF
LTB
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CD40LG
FASLG
CD70
TNFSF8
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CCL26
CCL27
CCL28
XCL1
XCL2
CX3CL1
TCFA
CTLA4
EPO
TPO
FLT3LG
KITLG
MSTI1
SFPI1
EDF
FGF2
VEGFA
VEGFB
ADIPOQ
SFRP1
LEP
GHRL
NAMPT
ANGPTL3
VCAM1
LGALS1
LGALS3
LGALS8
LGALS12
IGF2
ANXA1
TNFRSF1B
ILBR

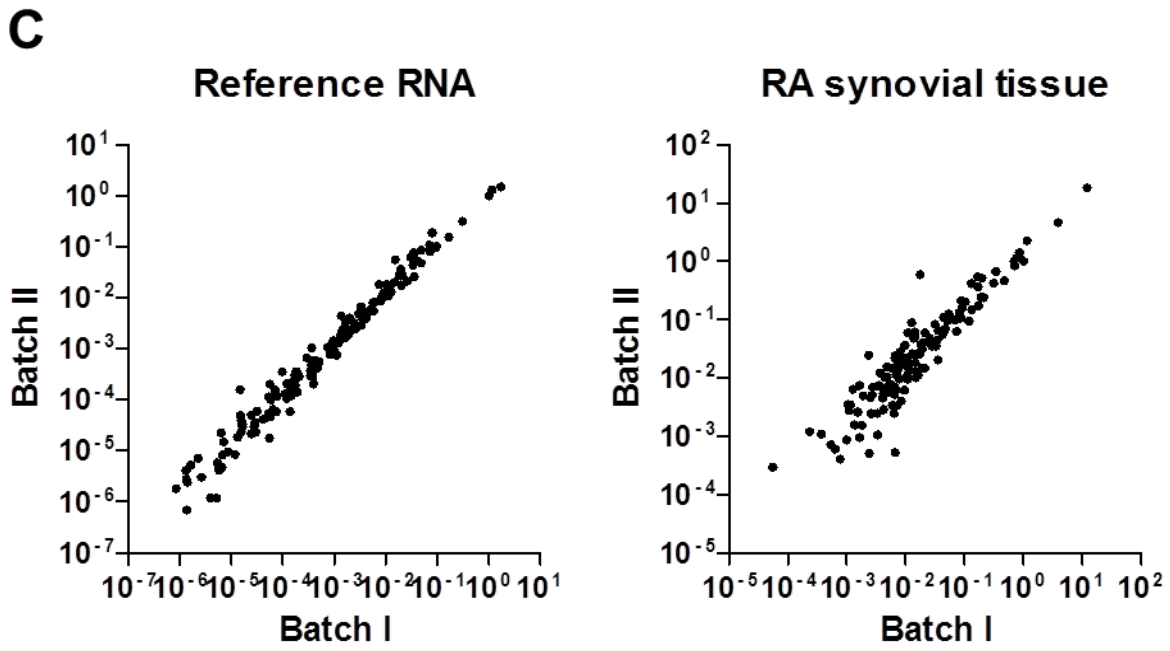


Figure 4.1 Method validation. (A) Cytokine mRNA expression in PBMC stimulated with anti-CD3/anti-CD28-coated beads was used as a positive control for the real-time PCR assays. (B) Cytokine mRNA expression in human reference RNA (Stratagene) was used as a positive control. (C) Comparison of cytokine mRNA expression in two batches of microfluidic cards using human reference RNA (left) and RNA extracted from a synovial tissue biopsy section (right). The expression of all genes detected in split samples using two cards from different batches is plotted .

Genes that were differentially expressed between groups were identified by performing the Kruskal-Wallis test followed by the Dunn's post-test between groups for each genes. Of the 135 cytokines analysed, 4 genes were found to be differentially expressed between groups, 16 genes were not detected, and 105 genes did not show significant differences between groups. The complete set of mRNA expression data is shown in Appendix 8.3.1. Genes not detected were IL-3, IL-4, IL-5, IL-9, IL-13, IL-17A, IL-17F, IL-19, IL-20, IL-22, IL-24, IL-25, IL-29, XCL2, RETN and EPO. Cytokines that were found to be differentially expressed between groups are shown in Figure 4.2 and Table 4.1. mRNA levels of IL-2 were significantly higher in established RA than resolving synovitis patients; IL-21 was higher in established RA than resolving synovitis patients and control subjects; CCL2 and CXCL13 were higher in established RA than controls; CXCL4 and CXCL7 were both higher in established RA and early RA compared to controls; and CXCL7 was also higher in resolving synovitis patients compared to controls.

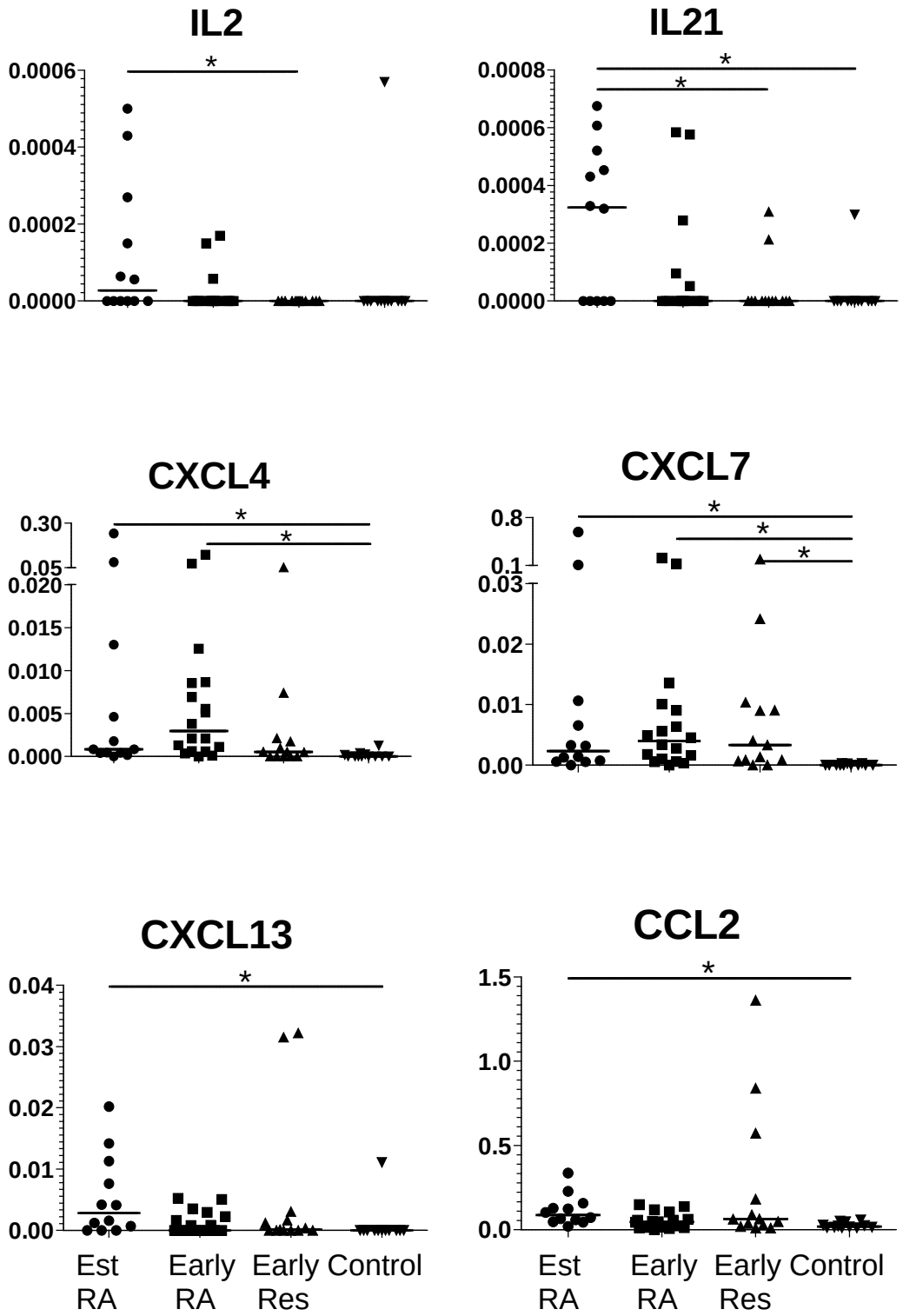


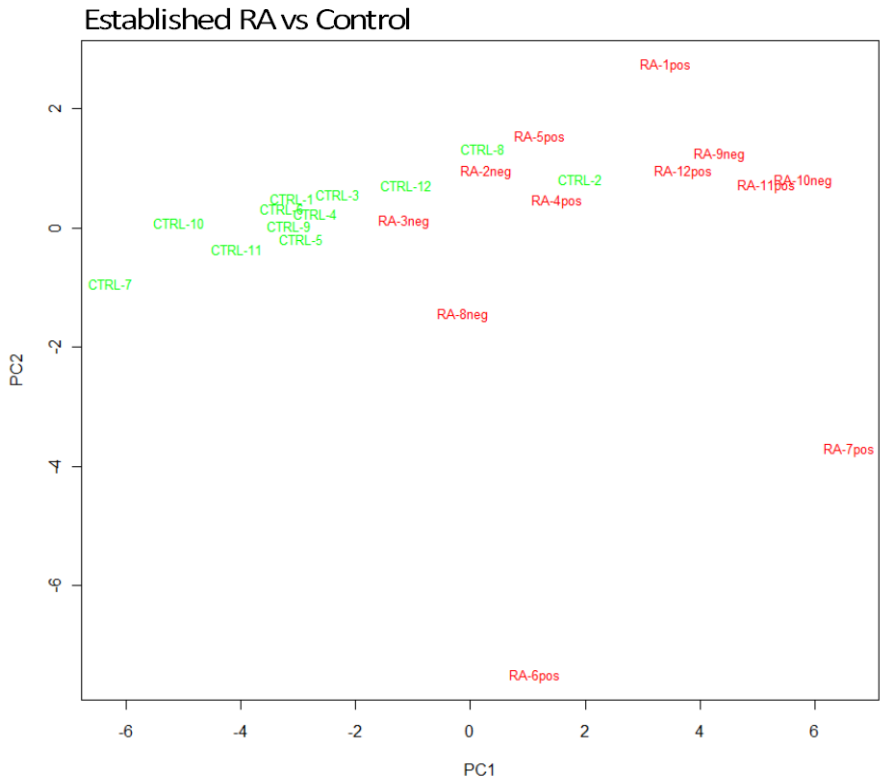
Figure 4.2 mRNA expression of cytokines differentially expressed between established RA, early RA, early resolving synovitis and control groups. Established RA (circle), early RA (squares), early resolving synovitis (upward triangles), and controls (downward triangles), Expression is quantified relative to GAPDH. Kruskal Wallis test with Dunn's post hoc test adjusted for multiple comparisons. *p<0.01.

Groups compared in Dunn's post test analysis	Differentially expressed genes
Est RA > Early resolving synovitis	IL-2, IL-21
Est RA > Control	IL-21, CXCL4, CXCL7, CXCL13, CCL2
Early RA > Control	CXCL4, CXCL7
Early resolving synovitis > Control	CXCL7

Table 4.1 Cytokines and chemokines differentially expressed in synovial tissue of early and established arthritis patients and controls.

The synovial tissue cytokine mRNA expression data was also analysed using principle component analysis (PCA). PCA is an algorithm commonly used to represent relationships between samples which seeks to reduce the dimensionality of a data set while retaining as much as possible of the variation present within the original data. In this analysis, samples are clustered into groups displayed in two dimensions. The axis of the first principal component (x-axis) represents the direction incorporating the maximum variability of all genes. The remaining variation within the data is represented in the direction of the second principal component (y-axis). Samples are thus visualised in "principal component space" by their expression of the most variable genes which make up the principal components. PCA was used to analyse relationships between cytokine expression in the synovium of patients with early RA, resolving synovitis, established RA and controls, as shown in Figure 4.3. Using genes that were differentially expressed by >1.5 fold, pair-wise comparisons were made between each of the groups. Comparison of established RA and early RA showed a degree of clustering by each of the two groups that could enable them to be separated visually along the first component, whereas early RA and early resolving synovitis patient groups did not cluster separately. Early resolving synovitis and established RA patients showed some degree of separation along the first principle component. When the patient groups were compared to the

controls, the control subjects tended to cluster separately from RA patients or early RA patients. In contrast, early resolving synovitis patients and control individuals did not cluster separately. Together these results indicate that based on synovial cytokine expression, it may be possible to distinguish established RA and early RA from each other and from the control group, whereas early resolving synovitis patients could not be readily distinguished from patients with early RA or controls. However, the sample size is small and more samples will be needed to assess the robustness of the differences observed.


Established RA vs Control
RA-1pos
RA-5pos
CTRL-8
RA-9neg
RA-2neg
RA-12pos
CTRL-2
RA-10neg
RA-11pos
CTRL-12
CTRL-3
RA-4pos
CTRL-1
CTRL-4
RA-3neg
CTRL-10
CTRL-9
CTRL-5
CTRL-11
CTRL-7
RA-8neg
RA-7pos
RA-6pos
PC2
PC1
2
0
-2
-4
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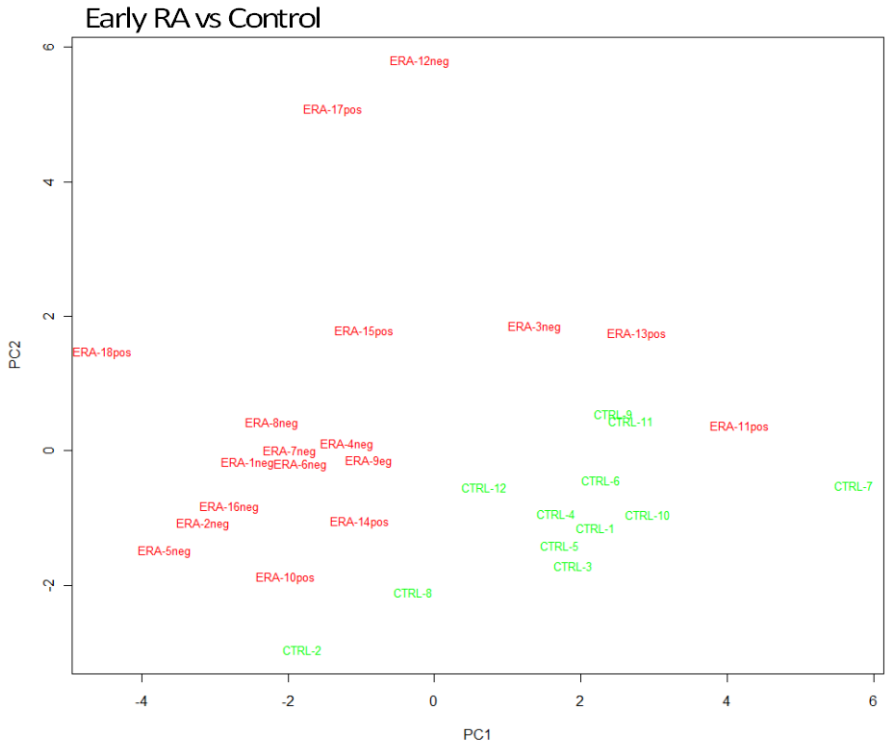
2
4
6


Early RA vs Control
ERA-12neg
ERA-17pos
ERA-15pos
ERA-3neg
ERA-13pos
ERA-18pos
CTRL-9
CTRL-11
ERA-11pos
ERA-8neg
ERA-4neg
ERA-7neg
ERA-1neg
ERA-6neg
ERA-9eg
CTRL-12
CTRL-6
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ERA-2neg
ERA-14pos
CTRL-4
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ERA-5neg
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ERA-10pos
CTRL-8
CTRL-2
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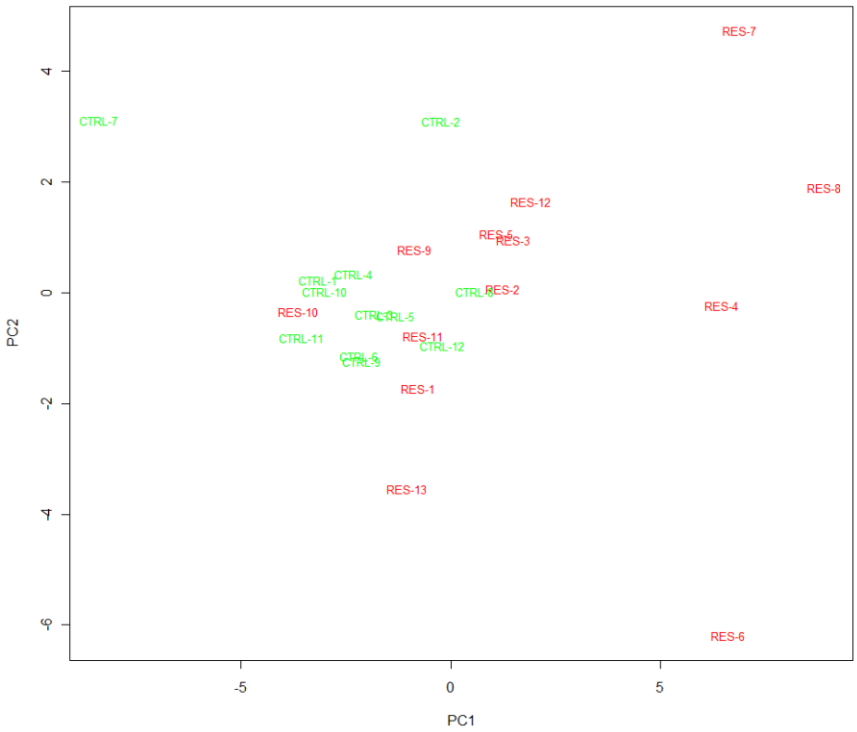
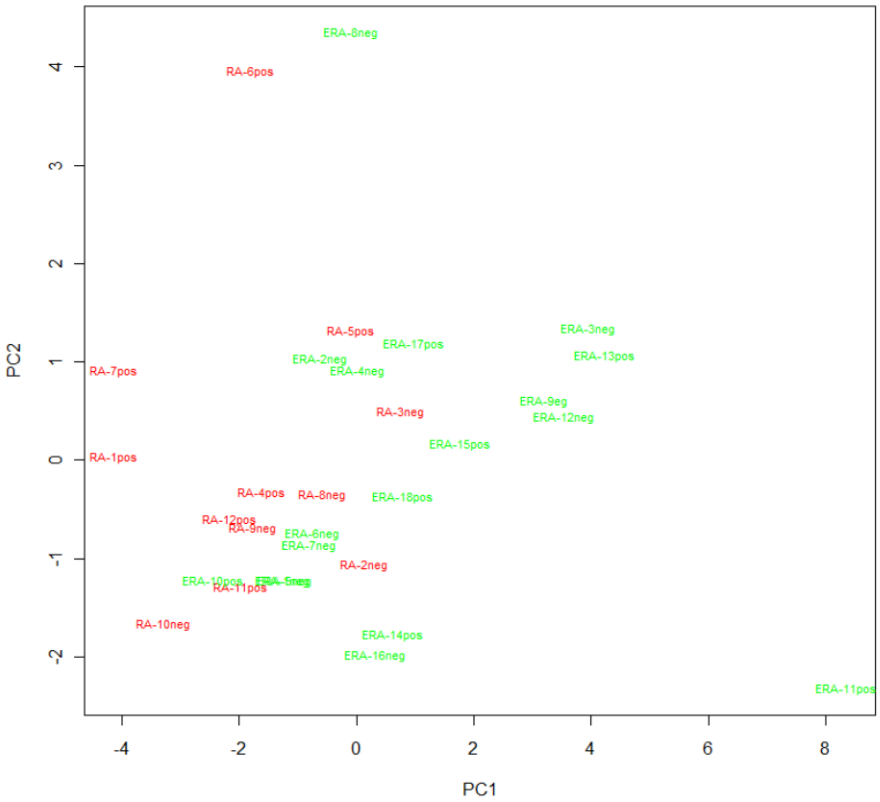
Early Resolving vs Control



Early RA vs Established RA



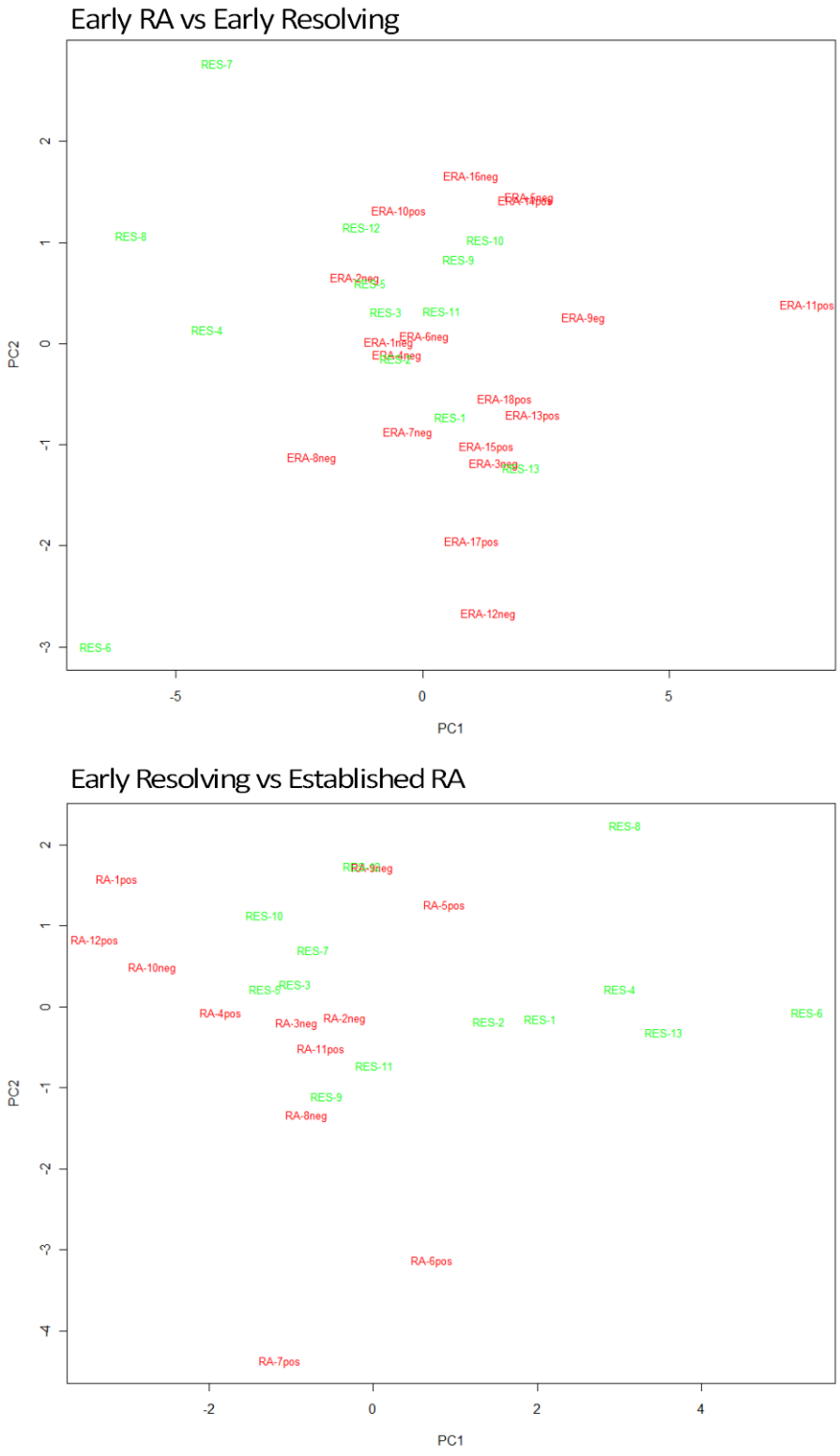


Figure 4.3 Comparison of synovial tissue cytokine expression in early RA, early resolving synovitis, established RA and controls. Principle component analysis was performed using genes showing >1.5 fold change. Red and green labelling is used to denote the different groups being compared. Established RA (RA), early RA (ERA), early resolving synovitis (RES), control (CTR).

The presence of circulating ACPA distinguishes two distinct subsets of RA, mainly based on differences in genetic and environmental associations. In this study, established and early RA patients were split into ACPA-positive and ACPA-negative groups, and differences in gene expression were calculated using Mann-Whitney tests. All data comparing cytokine expression in ACPA-positive and ACPA-negative patients is shown in Appendix 8.3.2. Of the 135 cytokines analysed, 1 gene showed a significant difference between the two groups, shown in Figure 4.4. In established RA patients, expression of CXCL16 was higher in the ACPA-positive group than the ACPA-negative group.

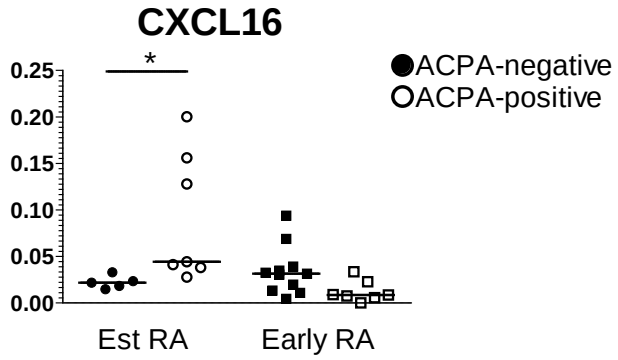


Figure 4.4 mRNA expression of CXCL16 in ACPA-positive and ACPA-negative patients. ACPA-negative patients (closed symbols) and ACPA-positive patients (open symbols) with established RA (circles) or early RA (squares). Expression quantified relative to GAPDH. Mann-Whitney unpaired t-test; p*<0.01.

4.3 Cytokine expression in sorted synovial fluid cell populations in early and established arthritis

In addition to examining cytokine expression in whole sections of synovial tissue, we also assessed cytokine expression in the five cell populations sorted from synovial fluid of early synovitis and established RA patients. By enriching specific populations, this approach has the benefit of detecting cytokines that may not have been detected in the whole tissue as their expression may have been obscured by that of larger synovial tissue populations. Cell populations were sorted from synovial fluid and peripheral blood from 5 early synovitis patients who progressed to RA, 4 early synovitis patients whose disease resolved and 10 patients with established RA. Cell populations were also sorted from peripheral blood from 5 healthy controls. CD4 and CD8 T cells, B cells, macrophages and neutrophils were isolated from each synovial fluid or peripheral blood sample as described previously. Often only small volumes of synovial fluid could be aspirated from the joints of patients with early synovitis. Due to the low cell numbers in the samples obtained, and the requirement for isolated populations to have ≥95% purity, there were relatively few samples from early synovitis patients which were suitable for using in the study. Microfluidic cards containing assays for 41 cytokines were used to determine mRNA expression by real-time PCR (see Appendix Table 8.1), and expression of each gene was normalised to 18S. Genes that were not detected were IL-4, IL-13, G-CSF, CCL11, CXCL12 and EGF.

To determine significant differences between patient groups, the Kruskal-Wallis test were performed to see if there were differences in gene expression within each sorted cell population. The Dunn's post-test was used to determine which groups differed significantly from each other. The complete set of mRNA expression data from synovial fluid and

peripheral blood sorted cells is shown in Appendix 8.3.3. Genes that were differentially expressed in synovial fluid cell populations between patient groups are shown in Figure 4.5. mRNA expression of IFN-β1 by CD8 T cells was significantly higher in early synovitis patients whose disease resolved than early synovitis patients who developed RA and patients with established RA. The level of IFN-β1 produced by CD8 T cells in early resolving synovitis patients were similar to levels produced by neutrophils in established RA patients. However, as there was only one synovial fluid neutrophil sample available in the early resolving synovitis group, it is not possible to compare expression in neutrophils and CD8 T cells. Levels of IL-10 in CD8 T cells and VEGF-α in B cells were higher expressed in established RA synovial fluid cell populations compared to early resolving synovitis patients.

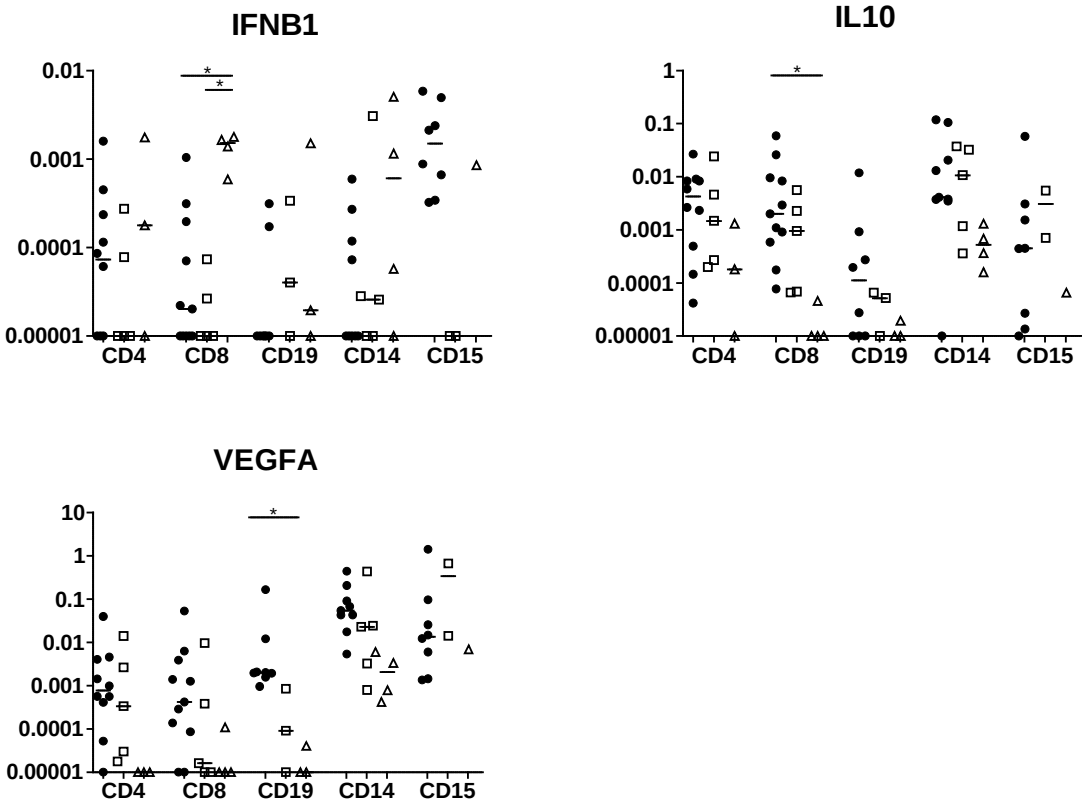


Figure 4.5 mRNA expression of genes differentially expressed between established RA, early RA and early resolving synovitis in cell populations sorted from synovial fluid. Established RA (circles), early RA (squares), early resolving synovitis (upward triangles).CD3+CD45RO+CD4+ T cells, CD3+CD45RO+CD8+ T cells, CD19+ B cells, CD14+ macrophages and CD15+ neutrophils were sorted from synovial fluid. Expression was quantified relative to 18S. Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons; *p<0.01.

Groups compared in Dunn's post test analysis	Differentially expressed gene	Cell population in which gene differentially expressed	Dunn's post test result
Early resolving synovitis vs Early RA	IFN-β1	CD8 T cell	**
Early resolving synovitis vs Established RA	IFN-β1	CD8 T cell	*
	IL-10	CD8 T cell	*
	VEGF-α	B cell	*

Table 4.2 Cytokines differentially expressed in synovial fluid cell populations from early and established arthritis patients. Dunn's post test analysis following Kruskal-Wallis test: * $p<0.05$, ** $p<0.01$.

4.4 Discussion

We hypothesised that within the first three months of onset of synovitis, patients destined to develop RA would show a distinct synovial pathology, reflected in cytokine expression, which would enable them to be distinguished from early synovitis patients with self-limiting disease. In sorted cells from the synovial fluid, we found that IFN-β was expressed at higher levels in CD8 T cells from patients with resolving synovitis compared to patients with early RA. As IFN-β is produced in direct response to virus infection, this may indicate a heightened anti-viral response in resolving synovitis patients whose disease was likely caused by a viral infection. Of the three resolving synovitis patients, one had post-streptococcal resolving synovitis, one patient had unclassified resolving synovitis, and data for the other patient is not available.

Cytokine mRNA profiling of synovial tissue from patients with resolving synovitis and early RA did not reveal any significant differences between the two groups, suggesting that at the level of the cytokines studied, the synovium of patients destined to develop RA largely resembles those with disease destined to self-limit. Assessing cytokine expression within the whole synovial tissue biopsy section may have concealed differences in expression as a mixture of synovial cell populations were studied, and production of cytokines by cell populations which are only found at a low frequency in the synovium may not have been identifiable. Furthermore, it should be noted that the early resolving group was very heterogeneous. In addition, these patients may have been seen at different stages of their disease course; for example, one patient may have been seen at the peak of an inflammatory flare, whereas another patient may have been seen during the resolution phase of inflammation; however this information was not systematically collected. The heterogeneity

of this group of patients may help to explain why so few significant differences were observed when compared to patients with early RA. In the future it would be interesting to look at these findings in relation to the clinical phase of the resolving synovitis.

Overall, comparison of early synovitis patients with self-limiting disease or that leading to RA revealed that cytokine expression in the synovium in these groups is largely similar. This finding implies that the synovial cytokine expression observed is largely reflective of general inflammation. If cytokine production can be considered to some degree to represent the cell populations present in the synovium, these findings may be considered broadly consistent with the finding that no clear histological differences are observed between early synovitis patients with self-limiting synovitis and patients who go on to develop RA (Schumacher and Kitridou 1972). Furthermore, the histopathological features of parallel biopsies were analysed in paraffin-embedded tissue stained with haematoxylin and eosin, and it was found that patients with early and resolving RA were similar in terms of patterns of leukocyte infiltration and lining layer thickness (data not shown). These findings are compatible with my results which indicate that the features of inflammation are broadly similar in both groups. Here, we studied synovitis patients within the first 3 months after symptom onset. The similarity of patients who developed RA and patients with self-resolving disease suggests that if there is a "switch to persistence" leading to development of RA, this occurs outside the time frame studied, or is simply not reflected in the cytokine expression by synovial populations. Indeed, evidence to support the former concept is provided by the observation that immunological abnormalities precede the development of clinically apparent RA. This indicates that due to genetic predisposition for development of RA, for example, it is determined during the pre-clinical phase whether an individual will develop RA following development of synovitis which may be triggered by a non-specific stimulus such as minor trauma or infection.

Therefore in patients who develop RA and patients whose synovitis resolves, it is possible that the same initiating factors are responsible for triggering inflammation, accounting for the observation that in the first 3 months after symptom onset, inflammation in the synovium, as reflected at the level of cytokine expression, is broadly similar in the two groups of patients. As alluded to previously, it is possible that the pathological differences in early RA and resolving synovitis patients are not reflected in synovial cytokine expression. While cytokines clearly have important roles in regulating cellular infiltration in the RA synovium by their ability to recruit and maintain cells at sites of inflammation, multiple additional factors are responsible for the persistence of cells in the synovium such as pro-inflammatory pathways involving lipid mediators, anti-apoptotic pathways, and hormonal influences. In patients with disease destined to self-limit compared with those with disease destined to persist, it may be more informative to compare features of resolution of inflammation, as these may be more important in determining outcome than the cytokines and related molecules assessed in this study. Another important factor determining the development of RA may involve phenotypic changes in the stromal cells of the synovium, and these changes would not necessarily be observed by assessing the genes selected in the panel investigated. Therefore there remains a need to identify persistent versus resolving disease to inform identification of therapeutic strategies and biomarkers of outcome for use in clinical practice.

Another important question we wished to address was whether cytokine expression differs in the early and established phases of RA. In the synovial tissue, cytokine mRNA expression was not significantly different in patients with established RA and early RA These results are compatible with an existing body of literature which suggests that that features of synovial inflammation in patients with early RA are similar to those observed in longstanding disease, in terms of cell infiltration and expression of cytokines, chemokines, granzymes, adhesion

molecules and MMPs (Baeten *et al.* 2000; Smeets *et al.* 1998b; Tak *et al.* 1997; Tak *et al.* 1995). Overall, these studies suggest that synovial inflammation observed in early arthritis is representative of chronic inflammation seen in established disease. A previous study of patients with recent-onset arthritis reported that distinct cytokine profiles could be found in different phases of disease (Raza *et al.* 2005), which differs from the results of the present study. Differences in study design may account for some of the discrepancies observed, for example, the study by Raza and colleagues measured protein expression of cytokines in synovial fluid, whereas in this study we measured expression of cytokines at the mRNA level.

It should be noted that whereas the "duration" of RA is timed by many groups in relation to either the first patient-reported joint swelling or the date of initial fulfilment of classification criteria for RA, in the Birmingham cohort, duration is timed from the date of the first symptoms (including joint pain and stiffness) attributed to inflammatory joint disease by the assessing rheumatologist. Patients in our cohort included in the "early RA" category may well not have had disease classified as RA at the time of sample collection – although they would have developed RA on follow-up assessment. The patients included in this study thus represent a unique cohort in the study of early inflammatory arthritis, capturing a much earlier phase of disease than has been studied by other early arthritis centres. This does however make it difficult to compare results between studies; a new EULAR initiative is addressing this issue of dating to onset of disease to aim for greater clarity. It appears that within the three-month window following symptom onset we have studied, it is not possible to observe a synovial cytokine profile in early RA that is distinct from established RA. Indeed, evidence suggests that important immunological changes occur *prior* to the development of clinical symptoms of RA. In a recent study it was reported that the levels of several cytokines, cytokine receptors and chemokines were significantly increased in the blood of asymptomatic

individuals who went on develop RA (Kokkonen *et al.* 2010). Genes that were significantly upregulated in these individuals compared to healthy controls included IL-1β, IL-2, IL-6, IL-1Ra, TNF-α, IFN-γ, IL-12, IL-4, IL-10, IL-7, GM-CSF, G-CSF, CCL2 and CCL3. When cytokine expression was assessed in the same individuals after disease onset, expression of the majority of the analysed variables was increased further, but interestingly, levels of TNF-α, IL-5, IL-7, IL-13, GM-CSF, G-CSF, FGF-2, CCL3, MIF and IL-17 were not significantly changed. These results support the concept of the immune system being activated before development of RA becomes clinically apparent, and may explain why we do not detect significant changes in cytokine expression in early and established disease.

It is recognised that ACPA-positive and ACPA-negative RA patients differ in respect to genetic association, degree of radiological destruction and outcome, and we compared cytokine expression in ACPA-positive and ACPA-negative groups to determine whether expression patterns would reflect distinct pathogenic mechanisms in these patients. ACPA-positive patients with early RA were found to express higher levels of CXCL16, a chemokine that promotes angiogenesis and is expressed by a number of different cell types including leukocytes and endothelial cells. CXCL16 has previously been implicated in mononuclear cell chemotaxis in RA (Ruth *et al.* 2006), but has not previously been specifically associated with ACPA-positive RA. The majority of cytokines were expressed at similar levels in the two patient subgroups. These similarities observed between ACPA-positive and ACPA-negative patients in the early RA group suggest that irrespective of ACPA status and potentially differing aetiology, in all patients ultimately diagnosed as having RA the mechanisms leading to chronic synovial inflammation converge on a final common pathway which is reflected in the similar cytokine expression profiles observed. In keeping with this notion, a study of RA

patients presenting within two years of symptom onset suggested no clinical phenotypic differences according to ACPA status (van der Helm-van Mil *et al.* 2005), and similarly within three months of symptom onset, clinical presentation is similar in patients with or without ACPA (Cader *et al.* 2010). It is interesting that although ACPA-positive patients show greater radiological destruction than ACPA-negative patients on follow-up (van der Helm-van Mil *et al.* 2005), we did not detect differences in cytokines in ACPA-positive and ACPA-negative patients within the established RA group.

Histological assessment of synovial biopsies from early RA patients included in this study showed that the type of infiltrate, lining layer thickness and vascularity look remarkably similar between the ACPA-positive and ACPA-negative patients (data not shown) which is consistent with the similar cytokine profiles observed. A study examining differences in synovial tissue infiltrates between ACPA-positive and ACPA-negative patients found ACPA-positive patients had a thinner synovial layer and greater infiltration of T cells (van Oosterhout *et al.* 2008). Interestingly, most parameters were the same in both groups, including infiltration of macrophages, B cells, neutrophils, plasma cells and germinal centre-like structures. These findings may be considered to be broadly consistent with our results which indicate that ACPA-positive and ACPA-negative RA patients have similar synovial cytokine expression profiles.

In the study comparing cytokine expression in sorted synovial fluid cell populations, a minimum of 10,000 cells were used per each sample. However, in the study comparing synovial tissue cytokine mRNA expression profiles, a cut-off for a minimal amount of material was not applied. It is therefore possible that in samples containing a relatively low

amount of RNA, genes with a low level of expression may have not been detected. This should be taken into consideration when comparing the expression of weakly-expressed genes in samples with variable amounts of RNA.

In the synovial tissue cytokine mRNA expression study, the dataset was analysed by performing Kruskal-Wallis tests to determine significant differences between groups for each gene, and PCA was used to cluster samples based on their cytokine gene expression profile. However, these methods do not take into account the relative significance of individual variables or groups of variables which may be important in differentiating between different patient groups. Therefore it will be important to apply alternative methods to analyse these data, for example machine-based learning approaches such as relevance learning vector quantitization, or classification tree–based methods, such as Random Forests analysis, which can identify which features are most useful in classifying different groups. The use of such approaches may be informative in defining which cytokines, or combinations of cytokines, may distinguish different patient groups. In addition, it would be important to repeat the experiment in an independent cohort and use the data as a test set to verify the findings made in this study which would serve as a training set; this would verify the significance of the findings made in the present study.

In summary, we have identified cytokine genes which are differentially expressed in the synovial tissue and synovial fluid of patients with early self-limiting synovitis, early RA and established RA. However the majority of cytokines investigated showed similar levels of expression in the different patient groups. The differences in mRNA expression reported

between early RA and self-limiting synovitis, and between early and established RA will be interesting to follow up in the future.

5 RANKL EXPRESSION BY B CELLS IN RA

5.1 Introduction

One of the hallmarks of RA is bone destruction mediated by the action of osteoclasts, a population of large, multinucleated cells derived from myeloid lineage precursor cells. The process of osteoclast differentiation is dependent on the TNF family member RANKL in addition to the growth factor, M-CSF, and as recently described, IL-34 (Chen *et al.* 2011). Binding of RANKL to its cell-bound receptor molecule RANK, triggers differentiation and activation of osteoclasts (Kong *et al.* 1999b; Lacey *et al.* 1998). The importance of RANKL-driven osteoclast-mediated bone destruction in RA is demonstrated by the effectiveness of denosumab, an anti-RANKL antibody, to suppress erosive progress in RA (Cohen *et al.* 2008). In a study of cytokine mRNA expression in RA synovial fluid cells we found that RANKL is expressed predominantly by the B cell population. Previous studies have reported expression of RANKL by T cells, fibroblasts and chondrocytes in RA, but this is the first report of RANKL production by RA B cells. The importance of the B cell lineage in RA pathogenesis is highlighted by the effectiveness of rituximab in suppressing the progression of joint damage in RA. Our observation of high mRNA levels of RANKL in B cells in RA raises the possibility that expression of this cytokine by B cells could contribute directly or indirectly to promoting bone erosion in RA. However, from our RANKL mRNA expression data from sorted synovial fluid cell populations, it is not clear whether a small proportion of B cells express high levels of RANKL, or whether RANKL is expressed equally among the whole B cell population. Due to its potential clinical relevance, this observation warranted further investigation.

RANKL mRNA expression has been described in B cells as part of an expression profile defining a subset of memory B cells that normally reside in epithelial tissue-based niches (Ehrhardt *et al.* 2008). The relevance of this RANKL-expressing B cell subset has yet to be explored in the context of RA. Classification of B cell developmental stages is commonly based on the expression of three surface markers. In humans, B cells are generated in the bone marrow, where they mature from stem cells to naïve B cells. Naïve B cells leave the bone marrow and migrate via the circulation to secondary lymphoid tissues where further development takes place. In secondary lymphoid tissues, antigen-specific memory B cells or plasma cells are formed. Plasma cells, or "effector B cells", are responsible for the production of antibody. Memory B cells are precursors capable of generating and replenishing the plasma cell compartment, and may also be responsible for the generation of distinct subsets of effector B cells. In addition, memory B cells also have important roles in antigen presentation, T cell and dendritic cell regulation, and cytokine production.

B cells can be classified based on relative expression of IgD and CD38; this so-called Bm1-Bm5 classification system can be used to identify the following subsets in the human tonsil: virgin naïve cells (Bm1: IgD+CD38-), activated naïve cells (Bm2: IgD+CD38+), pre-GC cells (Bm2'; IgD+CD38++), GC cells (Bm3-centroblasts and Bm4-centrocytes: both IgD−CD38++), and memory cells (Bm5: IgD−CD38+/−). The Bm1-Bm5 classification system recognizes similar subsets in the peripheral blood. However, this classification, although a useful point of reference, has significant limitations, for example Bm1 and Bm2 cells also contain unswitched memory cells and Bm2' also contains transitional cells. B cells are also commonly classified based on their expression of the surface markers IgD and CD27 as naïve (IgD+CD27-), pre-switch memory (IgD+CD27+) or post-switch memory (IgD-CD27+), and "double-negative" (IgD-CD27-) B cells. CD27 is a marker of activation which

was initially regarded as a universal marker of human memory cells. However, it now recognised that there are some memory B cells which do not express CD27. This CD27- population carries somatically mutated immunoglobulin genes, has a CD24/CD37 expression profile characteristic of memory cells, and can produce antibodies specific for previously encountered antigens (Fecteau *et al.* 2006; Wirths and Lanzavecchia 2005). It is thought that CD27- memory B cells are likely to derive from extra-follicular responses or incomplete germinal centre reactions. Alternatively, they may be derived from activated CD27+ B memory cells that have lost their CD27 expression (Sanz *et al.* 2008).

In the RA synovium there are increased numbers of CD27+ memory B cells compared to peripheral blood (Souto-Carneiro *et al.* 2009). In RA synovial fluid there are increased frequencies of IgD-CD27+ and IgD-CD27- B cells, and a reduced frequency of IgD+CD27- B cells compared to peripheral blood (Michelutti *et al.* 2011). Abnormalities in B cell subsets have also been reported in the peripheral blood of RA patients compared to healthy individuals. Studies have reported an increased frequency of IgD-CD27+ and a reduced frequency of IgD+CD27+ peripheral blood B cells in RA compared to healthy donors (Fekete *et al.* 2007; Souto-Carneiro *et al.* 2009).

The use of B cell depletion therapy in patients with RA has provided a useful tool for elucidating the role of B cells in RA pathogenesis. Rituximab is a chimaeric monoclonal antibody directed at CD20, a transmembrane protein expressed exclusively on B cells, including pre-B cells, naïve, mature, and activated B cells, B cell blasts, but not on early B cell progenitors or plasma cells. Rituximab is used for TNF blockade-refractory disease and is effective in improving disease symptoms and reducing radiographic progression in RA

(Cohen *et al.* 2006; Keystone *et al.* 2009). Treatment with rituximab significantly reduces levels of anti-CCP, IgA-RF, IgG-RF, and IgG antibodies compared to their corresponding total serum immunoglobulin classes (Cambridge *et al.* 2003). A reduction in CRP levels also parallels the decreases in autoantibody levels, whereas levels of antimicrobial antibodies do not change significantly (Cambridge *et al.* 2003). The mechanism by which rituximab produces its clinical effect is not well understood at present. Interference with B cell functions such as antigen presentation and cytokine production may be involved.

Rituximab is usually effective in inducing a near complete depletion of CD20+ B cells from peripheral blood, whereas B cell depletion in the synovial tissue is highly variable between patients (Boumans *et al.* 2011). Rituximab induces a transient depletion of B cells in the peripheral blood within a few hours (Leandro *et al.* 2006). The mean time frame of B cell return is 8 months, but this is highly variable between patients (Leandro *et al.* 2006). Repopulation occurs firstly by immature B cells, followed by naïve B cells. There is delayed recovery of memory B cells, and these cells may remain at reduced levels for over two years (Moller *et al.* 2009; Roll *et al.* 2008; Roll *et al.* 2006). Interestingly, the extent of memory B cell repletion is associated with clinical response: early clinical relapse is associated with a higher proportion of CD27+ memory B cells before therapy, and is also associated with repopulation with a higher proportion of IgD+CD27+ or IgD-CD27+ memory B cells (Leandro *et al.* 2006; Moller *et al.* 2009; Roll *et al.* 2008). As the expression of RANKL by B cells in RA is a novel observation which remains to be further investigated, we sought to determine protein expression of RANKL in B cells in synovial fluid and tissue and to investigate the differentiation and activation status of B cell subsets responsible for producing RANKL.

5.2 Identification and characterisation of RANKL-producing B cells in synovial fluid

In sorted cell populations from RA synovial fluid, high expression of RANKL mRNA was detected in the B cell population (see Figure 3.3). In this initial study, mRNA gene expression data were normalised to levels of 18S rRNA. In following up this observation, to exclude potential bias which may have arisen from normalisation to 18S as a result of varying levels of rRNA in different cell types, the mRNA data for RANKL was normalised to the number of cells in each sorted cell sample, as shown in Figure 5.1. Confirming our original observation, PCR data normalised to cell numbers identified synovial fluid B cells as the dominant producers of RANKL mRNA, while a smaller proportion of CD4 T cells also showed RANKL mRNA expression.

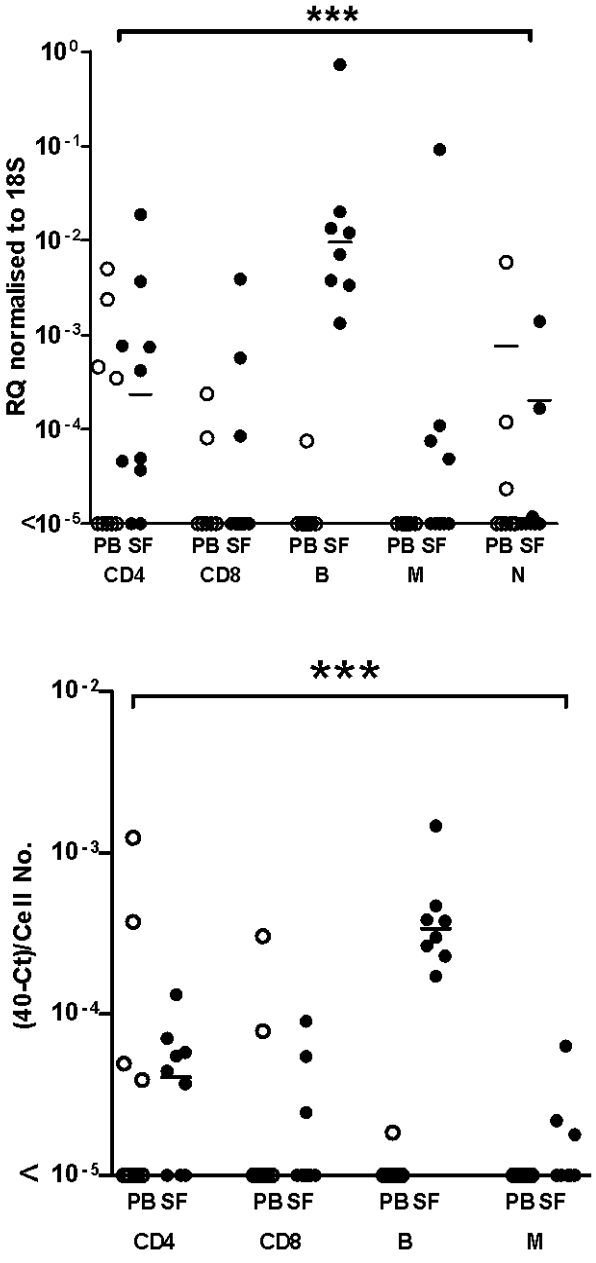


Figure 5.1 mRNA expression of RANKL in SF and PB cell populations. (A) RANKL expression normalised to 18S in CD4 and CD8 T cells, B cells, macrophages and neutrophils from PB (open circles) and SF (closed circles). (B) RANKL expression normalised to cell number in each cell population sorted using FACS. Kruskal-Wallis test; *** $p<0.005$. CD4: CD3+CD4+CD45RO+ T cells; CD8: CD3+CD8+CD45RO+ T cells; B: CD19+ B cells; M: CD14+ macrophages; N: CD15+ neutrophils.

The observation of RANKL expression in B cells was subsequently extended to the protein level in synovial fluid and peripheral blood of RA patients. Matched synovial fluid and peripheral blood samples were taken from eleven patients with RA. The clinical details are shown in Appendix Table 8.6. Surface expression of RANKL on CD19+ B cells in synovial fluid and peripheral blood was determined by flow cytometry. In synovial fluid the B cell population represented a smaller proportion of mononuclear cells compared with the peripheral blood (median 3.41% synovial fluid vs 9.05% peripheral blood) (Figure 5.2). The surface markers IgD and CD27 were used to divide CD19+ synovial fluid and peripheral blood B cells into naïve (IgD+CD27-), pre-switch memory (IgD+CD27+), and post-switch memory (IgD-CD27+ and IgD-CD27-) B cell subpopulations. As shown in Figure 5.2, the majority of B cells in synovial fluid were IgD-CD27+ (median 33%) or IgD-CD27- (median 28%). The relative proportions of IgD-CD27- and IgD-CD27+ B cell subsets in the synovial fluid were significantly higher than in peripheral blood ($p=0.03$ and $p=0.04$ respectively). The predominant B cell subset in peripheral blood was IgD+CD27- (median 54%) and the relative size of this population was significantly larger in peripheral blood than in synovial fluid ($p=0.03$).

RANKL expression was detected in a significantly higher proportion of B cells in synovial fluid compared to peripheral blood (median 14.0 vs 1.89, $p=0.009$) (Figure 5.3). Synovial fluid B cells expressing RANKL were further characterised by their expression of IgD and CD27 in seven samples which had a sufficient cell number for analysis. As the frequency of RANKL-expressing B cells in peripheral blood was very low, their phenotype was not characterised due to low cell numbers. In the synovial fluid, RANKL-producing B cells were mainly IgD-CD27- (median 64%) and a smaller proportion was IgD-CD27+ (median 19%) (Figure 5.3). The relative frequency of the IgD-CD27- population was significantly higher in

the RANKL-expressing B cell population compared to the RANKL-negative fraction of B cells, whereas the relative IgD-CD27+ population was significantly higher in the RANKL-negative B cell population compared to the RANKL-positive fraction of B cells (Figure 5.3). However, there was considerable variation between the patients studied. The percentage of RANKL+ B cells in all of the subsets defined by expression of IgD and CD27 was higher in the synovial fluid compared to the corresponding population in the peripheral blood (Figure 5.3C).

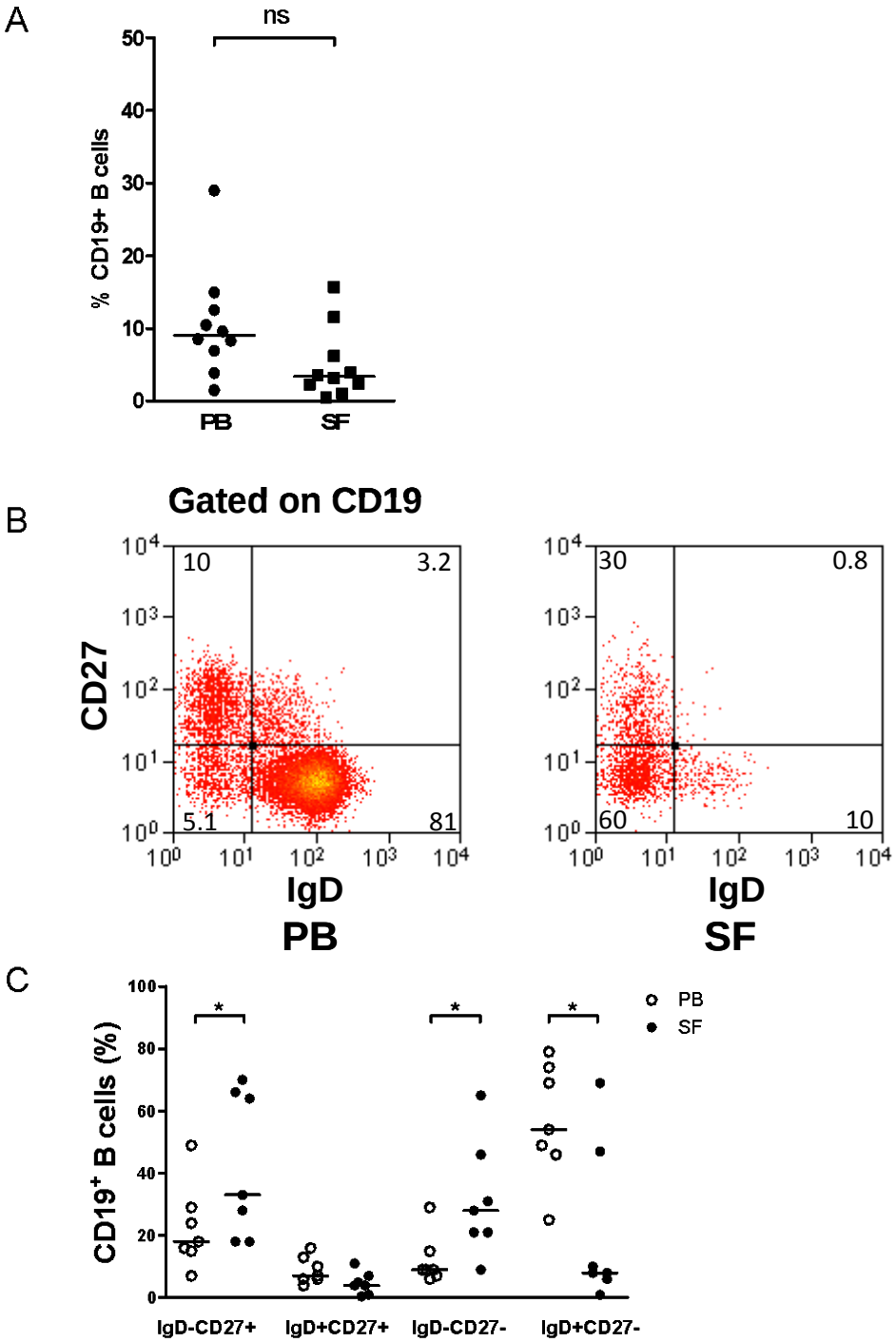


Figure 5.2 Distribution of B cell populations in RA peripheral blood and synovial fluid. (A) Percentage of CD19+ B cells in mononuclear cell fractions of peripheral blood and synovial fluid; n=11. Wilcoxon matched pairs test; ns = non-significant. (B) Representative image showing staining of B cell subsets based on expression of IgD and CD27 by CD19+ cells in peripheral blood and synovial fluid. (C) Frequency of B cell subsets gated on CD19 present in peripheral blood (open circles) and synovial fluid (closed circles); n=7. Wilcoxon matched pairs test; * $p<0.05$.

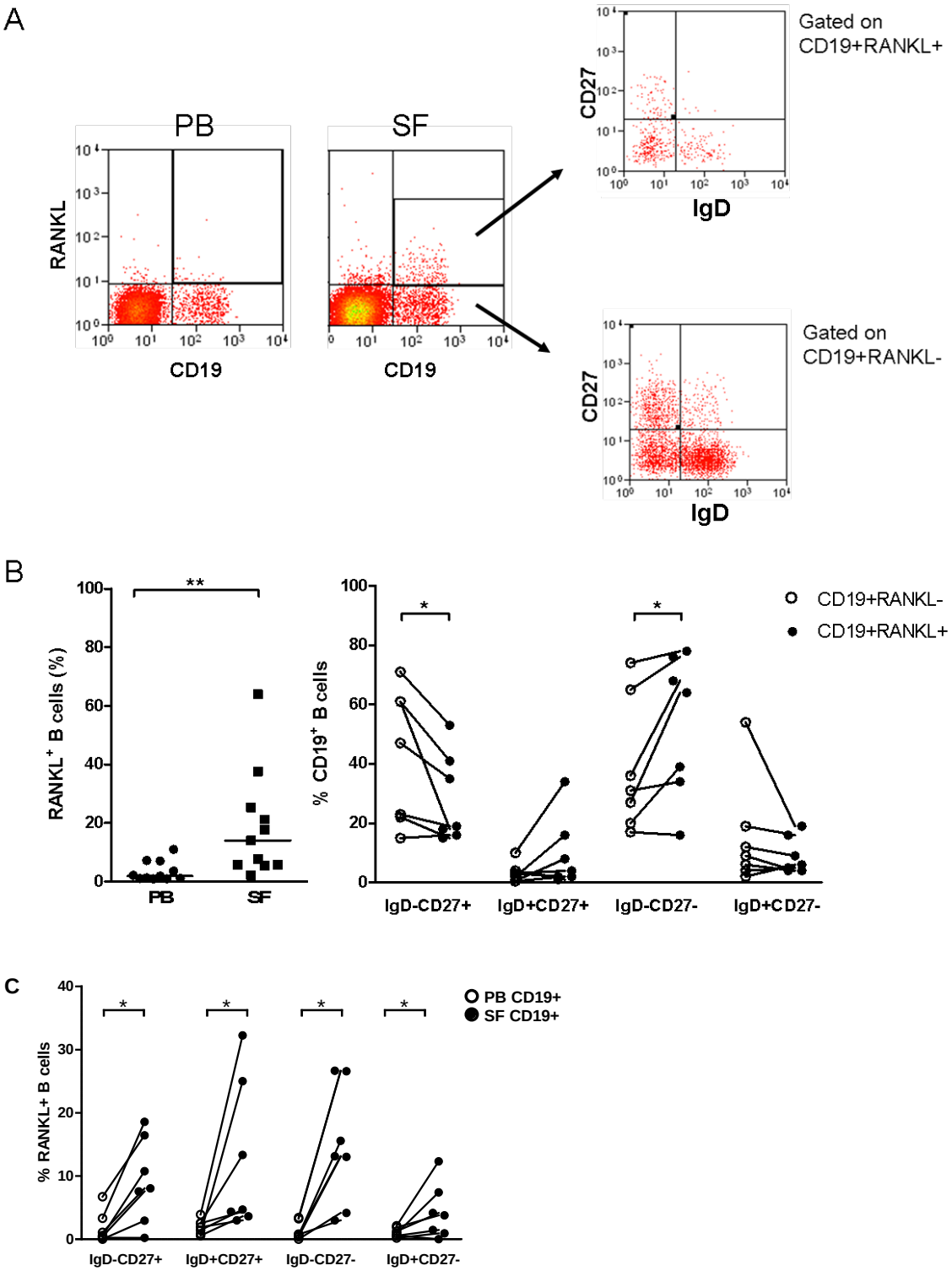


Figure 5.3 RANKL production by B cells in peripheral blood and synovial fluid. (A) Representative flow cytometry plot showing RANKL-expressing CD19+ B cells in peripheral blood and synovial fluid. IgD and CD27 expression of RANKL+ and RANKL- B cells in synovial fluid. (B) Percentage of RANKL+ B cells in mononuclear cell fraction of peripheral blood and synovial fluid (n=11) (left). B cell subsets based on IgD and CD27 expression within RANKL- (open circles) and RANKL+ (closed cells) B cell populations in synovial fluid. (C) Percentage of RANKL+ B cells in B cell subsets defined by IgD/CD27 expression in peripheral blood (open circles) and synovial fluid (closed circles). Wilcoxon matched pairs test; *p<0.05, **p<0.001.

5.3 RANKL production by B cells in synovial tissue and the effect of B cell depletion therapy

As the synovium is the primary site of inflammation in RA, we investigated protein expression of RANKL by B cells in the synovial tissue. Biopsies from four RA patients were stained for RANKL and CD19 and assessed using immunofluorescence microscopy. As shown in Figure 5.4, RANKL expression was found in CD19+ B cells in the synovium.

In light of the observation that B cell depletion is effective in reducing synovial inflammation and progression of joint destruction in RA, we wished to investigate whether there was an association between the presence of B cells in the synovium and expression of RANKL and if depletion of synovial B cells correlated with a reduction in RANKL. Synovial tissue biopsies from RA patients taken at baseline and 4 and 16 weeks after rituximab treatment were provided by Professor Paul Peter Tak, Academic Medical Centre/University of Amsterdam. Clinical details of the patients studied are shown in Appendix Table 8.7. Synovial tissue was stained for RANKL and the B cell marker CD22. Sections were analysed by immunofluorescence microscopy and the expression of both markers was quantified using digital image analysis. 13 patients from whom biopsies were available from all three time points were included in the study. Following staining, two patients were excluded from analysis due to poor quality of the tissue.

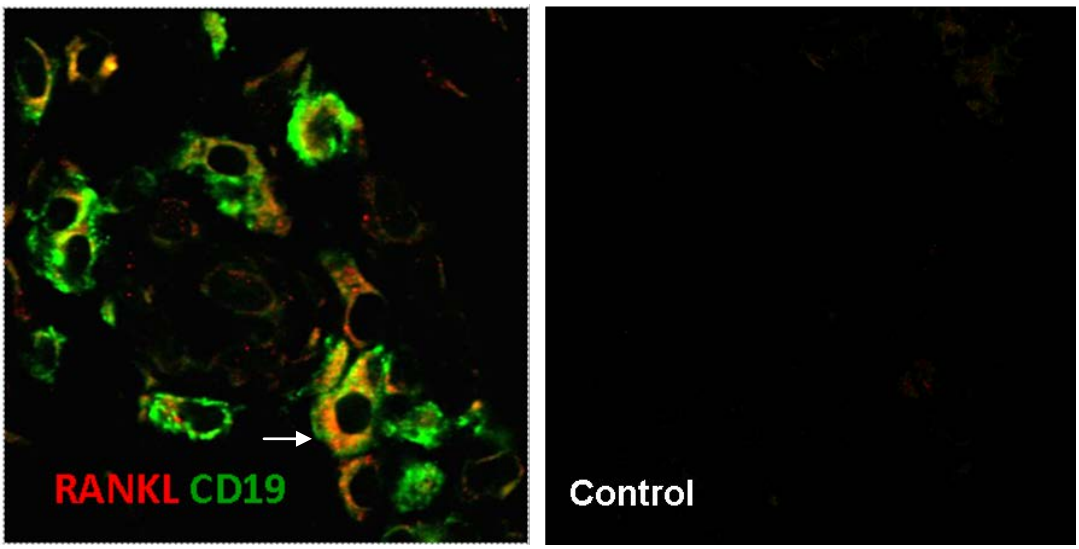


Figure 5.4 RANKL expression by synovial tissue B cells. Synovial tissue sections from RA patients were stained for CD19 and RANKL (left) or matched negative control antibodies (right). RANKL is shown in red; CD19 is shown in green. Expression of the B cell antigen CD19 was detected on the cell surface. RANKL expression was localised predominantly beneath the cell membrane, indicating intracellular expression of RANKL by B cells (indicated by arrow). Co-expression of CD19 and RANKL, indicated by yellow staining, shows membrane expression of RANKL by B cells. Immunofluorescence staining was visualised using a confocal microscope and viewed at a final magnification of X400. Images are representative of five samples.

RANKL and CD22 expression was quantified in the whole synovial biopsy section at the three time points for each patient. This approach was taken in order to exclude bias which may arise if specific areas of synovial tissue to be quantified are selected by the assessor. The synovial tissue sections assessed were comprised of at least six biopsies taken from different areas of the joint to reduce sampling variation. Representative images of stained sections are shown in Figure 5.5. There was a significant reduction in RANKL at 4 weeks and 16 weeks after rituximab compared to baseline ($p=0.01$ and $p=0.03$ respectively) (Figure 5.6A). There was no statistically significant decrease in CD22 at 4 or 16 weeks compared to baseline (Figure 5.6A). Since the clinical response to rituximab treatment is variable, we determined if changes in RANKL and CD22 were related to clinical improvement. Patients were grouped as responders (n=6) and non-responders (n=5). Patients were classed as responders if they showed a decrease in DAS28 of $\geq$1.2 at 24 weeks (Smolen *et al.* 2007), and had a good or moderate response according to the EULAR response criteria (van Gestel *et al.* 1998). One patient did not have a decrease in DAS28 of at least 1.2 at 24 weeks but had a moderate response according to the EULAR response criteria, and in this study this patient was classified as a non-responder. As reported previously in this patient cohort, clinical response was not predicted by the baseline number of B cells, and the decrease in synovial B cells between baseline and 4 weeks or between 4 and 16 weeks was not significantly different between the responders and non-responders (Thurlings *et al.* 2008). We did not observe a significant reduction in CD22 at 16 weeks compared to baseline in either group (Figure 5.6B and C). However, in the group of responders but not non-responders, total RANKL was significantly reduced at 16 weeks post-treatment compared to baseline (Figure 5.6B and C).

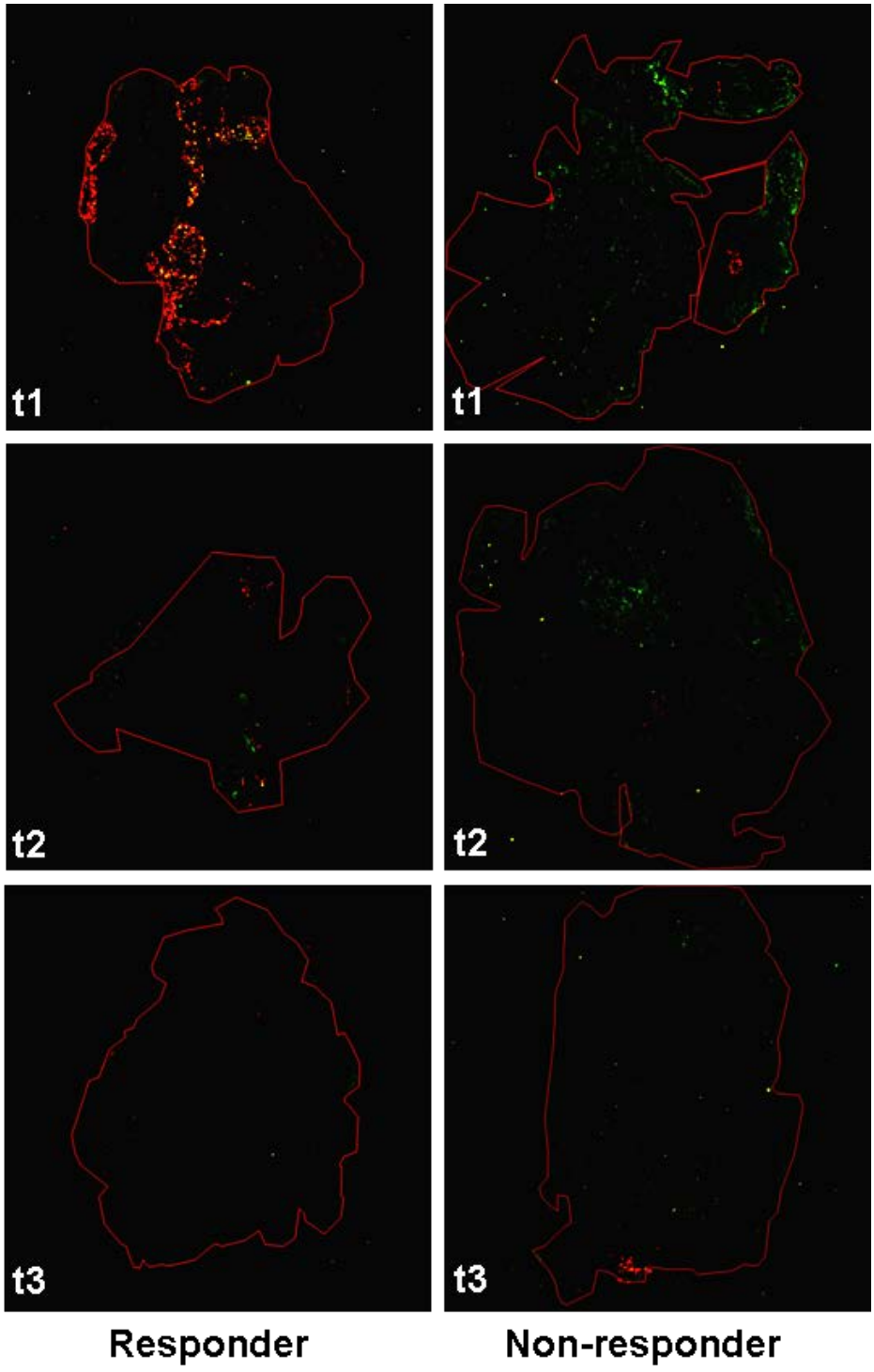


Figure 5.5 RANKL and CD22 synovial tissue expression before and after rituximab. Representative images of synovial tissue obtained at baseline (t1), 4 weeks post-infusion (t2) and 16 weeks post-infusion (t3) stained for RANKL (red) and CD22 (green) from patients classified by DAS28 improvement as a responder (left) or non-responder (right).

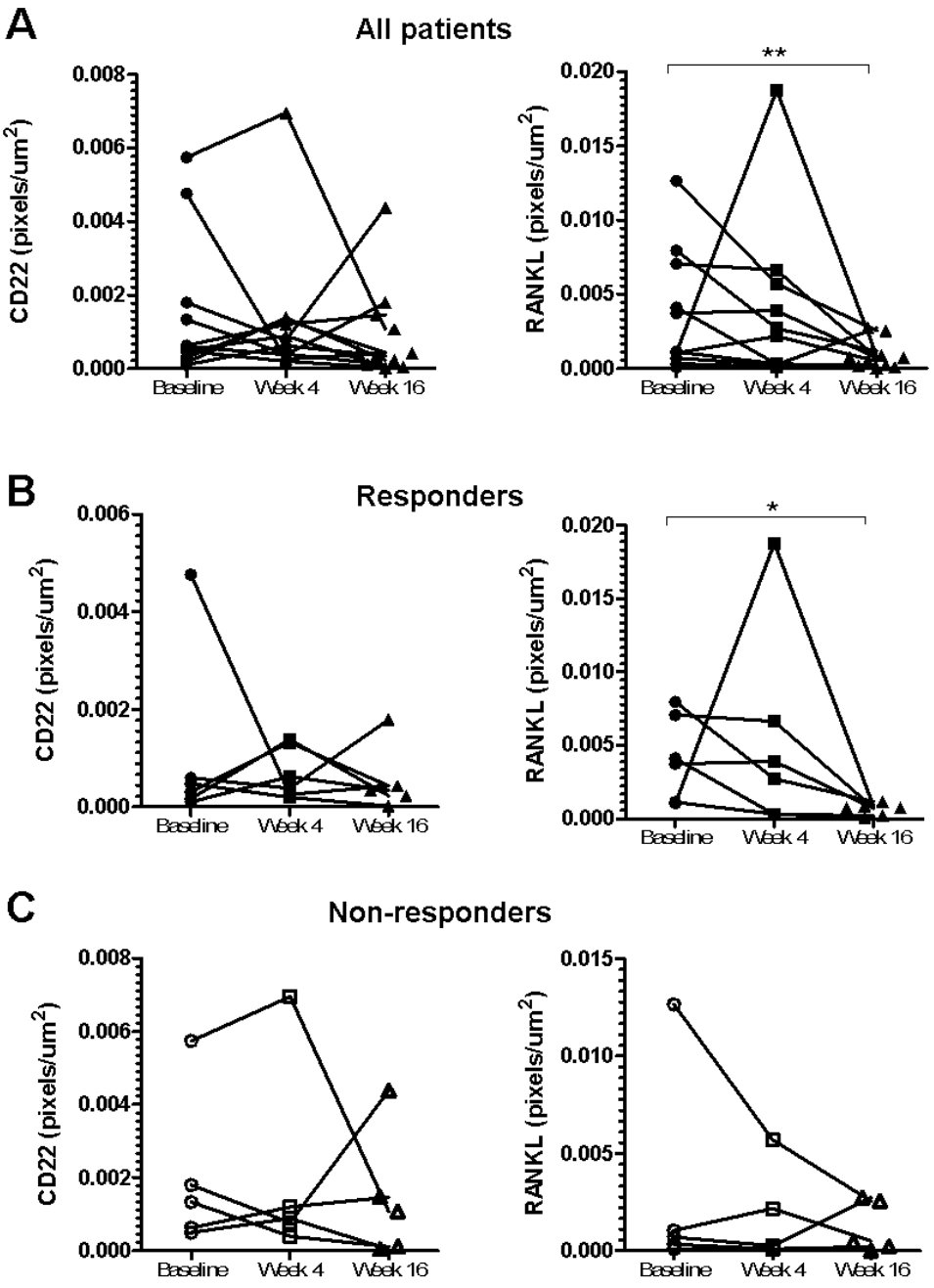


Figure 5.6 Changes in the B cell marker CD22 and RANKL expression in the synovium after rituximab treatment. Synovial tissue sections taken at baseline and 4 and 16 weeks after rituximab were stained for CD22 and RANKL by immunofluorescence. Expression of each marker in the whole section was quantified by digital image analysis and expressed as pixels per unit area. (A) Synovial CD22 and RANKL expression at baseline (t1), 4 weeks post-treatment (t2) and 16 weeks post-treatment (t3) in all patients. Patients were classified as responders or non-responders based on their DAS28 score at week 24. (B) Synovial CD22 and RANKL expression at each time point in responding patients. (C) Synovial CD22 and RANKL expression at each time point in non-responding patients. Friedman repeated-measures non-parametric tests with Dunn's post-tests; *$p<0.05$, **$p<0.001$.

5.4 Discussion

The aim of these experiments was to investigate RANKL protein expression by B cells in RA. We have shown that RANKL is expressed by B cells in synovial fluid and synovial tissue. Furthermore we observed that synovial RANKL levels decrease after B cell depletion therapy. The identification and characterisation of RANKL-producing B cells in RA is important as RANKL expression by B cells has not been previously reported in RA and implicates new potential roles for this cell type in RA pathogenesis.

We observed increased frequencies of IgD-CD27- and IgD-CD27+ B cell subsets in synovial fluid compared to peripheral blood in agreement with findings from a recent study (Michelutti *et al.* 2011). RANKL production by B cells was ascribed predominantly to the IgD-CD27- subset, and to a lesser extent, the IgD-CD27+ subset. It is of interest that both of these populations are expanded in the synovial fluid. In addition, it has been reported that CD27+ memory B cells are increased in the RA synovium (Souto-Carneiro *et al.* 2009). The IgD-CD27+ B cell subset represents a population of switched B cells which have undergone somatic hypermutation, and the IgD-CD27- population is also thought to represent a memory B cell subset. In addition to being expanded in the synovial fluid in RA, the population of IgD-CD27- B cells is significantly expanded in the peripheral blood of patients with SLE (Anolik *et al.* 2004; Rodriguez-Bayona *et al.* 2010; Wei *et al.* 2007), and the appearance of this subset correlated with disease activity. The IgD-CD27- memory population is also expanded in healthy subjects exposed to respiratory syncytial virus (RSV) (Sanz *et al.* 2008). The functional relevance of the expansion of this population in RA and SLE has yet to be investigated. Our observation that RANKL is mainly produced by memory B cells is of interest in light of the observation that a higher fraction of memory B cells and lower fraction

of immature transitional B cells during reconstitution after B cell depletion therapy correlates with earlier relapse of disease (Leandro *et al.* 2006; Palanichamy *et al.* 2008).

We have identified RANKL-expressing B cells in the synovium, the primary site of inflammation in RA. In light of this observation, the ability of RANKL to mediate bone erosion implicates a role for B cells in joint destruction. Although not previously described in RA, RANKL expression by B cells associated with bone destruction has been reported in other diseases. In periodontal disease, B cells have been found to constitute the major source of RANKL in the gingival tissue, and this expression by B cells is associated with osteoclast-mediated bone resorption (Kawai *et al.* 2006). RANKL production has been described in plasma cells in osteolytic myeloma bone disease (Heider *et al.* 2003), indicating a role for terminally differentiated B cells in bone degradation via RANKL. In addition, B cell lymphomas can also produce RANKL where B cell expression of RANKL is associated with bone loss and hypercalcemia (Shibata *et al.* 2005). It remains to be determined whether B cells have a similar role in mediating bone destruction in RA.

In addition to its role in mediating bone resorption, studies using RANKL-deficient mice have suggested that RANKL also has a critical role in the differentiation of T and B cells and is required for development of lymph nodes (Kong *et al.* 1999a). The role of RANKL in lymphocyte development and its production by B cells may be relevant to the development of ectopic lymphoid structures in RA. Germinal centre-like structures can be found in the inflamed RA synovium where they can participate in the local immune response and maintain autoreactive lymphocytes. It has been shown that B cells in synovial lymphoid aggregates undergo affinity maturation and somatic hypermutation (Gause *et al.* 1995; Randen *et al.* 1992). B cells are recognised as having an important role in the development of these ectopic

lymphoid structures due to their surface expression of LT-α. In a study using synovial biopsies it was demonstrated that activation of CD4 T cells is dependent on the presence of B cell follicles (Takemura *et al.* 2001b). It is possible that synovial B cells could also contribute to lymphocyte development in the RA synovium via production of RANKL. Lymphoid neogenesis has also been described in subchondral bone marrow adjacent to the joint in RA (Jimenez-Boj *et al.* 2007; Jimenez-Boj *et al.* 2005). Lymphoid aggregates were predominantly composed of CD27+ B cells, and B cells expressed bone morphogenic protein BMP-6 and BMP-7 which stimulate formation of new bone. An accumulation of osteoblasts and osteoid deposition in the endosteal bone next to the subcortical bone marrow aggregates implied a protective role for B cells. Animal models of arthritis have also postulated a protective role for B cell bone marrow infiltrates (Gortz *et al.* 2004; Hayer *et al.* 2008), although a study by Bugatti and colleagues described bone marrow lymphoid neogenesis associated with bone erosion (Bugatti *et al.* 2005). It remains unclear whether B cells present in bone marrow aggregates contribute to or protect against bone erosion, but may depend on the developmental stage and activation state of the B cell subset. For example, RANKL expression has been described in memory B cell subsets, whereas B cell precursors can produce OPG (Ehrhardt *et al.* 2008; Li *et al.* 2007). The potential for RANKL-producing B cells in bone marrow aggregates to promote bone erosion remains an interesting possibility.

We observed that levels of RANKL in the synovium decrease after B cell depletion therapy. In addition, baseline expression of RANKL was found to correlate with bone erosion and joint space narrowing as measured by the Sharpe/van der Heijde score (SHS) (Boumans *et al.*, 2011). This observation supports the notion that RANKL production by synovial B cells could have a direct role in promoting joint destruction. However, since we have not looked at the impact of rituximab on RANKL in B cells specifically, it remains possible that the

decrease in RANKL observed may be due to effects of rituximab on other cell types. We did not observe a significant reduction in synovial B cells, which is in keeping with previous reports that B cell depletion in the synovium is highly variable between patients (Thurlings *et al.* 2008). Immunohistochemical analysis of biopsies from the same cohort as examined in the present study showed that rituximab treatment resulted in a decrease in synovial T cells, lymphocyte aggregates, germinal centres and macrophages, and plasma cell depletion in responding patients (Thurlings *et al.* 2008). This indicates that B cells have an important role in maintaining inflammatory cells in the synovium. B cells could potentially contribute via production of RANKL which prolongs the survival of dendritic cells. Dendritic cells are present in the RA synovium and could have a role in perpetuating synovial inflammation by presenting autoantigens to T cells. Immature dendritic cells express both RANK and RANKL and are thus able to provide an autocrine survival signal; however, peripheral maturation leads to down-regulation of RANKL, suggesting that an independent source of RANKL may be required for dendritic activation (Cremer *et al.* 2002).

RANKL is produced in membrane-bound and soluble forms, both of which are capable of stimulating osteoclast differentiation *in vitro*. The soluble form can be generated by proteolytic cleavage of the membrane-bound protein, or by alternative splicing. It is thought that transcription of a RANKL gene that does not encode a trans-membrane domain may be preferentially produced in malignant cell types. It would be interesting to determine intracellular expression of RANKL in B cell subsets, and soluble expression of RANKL by B cells in addition to the membrane expression that we have previously detected. Future experiments include detection of intracellular RANKL expression in synovial fluid B cells by flow cytometry, following fixing and permeabilisation of the cell membrane, or using immunofluorescence microscopy to detect intracellular vs surface expression of RANKL by

sorted synovial fluid B cells. It would also be important to determine whether RANKL is released from the cell surface, which could be addressed by stimulating synovial fluid B cells then using ELISA to detect soluble RANKL in the supernatant.

In summary, the identification of RANKL-producing B cells in the RA synovium and characterisation of the memory B cell subsets expressing RANKL is of importance due to the functional relevance of this cytokine in the pathogenesis of RA. These experiments thus provide an important basis for determining the functional role of RANKL expression by B cells in RA.

6 GENERAL DISCUSSION

6.1 Cytokine production in RA

Cytokines play a critical role in influencing the immune response, being involved in almost every aspect of immunity and inflammation, from the induction of an innate immune response to the generation of cytotoxic T cells and the production of antibodies by the adaptive immune system. Many cytokines are found in the chronically inflamed synovium of patients with rheumatoid arthritis, and over the years there have been many studies undertaken to address the roles of cytokines in the disease processes. In the literature there is much evidence to suggest that cytokines play a central role in RA pathogenesis, and this concept is supported by the demonstration that cytokines are effective therapeutic targets in current clinical practice. Despite the large number of studies investigating the expression and functions of many cytokines in RA, there has been no attempt made to systematically determine the expression of multiple cytokines in the predominant cell populations present in the inflamed RA synovium. In this thesis I have presented data showing cytokine expression profiles determined directly *ex vivo* in the major cell populations isolated from RA synovial fluid. This work provides important confirmation of cytokine expression data from studies which were performed using *ex vivo* stimulation or *in vitro* culture of RA synovial cells, and has also resulted in several novel observations which will be discussed further in this chapter.

Chronic inflammation of the synovium in RA is characterised by interactions between cells of the adaptive immune system, the innate immune system, and resident stromal cells. Characterising cytokine production by these cell types can provide insight into disease processes. Indeed, efforts have been made to determine cytokine production in the RA

synovium and synovial fluid since the necessary molecular techniques became available in the late 1980s. At the time of these studies, the prevailing hypothesis implicated Th1 cells as the cell type responsible for driving RA, and the detection of relatively low concentrations of IFN-γ was an unexpected observation. In recent years, Th17 cells have been proposed as central mediators of RA pathology, although their role in RA is still a matter of debate. IL-17 synergises with IL-1β by inducing RANKL and its receptor, RANK, which induces osteoclast differentiation (Lubberts *et al.* 2003). IL-17 also synergises with TNF-α to induce pro-inflammatory cytokines and chemokines from monocytes and synovial fibroblasts (Chabaud *et al.* 1998; Jovanovic *et al.* 1998; Katz *et al.* 2001). In this work, both IFN-γ and IL-17 were found to be expressed by established RA synovial fluid CD4 T cells. It remains to be determined whether these CD4 T cells represent Th1 and Th17 cell subsets as have been previously reported in RA (Chabaud *et al.* 1999; Kotake *et al.* 1999; Raza *et al.* 2005; Yamada *et al.* 2011; Ziolkowska *et al.* 2000), or whether these cells express both IFN-γ and IL-17 as has been described in a Th17/1 subset of T cells (Annunziato *et al.* 2007). The concept that T cells have a remarkable degree of plasticity has revolutionised our understanding of cell lineages, which were originally ascribed a unique transcription factor and a terminally differentiated effector phenotype. Cells with an intermediate phenotype sharing master transcription factors from apparently distinct cell types such as Th17/1 cells are detectable in humans, and regulatory T cells can convert to Th17 cells (Koenen *et al.* 2008; Lee *et al.* 2009; Manel *et al.* 2008). Therefore it would be of interest to further characterise the T cell subsets we have identified as producing IL-17 and IFN-γ in RA synovial fluid, although many of the recently-described subsets are defined by their effector function, i.e. cytokine production or suppression, and very few surface markers are defined that would allow us to safely distinguish these subsets without *ex vivo* stimulation.

B cells are regarded as an important cell type in RA due to their ability to produce antibody and present antigen, but little is known about direct cytokine production by B cells in RA. In this study, RA synovial fluid B cells were found to express high levels of mRNA for pro-inflammatory cytokines including IL-12 and IL-23, indicating a potential role in promoting the differentiation of Th1 and Th17 cell subsets in the RA synovium, which as discussed may have important roles in promoting inflammation and joint destruction. We also observed high levels of RANKL expression in RA B synovial fluid cells (Yeo *et al.* 2011), suggesting a role in promoting osteoclast-mediated bone erosion, and this finding is discussed in further detail in 7.2.

In the first studies of cytokine expression in RA, a striking observation was that macrophage and fibroblast products were abundant in the RA synovium. In this study RA synovial fluid macrophages were found to express high levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-1α, in addition to other synovial fluid populations. Importantly, this work has highlighted neutrophils as a prominent source of the type II IFNs, IFN-α1 and IFN-β1, suggesting a role in activating and maintaining inflammatory cell populations in the RA synovium. We observed that RA synovial fluid neutrophils also express high levels of mRNA for IL-1β, CCL3 and CXCL8, indicating a direct pro-inflammatory role for this cell type in RA and indicating their ability to attract other leukocytes to the synovial fluid, thus perpetuating the cycle of inflammation.

6.2 B cells as a source of RANKL in RA

Destruction of bone, manifesting as erosions on radiographs, is a major effect of RA, and joint destruction may occur very early in the disease course (Plant *et al.* 1998). RA causes local erosions and juxta-articular and general osteopenia. In this thesis I have presented evidence that B cells in the synovial fluid and synovial tissue of patients with RA produce RANKL, a critical factor for osteoclast differentiation. Expression of RANKL implies a direct role for B cells in promoting osteoclastogenesis leading to joint destruction, a concept supported by previous reports of bone destruction driven by B-cell RANKL production in periodontitis, and in transformed B cells and plasma cells of B cell lymphomas and multiple myeloma respectively. Several cell populations have previously been reported to produce RANKL in the RA synovium, including T cells, chondrocytes, and synovial fibroblasts which are present at the site at which the pannus tissue invades the bone (Crotti *et al.* 2002; Gravallese *et al.* 2000; Shigeyama *et al.* 2000; Skoumal *et al.* 2005). The functional relevance of the B cell population contribution to RANKL expression in the RA synovium has yet to be explored. A growing body of evidence suggests an important role for B cells in regulating osteoclastogenesis through their ability to produce RANKL, (Choi *et al.* 2001; Manabe *et al.* 2001), OPG (Li *et al.* 2007), and TGFβ (Weitzmann *et al.* 2000).

RANKL was found to be expressed by B cells in the synovial fluid of patients with established RA and early RA but not with resolving synovitis, indicating that RANKL expression may be specific for RA. This observation requires further analysis as we have only investigated a small number of patients with resolving disease so far. However, other studies have reported expression of RANKL by activated B cells (Choi *et al.* 2001), and mRNA expression of RANKL has been reported in memory B cells from healthy individuals (Ehrhardt *et al.* 2008). It would be of interest to investigate whether RANKL expression by B

cells plays a role in other autoimmune pathologies associated with osteopenia and osteoporosis such as SLE, type I diabetes, autoimmune thyroid disease and celiac disease, in which bone impairment may occur as a consequence of immunoregulatory imbalance. Interestingly, RANKL is upregulated in B cells and T cells isolated from the bone marrow of post-menopausal women compared with pre-menopausal women or post-menopausal women treated with estrogen (Eghbali-Fatourechi *et al.* 2003), and it has been suggested that lymphocyte expression of RANKL could play a role in the elevated bone resorption associated with loss of sex steroids. This may be pertinent to the pathogenesis of RA which is 2-3 times more prevalent in women than men and is most common in those aged 40 to 60, an age range which coincides with that during which women commonly enter menopause. Hormone replacement therapy administered to post-menopausal women with RA has been found to increase and maintain bone mineral density, indicating a protective role of estrogens against bone loss. In addition, studies have shown that B cell precursors are able to differentiate into osteoclasts *in vitro* (Sato *et al.* 2001). It has been suggested that by stimulating B cell lymphopoiesis, estrogen deficiency could enhance osteoclastogenesis by increasing the number of B cell precursors with the potential to differentiate into osteoclasts (Li *et al.* 2007). It would be interesting to explore a potential association between the influence of estrogens and RANKL expression by lymphocytes, including B cells, in patients with RA. Another aspect which would be interesting to investigate is whether RANKL is expressed by B cells in the B cell aggregates which are found at erosive sites at the interphase between synovial tissue and bone marrow in RA (Bugatti *et al.* 2005). While the authors suggest an erosive role for B cells in RA, other studies have proposed that B cells in the bone marrow in animal models of RA may have a protective effect: B cell infiltration in the bone marrow in RA has been linked to a shift in bone homeostasis toward osteoblasts and new bone formation (Gortz *et al.* 2004; Jimenez-Boj *et al.* 2005), and in an experimental model of

arthritis using TNF-transgenic mice lacking an essential molecule for B cell development, Btk, bone marrow infiltration by B cells was significantly diminished (Hayer *et al.* 2008). It would be interesting to investigate whether RANKL-expressing B cells are found in the bone marrow in RA in relation to the hypothesis that RA starts in the bone marrow and migrates to the synovium (the so-called "inside-out" hypothesis) rather than originating in the synovial membrane and promoting invasion into cartilage, bone and bone marrow space (the "outside-in" hypothesis) (Schett and Firestein 2010).

RANKL was found to be produced predominantly by IgD-CD27- and IgD-CD27+ memory B cell subsets in RA synovial fluid. Memory B cell subsets are expanded in the RA synovium compared to peripheral blood (Michelutti *et al.* 2011; Souto-Carneiro *et al.* 2009), supporting the concept of RANKL produced by B cells mediating local joint destruction. Our observation that B cells produce RANKL in RA is important in the context of understanding current therapies used in RA, rituximab and denosumab, which target B cells and RANKL respectively. Interestingly, the presence of IgD-CD27- memory B cells correlates with disease activity in SLE (Wei *et al.* 2007), highlighting a potential pathogenic role for this B cell compartment. Furthermore, levels of RANKL in the RA synovium were found to decrease following treatment with rituximab, suggesting that B cell depletion at least has an indirect effect, if not direct, in inhibiting bone erosion by osteoclasts. The memory B cell compartment is considered important in mediating chronic autoimmunity due to its properties of long lifespan, prompt and enhanced responses to activation, and ability to stimulate T cells. Thus, once tolerance is broken in the B cell compartment, memory responses would continue to persist, being easily reactivated in the presence of self-antigen, and could serve to further diversify and amplify the autoimmunity. RANKL was mostly expressed by the IgD-CD27- memory B cell subset, a population suggested to derive from extra-follicular responses or

incomplete germinal centre reactions (Sanz *et al.* 2008). This is interesting in light of the observation that the ACPA have a significantly lower avidity than antibodies to the recall antigens tetanus toxoid and diphtheria toxoid, suggesting that avidity maturation in germinal centres in the ACPA response differs from that which occurs in conventional B cell responses (Suwannalai *et al.* 2010). Evidence of lymphoid aggregation and germinal centre-like structures has been observed in the RA synovium, suggesting that the memory cells which produce RANKL may be produced locally in the synovium. Recent unpublished evidence from studies in mice suggests that RANKL is expressed by long-lived plasma cells in T cell-dependent responses and has a role in inducing and maintaining long-lived plasma cells (M. Cancro, presented at 17th Germinal Centre Conference 2011). These data may fit with our observation in that memory B cells found to be expressing RANKL in our study may represent B cells differentiating towards a long-lived plasma cell fate. A further avenue to be explored concerns the putative role of RANKL in lymphocyte differentiation and lymph node development (Kong *et al.* 1999b). Interpretation of results from studies using RANKL-deficient mice needs to take into account the fact that these mice lack a bone marrow compartment. Nevertheless, it would be interesting to investigate in the context of human RA whether RANKL expression by B cells has a role in maintaining lymphocytes and lymphoid aggregates in the RA synovium, especially in light of the observation that B cells appear to have a role, which may be indirect, in maintaining inflammatory cell populations in the RA synovium (Thurlings *et al.* 2008). RANKL has been reported to be involved in activating dendritic cells (Cremer *et al.* 2002), a population which is present in the RA synovium and may thus have a role in activating T cells in the RA synovium.

6.3 Cytokine expression in early synovitis: early RA vs self-resolving disease

The potential outcomes of patients with early synovitis vary from self-limiting inflammation to severe destructive RA. We hypothesised that early synovitis patients with resolving disease vs early RA would differ in cytokine expression in synovial fluid cell populations and in the synovium. Chronic inflammatory diseases such RA can be considered to have both “public” features of inflammation that are common to a generic inflammatory response, and “private” features of inflammation that are specific for the site of inflammation and development of chronicity. By comparing cytokine profiles of the synovium of patients with different outcomes, we sought to determine whether particular cytokines would identify those pathological features that are unique to RA. Cytokine profiling of synovial fluid populations showed that IFN-β1 expression in CD8 T cells was higher in patients with early synovitis with resolving disease than synovitis patients who develop RA, suggesting a heightened anti-viral response in resolving synovitis patients. In synovial tissue, perhaps partly due to the large variation within patient groups, cytokine levels were not significantly different between early synovitis patients with resolving disease and those destined to develop RA. These results indicate that the same type of inflammatory response may be present in synovitis patients irrespective of their final outcome. This would be consistent with histological data from a previous study (Schumacher and Kitridou 1972) and our own preliminary histological observations. We did not detect differences in expression of cytokines which have been previously reported as upregulated in the synovial fluid of early RA compared to resolving synovitis patients (Raza *et al.* 2005), which may be due to different methods such as measurement of mRNA rather than protein expression, the site of sample collection, and measurement of cytokine expression in isolated synovial fluid populations rather than in the whole synovial fluid. Evidence suggests that treatment of RA within the first 3 months is

more effective than when commenced later in the disease, highlighting the need for the early identification of patients destined to develop RA. Our results suggest that it is not possible to distinguish by their synovial cytokine profile those early synovitis patients who will develop RA from those with resolving synovitis, and that other parameters need to be assessed to predict the outcome of synovitis patients at presentation. At present, treatment protocols are guided by prognostic algorithms that include clinical parameters and positivity for autoantibodies. Current research efforts aimed at predicting the fate of early synovitis patients include the assessment of clinical, radiological, genetic and biological data collated from multiple centres within the Pathobiology of Early Arthritis Cohort (PEAC) consortium. Data derived from studies using gene expression profiling, proteomics, metabonomics, genotyping, serum and synovial fluid cytokine and chemokine analysis, synovial tissue characterisation, ultrasound imaging and clinical phenotyping may provide predictors of disease evolution that will be useful in future clinical practice, and shed light on the underlying disease processes involved in perpetuation or resolution of inflammation in patients with diverging clinical outcomes.

6.4 Synovial cytokine expression in different phases of RA and patient subsets

Evidence suggests that different disease mechanisms may operate during the early and established phases of RA. In this work, higher levels of IFN-β were found in established RA patients, which may reflect a mechanism that sustains the survival of inflammatory cells in the synovium. Apart from this difference, cytokine expression was similar in the early and established phases of RA, indicating that in patients destined to develop RA, inflammation is already established within the first 3 months of symptom onset. These findings support the notion that intervention before onset of clinical signs and symptoms may be beneficial, as it may provide an opportunity to prevent synovial inflammation from becoming established. The inability of currently used therapies, such as anti-TNF therapy, to induce permanent remission that is maintained following drug withdrawal, may be explained if the arbiters of persistent inflammation have already been established at the time treatment is initiated and are not modulated by this treatment; in this situation, therapy is only able to suppress inflammation rather than cure the disease.

The presence of ACPA in a large proportion of patients with RA and the specificity of ACPA for RA implicates an autoimmune component at least in a proportion of patients with RA. It is still debated whether autoimmunity may arise as an inevitable consequence of a highly sensitive amplificatory immune system or if it only occurs as a result of an external stimulus. The striking similarities observed between ACPA-positive and ACPA-negative RA suggests that although the aetiology of disease may be different in these patient subsets, pathogenic mechanisms converge on a final common pathway resulting in the clinical entity we define as “RA”. Similarities between ACPA-positive and ACPA-negative patients with early RA fits

with the observation that at presentation, there are no apparent clinical phenotypic differences according to ACPA status (Cader *et al.* 2010; van der Helm-van Mil *et al.* 2005). However, similarities observed between ACPA-positive and ACPA-negative patients with established RA does not reflect the observation that ACPA-positive patients have a more erosive disease course, although cytokine expression reflecting these differences may be more apparent at the site of bone and cartilage destruction, i.e. the cartilage-pannus junction, rather than the sites biopsied from the synovium in this study.

Several pieces of evidence support the importance of pre-clinical abnormalities which precede development of RA. The presence of ACPA predicts development of RA, and there is evidence for epitope spreading prior to disease onset, with the extent of the repertoire being associated with development of arthritis in patients with arthralgia (van de Stadt *et al.* 2010). Positivity for rheumatoid factor is also strongly associated with the development of RA. mRNA expression profiling of peripheral blood cells from ACPA- and/or rheumatoid factor-positive patients with arthralgia found that signatures associated with development of arthritis were involved in IFN-mediated immunity, haematopoiesis, and chemokine/cytokine activity (van Baarsen *et al.* 2010). In addition, the upregulation of serum cytokines at the protein level (IL-1, TNF-α, IL-6, IL-2, IL-12, IFN-γ, IL-4, IL-10, CCL2, CCL3, GM-CSF and G-CSF) has been reported in another study which examined ACPA-positive individuals who went on to develop RA (Kokkonen *et al.,* 2010). Some studies have also reported increased levels of CRP prior to onset of RA (Aho *et al.* 2000; Nielen *et al.* 2004). Despite increasing insight into the pre-clinical phase of RA, the natural history of the disease remains unknown. Evidence supports an initial phase in which genetic and environmental interactions lead to a period of asymptomatic autoimmunity which later develops into clinically-apparent disease. However, the physical location where genetic and environmental factors interact leading to a break in

tolerance remains unknown. Due to the link between RA and smoking it has been suggested that this may occur at periodontal surfaces or the lung. Furthermore, the stimuli responsible for mediating the transition from a systemic immune disturbance into a joint-centric pathology are not clear at present. Studying disease processes in non-articular tissue such as the bone marrow and lymph nodes, and systems such as the neuroendocrine system, may be informative in furthering our understanding of the development of RA. Some of these issues are being addressed by collaborative efforts such as those within the EULAR study group on the pre-clinical and earliest clinically-apparent phases of RA. Ultimately, research into these areas may lead to the development of pharmacological or non-pharmacological interventions that could be used in the pre-clinical and earliest clinically-apparent phases of RA. For example, risk-factor modification in certain individuals such as cessation of smoking could prevent clinical development of the disease.

6.5 Future work

In this work I have described novel cytokine mRNA expression patterns in cell populations isolated from the synovial fluid of RA patients. For example, several pro-inflammatory cytokines were found to be expressed by synovial fluid B cells and neutrophils. These observations, made on the level of mRNA expression, need to be verified on the protein level, since mRNA expression may not be representative of protein production due to post-transcriptional and post-translational regulation of gene expression. To follow up the findings reported here, it would also be interesting to sort these populations from synovial tissue and confirm cytokine expression in cells present within the RA synovium itself. This could be achieved by digestion of synovial tissue, isolation of mononuclear cell populations on a Ficoll gradient, and sorting stained cell populations by FACS as I have done for synovial fluid

populations. In this study I have identified genes as being differentially expressed between different patient groups (early resolving synovitis, early RA and established RA). These observations also need to be confirmed on the protein level, for example by immunofluorescence staining of synovial tissue sections.

The cytokine expression profiling study of RA synovial fluid populations has enabled us to characterise sources of several cytokines. Using the data generated in this study, it would be of interest to employ bioinformatic strategies to propose a model of cell-cell interactions mediated by cytokines between the five largest populations in the synovial fluid in RA.

Further work remains to be done to characterise the B cell subsets producing RANKL in RA synovial fluid, and to extend this work to synovial tissue B cells. We are planning to use an extended panel of antibodies including RANKL, CD19, IgD, IgM, CD27, CD38, CD20, CD21, CD95, CD11c and CD138 to identify subsets of B cells and plasma cells in the synovial fluid and peripheral blood and to assess their level of RANKL expression. Immunofluorescence staining could also be used to distinguish these B cell and plasma cell populations in RA synovial tissue. RANKL-expressing B cell subsets could then be sorted from synovial tissue and synovial fluid to assess mRNA levels of RANKL expression. An aspect that remains to be addressed concerns the regulation of the expression and release of RANKL which is produced in both membrane-bound and soluble forms. This may be investigated by stimulating sorted B cells *in vitro* using anti-CD40 or anti-IgG, and assessing the effects of B cell stimulating cytokines such as BAFF, APRIL, IL-5, IL-6 and IL-10. In addition, functional assays need to be carried out to assess whether RANKL-producing B cells from patients with RA are capable of mediating bone-resorption by osteoclasts *in vitro.*

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8 APPENDIX

8.1 Custom gene sets

Table 8.1 Custom gene set for real-time PCR assays used to detect mRNA expression in synovial fluid and peripheral blood cell populations

No.	Assay ID	No.	Assay ID
1	IL1A-Hs00174092_m1	25	CCL5-Hs00174575_m1
2	IL1B-Hs00174097_m1	26	MIF-Hs00236988_g1
3	IL2-Hs00174114_m1	27	CCL11-Hs00237013_m1
4	IL4-Hs00174122_m1	28	TNF-Hs00174128_m1
5	IL5-Hs00174200_m1	29	LTB-Hs00242739_m1
6	IL6-Hs00174131_m1	30	TGFB1-Hs99999918_m1
7	IL7-Hs00174202_m1	31	TNFSF11-Hs00243522_m1
8	IL8-Hs00174103_m1	32	TNFSF13B-Hs00198106_m1
9	IL10-Hs00174086_m1	33	TNFSF13;TNFSF12-TNFSF13-Hs00182565_m1
10	IL11-Hs00174148_m1	34	IFNA1-Hs00256882_s1
11	18S-Hs99999901_s1	35	IFNG-Hs99999041_m1
12	IL12A-Hs00168405_m1	36	IFNB1-Hs00277188_s1
13	IL12B-Hs00233688_m1	37	CSF2-Hs00171266_m1
14	IL13-Hs00174379_m1	38	CSF3-Hs00236884_m1
15	IL15-Hs00174106_m1	39	CXCL12-Hs00171022_m1
16	IL17A-Hs00174383_m1	40	EGF-Hs01099999_m1
17	IL18-Hs00155517_m1	41	FGF2-Hs00266645_m1
18	IL21-Hs00222327_m1	42	VEGFA-Hs00173626_m1
19	IL22-Hs00220924_m1	43	LGALS3-Hs00173587_m1
20	IL23A-Hs00413259_m1	44	LGALS9-Hs00371321_m1
21	IL27-Hs00377366_m1	45	HSD11B1-Hs01547870_m1
22	CCL3-Hs00234142_m1	46	HSD11B2-Hs00388669_m1
23	CCL4;CCL4L1;CCL4L2-Hs00237011_m1	47	GAPDH-Hs99999905_m1
24	CCL2-Hs00234140_m1	48	IL6ST-Hs00174360_m1

Table 8.2 Custom gene set 1 for real-time PCR assays used to detect mRNA expression in synovial tissue

No.	Assay ID	No.	Assay ID
1	CSF3-Hs00236884_m1	25	TXLNA-Hs00233304_m1
2	CSF2-Hs00171266_m1	26	IL15-Hs00542562_m1
3	CSF1-Hs00174164_m1	27	IL16-Hs00189606_m1
4	IFNA1-Hs00256882_s1	28	IL17A-Hs00174383_m1
5	IFNA2-Hs00265051_s1	29	IL17F-Hs00369400_m1
6	IFNB1-Hs00277188_s1	30	IL18-Hs00155517_m1
7	IFNG-Hs00174143_m1	31	IL19-Hs00203540_m1
8	IL1A-Hs00174092_m1	32	IL20-Hs00218888_m1
9	IL1B-Hs00174097_m1	33	IL22-Hs00220924_m1
10	IL1RN-Hs00277299_m1	34	IL23A-Hs00372324_m1
11	18S-Hs99999901_s1	35	IL24-Hs01114274_m1
12	IL2-Hs00174114_m1	36	IL25-Hs00224471_m1
13	IL3-Hs00174117_m1	37	IL26-Hs00218189_m1
14	IL4-Hs00174122_m1	38	IL27-Hs00377366_m1
15	IL5-Hs00174200_m1	39	IL28A-Hs00820125_g1
16	IL6-Hs00985639_m1	40	IL29-Hs00601677_g1
17	IL7-Hs00174202_m1	41	IL32-Hs00992441_m1
18	IL8-Hs00174103_m1	42	IL33-Hs00369211_m1
19	IL9-Hs00174125_m1	43	LIF-Hs00171455_m1
20	IL10-Hs00174086_m1	44	OSM-Hs00171165_m1
21	IL11-Hs00174148_m1	45	LTA-Hs00236874_m1
22	IL12A-Hs00168405_m1	46	TNF-Hs00174128_m1
23	IL12B-Hs00233688_m1	47	LTB-Hs00242739_m1
24	IL13-Hs00174379_m1	48	TNFSF4-Hs00182411_m1

continued

No.	Assay ID	No.	Assay ID
49	CD40LG-Hs00163934_m1	73	CXCL5-Hs00171085_m1
50	FASLG-Hs00181225_m1	74	CXCL6-Hs00237017_m1
51	CD70-Hs00174297_m1	75	PPBP-Hs00234077_m1
52	TNFSF8-Hs00174286_m1	76	CXCL9-Hs00171065_m1
53	TNFSF9-Hs00169409_m1	77	CXCL10-Hs00171042_m1
54	TNFSF10-Hs00234356_m1	78	CXCL11-Hs00171138_m1
55	TNFSF11-Hs00243522_m1	79	CXCL12-Hs00171022_m1
56	TNFSF12-Hs00611242_m1	80	CXCL13-Hs00757930_m1
57	TNFSF13;TNFSF12-TNFSF13-Hs00182565_m1	81	CXCL14-Hs00171135_m1
58	TNFSF13B-Hs00198106_m1	82	CXCL16-Hs00222859_m1
59	TNFSF14-Hs00542477_m1	83	CCL1-Hs00171072_m1
60	TNFSF15-Hs00353710_s1	84	CCL2-Hs00234140_m1
61	TNFSF18-Hs00183225_m1	85	CCL3-Hs00234142_m1
62	CD40-Hs00386848_m1	86	CCL4;CCL4L1;CCL4L2-Hs00237011_m1
63	FAS-Hs00531110_m1	87	CCL5-Hs00174575_m1
64	TNFRSF18-Hs00188346_m1	88	CCL7-Hs00171147_m1
65	TGFB1-Hs00998133_m1	89	CCL8-Hs00271615_m1
66	TGFB2-Hs00234244_m1	90	CCL11-Hs00237013_m1
67	TGFB3-Hs00234245_m1	91	CCL13-Hs00234646_m1
68	MIF-Hs00236988_g1	92	CCL15;CCL14-Hs00234981_m1
69	CXCL1-Hs00236937_m1	93	CCL16-Hs00171123_m1
70	CXCL2-Hs00236966_m1	94	CCL17-Hs00171074_m1
71	CXCL3-Hs00171061_m1	95	CCL18-Hs00268113_m1
72	PF4-Hs00427220_g1	96	GAPDH-Hs99999905_m1

Table 8.3 Custom gene set 2 for real-time PCR assays used to detect mRNA expression in synovial tissue

No.	Assay ID	No.	Assay ID
1	IFNA1-Hs00256882_s1	25	IL20-Hs00218888_m1
2	IFNA2-Hs00265051_s1	26	IL22-Hs00220924_m1
3	IFNB1-Hs00277188_s1	27	IL24-Hs01114274_m1
4	KITLG-Hs00295067_s1	28	IL25-Hs00224471_m1
5	ADIPOQ-Hs02564413_s1	29	CCL1-Hs00171072_m1
6	TNFSF15-Hs00353710_s1	30	CCL7-Hs00171147_m1
7	CD248-Hs00535586_s1	31	CSF3-Hs99999083_m1
8	SOCS3-Hs00269575_s1	32	18S-Hs99999901_s1
9	IL28A-Hs00820125_g1	33	B2M-Hs00187842_m1
10	IL29-Hs00601677_g1	34	IFNG-Hs00174143_m1
11	GAPDH-Hs99999905_m1	35	IL2-Hs00174114_m1
12	MIF-Hs00236988_g1	36	IL23A-Hs00372324_m1
13	SUMO1-Hs02339312_g1	37	IL11-Hs00174148_m1
14	SRD5A1-Hs00971643_g1	38	IL12A-Hs00168405_m1
15	IL21-Hs00222327_m1	39	IL12B-Hs00233688_m1
16	IL1A-Hs00174092_m1	40	LTA-Hs00236874_m1
17	IL3-Hs00174117_m1	41	CSF2-Hs00171266_m1
18	IL4-Hs00174122_m1	42	CCL11-Hs00237013_m1
19	IL5-Hs00174200_m1	43	CCL25-Hs00171144_m1
20	IL9-Hs00174125_m1	44	PADI4-Hs00202612_m1
21	IL13-Hs00174379_m1	45	PADI2-Hs00247108_m1
22	IL17A-Hs00174383_m1	46	AOC3-Hs00186647_m1
23	IL17F-Hs00369400_m1	47	FAP-Hs00990806_m1
24	IL19-Hs00203540_m1	48	IL1F7-Hs00367199_m1

8.2 Patient clinical data

Table 8.4 Clinical data for established RA, early RA and early resolving synovitis patients. Synovial fluid and peripheral blood samples from these patients were used for experiments described in Chapter 3 and Chapter 4.

Outcome	Age	Gender	Symptom duration	ESR	CRP	RF	CCP	Treatment	TJC28	SJC28	DAS28 ESR
Early RA	66	M	6 weeks	25	83	positive	negative	nsaid	3	24	4.7
Early RA	48	F	2 weeks	4	102	negative	negative	diclofenac	8	6	3.5
Early RA	75	M	5 weeks	40	40	negative	negative	nil	27	26	8.3
Early RA	NA	F	7 weeks	60	40	negative	negative	nsaid	11	16	6.7
Early RA	41	F	6 weeks	54	40	positive	positive	diclofenac	28	18	8.3
Early Resolving	32	M	7 weeks	10	10	negative	negative	nsaid	1	1	2.9
Early Resolving	35	M	1 week	2	9	negative	negative	nil	1	3	1.6
Early Resolving	34	M	2 weeks	51	7	negative	NA	ibuprofen	1	1	NA
Est RA	66	F	6 yrs	81	92	negative	negative	hydroxychloroquine	1	3	4.4
Est RA	79	M	1 yr	17	16	positive	positive	prednisolone, methotrexate	5	5	4.5
Est RA	67	F	30 yrs	66	89	positive	positive	hydroxycholorquine, sulfasalazine	13	10	7.2
Est RA	59	F	1 yr	27	19	positive	positive	ibuprofen, prednisolone	3	3	5
Est RA	41	M	6 yrs	32	54	positive	positive	prednisolone, methotrexate, etanercept	3	8	5.1
Est RA	79	M	1 yr	48	119	positive	positive	nil	28	26	8.4
Est RA	75	F	40 yrs	55	49	positive	positive	methotrexate, sulphasalazine	2	3	4.9
Est RA	41	M	6 yrs	32	44	positive	positive	methotrexate, prednisolone, adalimumab	3	5	5.3
Est RA	55	F	5 yrs	124	221	positive	positive	methotrexate	14	8	7.5
Est RA	52	M	2 yrs	7	9	positive	negative	diclofenac	2	8	3.2
Est RA	73	F	18 yrs	7	7	positive	NA	meloxicam, methotrexate	9	10	4.6
Est RA	58	F	6 yrs	22	18	positive	positive	nabumetone, prednisolone	7	6	5.6

Erythrocyte Sedimentation Rate (ESR), C Reactive Protein (CRP), Rheumatoid Factor (RF), Cyclic Citrullinated Peptide Antibody (CCP), Tender Joint Count 28 (TJC), Swollen Joint Count 28 (SJC), Disease Activity Score 28 (DAS28), Established RA (Est RA), non-steroidal anti-inflammatory drug (NSAID), data not available (NA).

Table 8.5 Clinical data for established RA, early RA and early resolving synovitis patients. Synovial tissue samples from these patients were used for experiments described in Chapter 4.

Outcome	Age	Gender	Symptom duration (weeks)	ESR	CRP	RF	CCP	Treatment NSAID	Treatment Prednisolone	TJC28	SJC28	DAS28 ESR
Est RA	46	M	150	34	7	p	p	y	n	13	16	6.7
Est RA	61	F	30	8	9	n	n	n	n	15	6	4.9
Est RA	69	F	52	11	0	n	n	n	n	7	7	4.6
Est RA	57	M	14	56	16	p	p	y	n	21	14	7.1
Est RA	58	M	16	7	0	p	p	y	n	7	7	4
Est RA	56	F	52	10	0	p	p	y	n	12	3	5.5
Est RA	46	F	30	24	na	n	p	y	n	10	4	5.2
Est RA	64	M	26	13	17	n	n	y	n	14	16	5.4
Est RA	67	M	38	29	48	p	p	n	n	1	1	4.2
Est RA	22	F	52	81	79	n	n	y	n	6	6	6.4
Est RA	72	F	38	53	43	n	n	y	n	21	16	7.4
Est RA	52	F	38	26	52	p	p	y	n	20	8	6.5
Est RA	65	M	156	72	81	p	p	n	n	3	12	5.3
Early RA	70	F	5	68	26	n	n	y	n	4	5	6
Early RA	49	F	2	12	8	n	n	n	n	9	8	4.7
Early RA	45	F	10	24	12	n	n	y	n	3	3	3.8
Early RA	63	F	4	104	9	n	n	n	n	1	5	5.1
Early RA	48	F	2	4	102	n	n	y	n	8	6	3.5
Early RA	59	M	6	14	22	n	n	n	y	4	20	5
Early RA	44	F	5	18	10	n	n	y	n	3	2	3.8
Early RA	43	M	9	58	0	n	n	y	n	19	4	6.9
Early RA	56	M	10	5	0	p	n	n	n	14	21	5.2
Early RA	55	M	4	58	45	n	p	n	n	0	4	3.5
Early RA	42	F	2	54	40	p	p	y	n	28	18	8.3
Early RA	53	F	12	11	0	n	n	y	n	7	2	4.2

Outcome	Age	Gender	Symptom duration (weeks)	ESR	CRP	RF	CCP	Treatment NSAID	Treatment Prednisolone	TJC28	SJC28	DAS28 ESR
Early RA	62	F	12	40	5	p	p	y	n	4	2	4.3
Early RA	74	F	9	20	32	n	p	n	n	3	3	4.4
Early RA	58	F	7	27	0	p	p	y	n	5	9	4.5
Early RA	49	F	6	25	18	p	p	y	n	17	12	6.8
Early RA	48	M	4	63	38	p	p	y	n	8	9	6
Early Resolving	64	M	6	24	15	n	n	y	n	5	2	4.5
Early Resolving	40	F	4	5	0	n	n	y	n	7	7	3.9
Early Resolving	32	M	7	10	10	n	n	y	n	1	1	2.9
Early Resolving	33	M	4	51	14	n	n	y	n	12	9	6.7
Early Resolving	74	M	5	45	13	n	n	y	n	0	23	4.8
Early Resolving	72	M	8	5	0	n	n	n	n	7	4	3.6
Early Resolving	81	F	7	60	52	n	n	y	n	16	11	6.7
Early Resolving	45	F	1	4	0	n	n	n	n	5	5	4
Early Resolving	35	M	2	51	7	n	n	y	n	1	1	4.1
Early Resolving	35	M	1	2	9	n	n	n	n	1	3	1.6
Early Resolving	41	F	4	5	9	n	n	y	n	0	2	1.7
Early Resolving	28	M	6	18	8	n	n	y	n	2	1	4.5
Early Resolving	27	M	4	37	28	n	n	y	n	2	2	3.8

Erythrocyte Sedimentation Rate (ESR), C Reactive Protein (CRP), Rheumatoid Factor (RF), Cyclic Citrullinated Peptide Antibody (CCP), Tender Joint Count 28 (TJC), Swollen Joint Count 28 (SJC), Disease Activity Score 28 (DAS28), Established RA (Est RA), non-steroidal anti-inflammatory drug (NSAID), data not available (NA).

Table 8.6 Clinical data for RA patients. Synovial fluid and peripheral blood samples from these patients were used for experiments described in Chapter 5.

Outcome	Age	Gender	Symptom duration	ESR	CRP	RF	CCP	Treatment nsaid	TJC28	SJC28	DAS28 ESR
Est RA	41	M	6 yrs	26	37	positive	positive	prednisolone, methotrexate, adalimumab	2	6	4.2
Est RA	81	M	< 1 yr	21	9	positive	positive	nil	12	15	6.2
Est RA	67	F	6 yrs	86	84	negative	negative	methotrexate, hydroxychloroqine	5	8	5.8
Est RA	55	F	5 yrs	124	221	positive	positive	methotrexate	14	8	7.5
Est RA	82	M	1 yr	77	54	positive	positive	adalimumab (first injection 1/7 ago)	14	6	6.7
Est RA	52	M	2 yrs	7	9	positive	negative	diclofenac	2	8	3.2
Est RA	74	F	2 yrs	2	0	negative	positive	prednisolone, methotrexate	2	1	1.8
Early RA	73	f	9 weeks	20	32	negative	positive	nil	4	5	4.7
Est RA	66	F	10 yrs	45	32	positive	positive	nil	8	7	6.3
Est RA	47	M	9 yrs	41	26	positive	positive	nil	11	23	6.9
Est RA	82	M	1 yr	76	31	positive	positive	prednisolone, adalimumab	21	12	7.9

Erythrocyte Sedimentation Rate (ESR), C Reactive Protein (CRP), Rheumatoid Factor (RF), Cyclic Citrullinated Peptide Antibody (CCP), Tender Joint Count 28 (TJC), Swollen Joint Count 28 (SJC), Disease Activity Score 28 (DAS28), Established RA (Est RA).

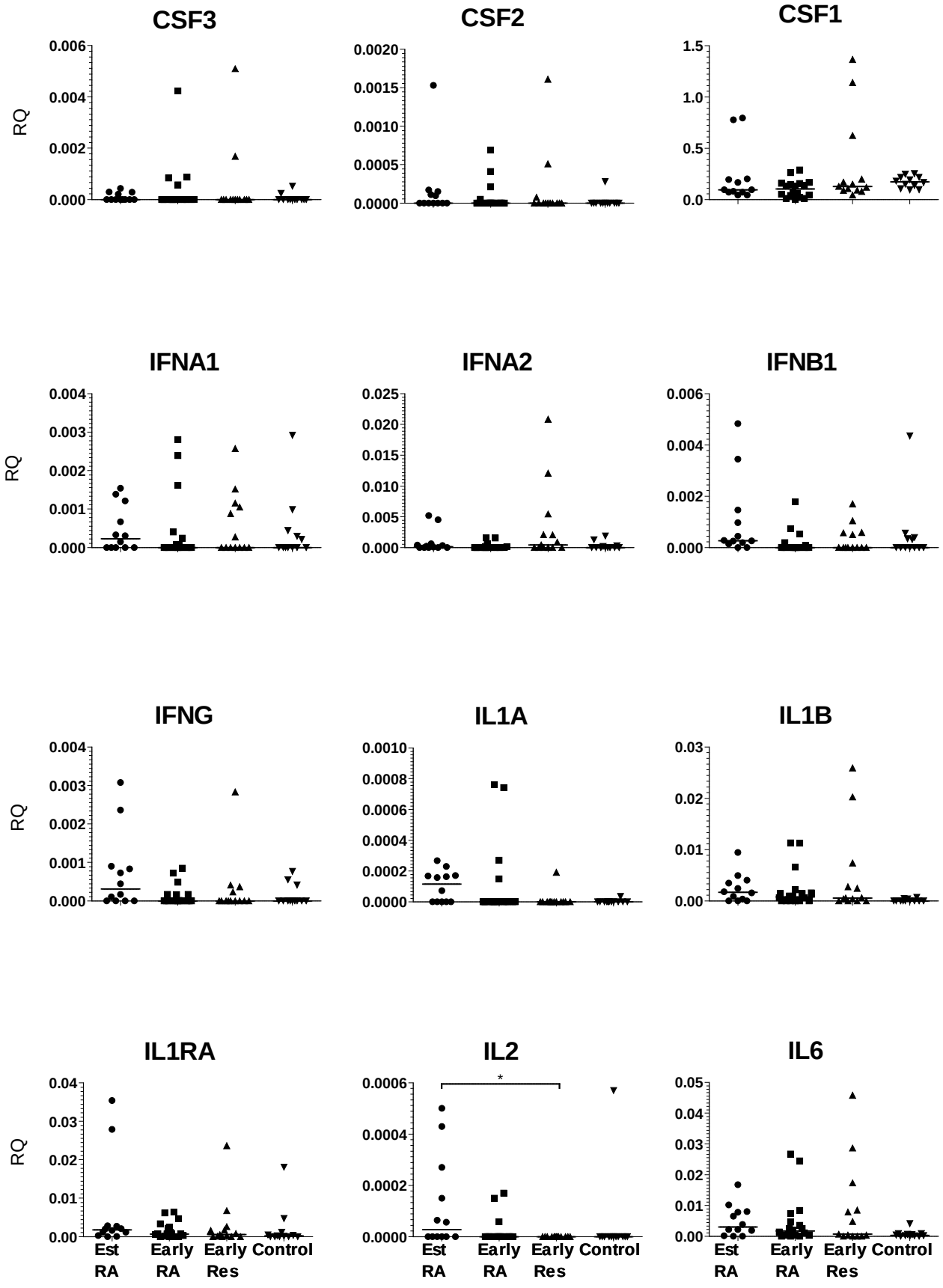
Table 8.7 Clinical data for established RA patients treated with rituximab. Synovial tissue samples from these patients were used for experiments described in Chapter 5.

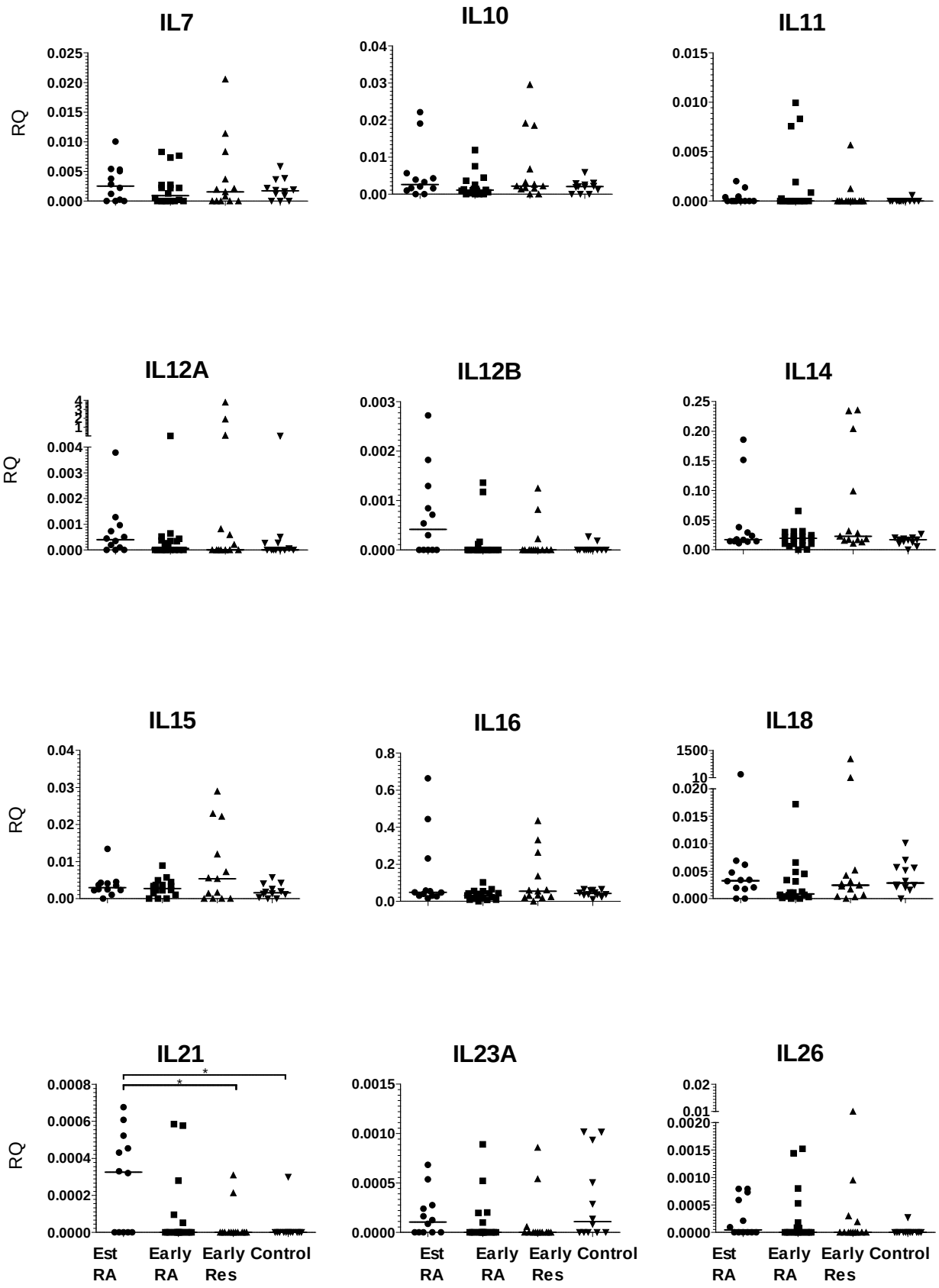
Age	Gender	Disease duration	ESR	CRP	SHS	RF	CCP	No. of failed DMARDs	No. of failed biologicals	MTX use (mg/week)	Prednisolone use (mg/day)
43	F	2	50	37.2	29	38	26	3	1	25	10
42	F	20	47	40.5	33	9	240	5	2	10	10
55	M	17	49	83	10	46	24	2	2	15	0
63	F	6	37	87	63	50	208	2	1	10	5
67	F	29	37	9.4	247	247	37	3	0	10	0
75	F	29	59	92.7	NA	117	1332	3	4	15	5
46	F	13	66	111.8	43	55	2370	8	4	15	10
60	M	14	57	15	80	417	126	4	1	10	0
64	F	6	11	7.4	29	8	68	2	3	30	10
61	F	8	16	5.2	0	3	1268	2	2	15	0
53	F	14	27	12.7	81	26	15	9	3	5	5
49	F	9	47	33.5	0	15	2709	6	2	10	8
65	F	7	45	58.6	169	26	516	4	1	20	0

Erythrocyte Sedimentation Rate (ESR), C Reactive Protein (CRP), Sharpe/van der Heijde score (SHS), Rheumatoid Factor (RF), Cyclic Citrullinated Peptide Antibody (CCP), disease-modifying anti-rheumatic drug (DMARD), methotrexate (MTX).

8.3 Cytokine mRNA expression data

8.3.1 Cytokine mRNA expression in synovial tissue from patients with resolving synovitis, early RA, established RA and controls

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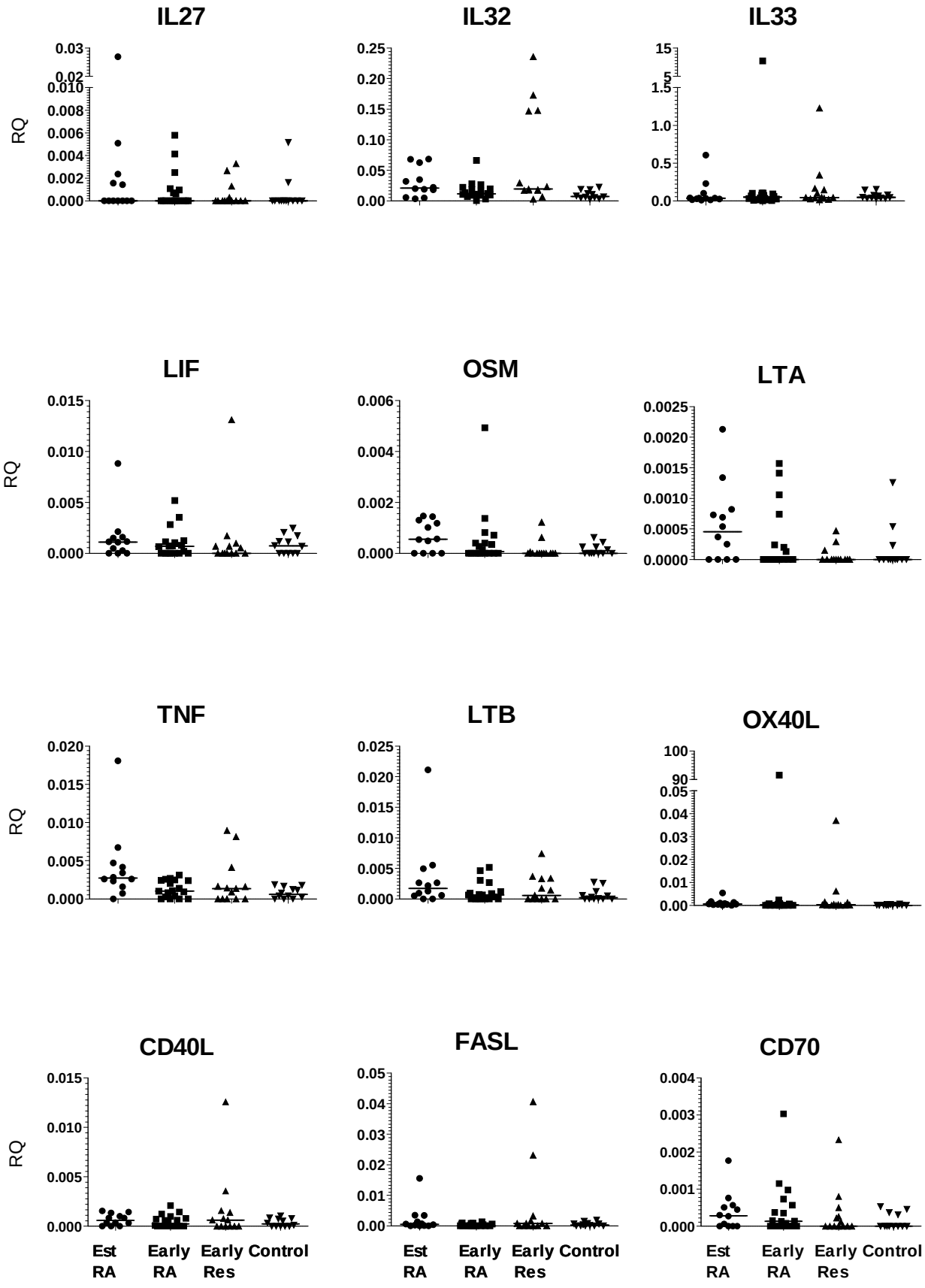
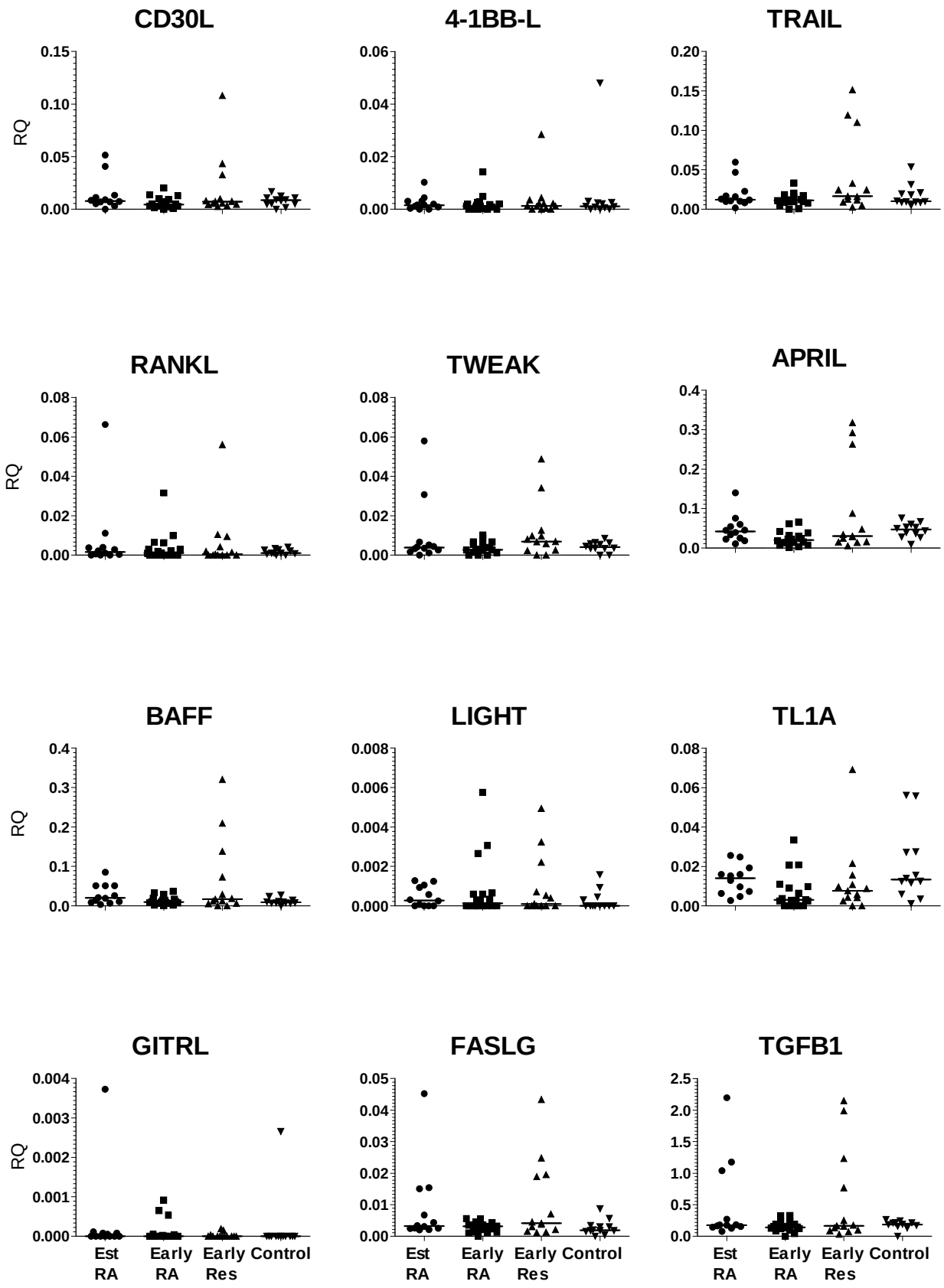
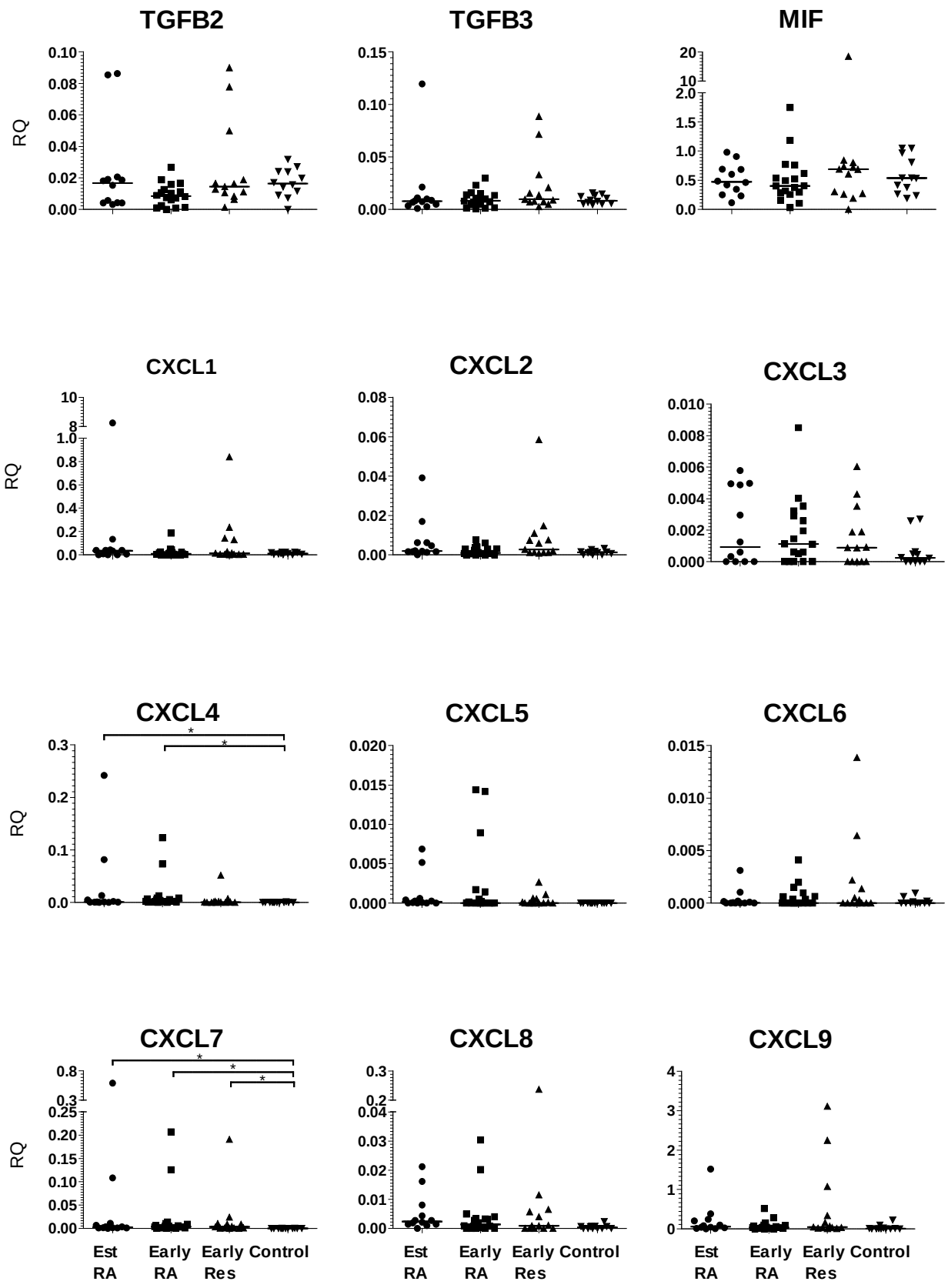
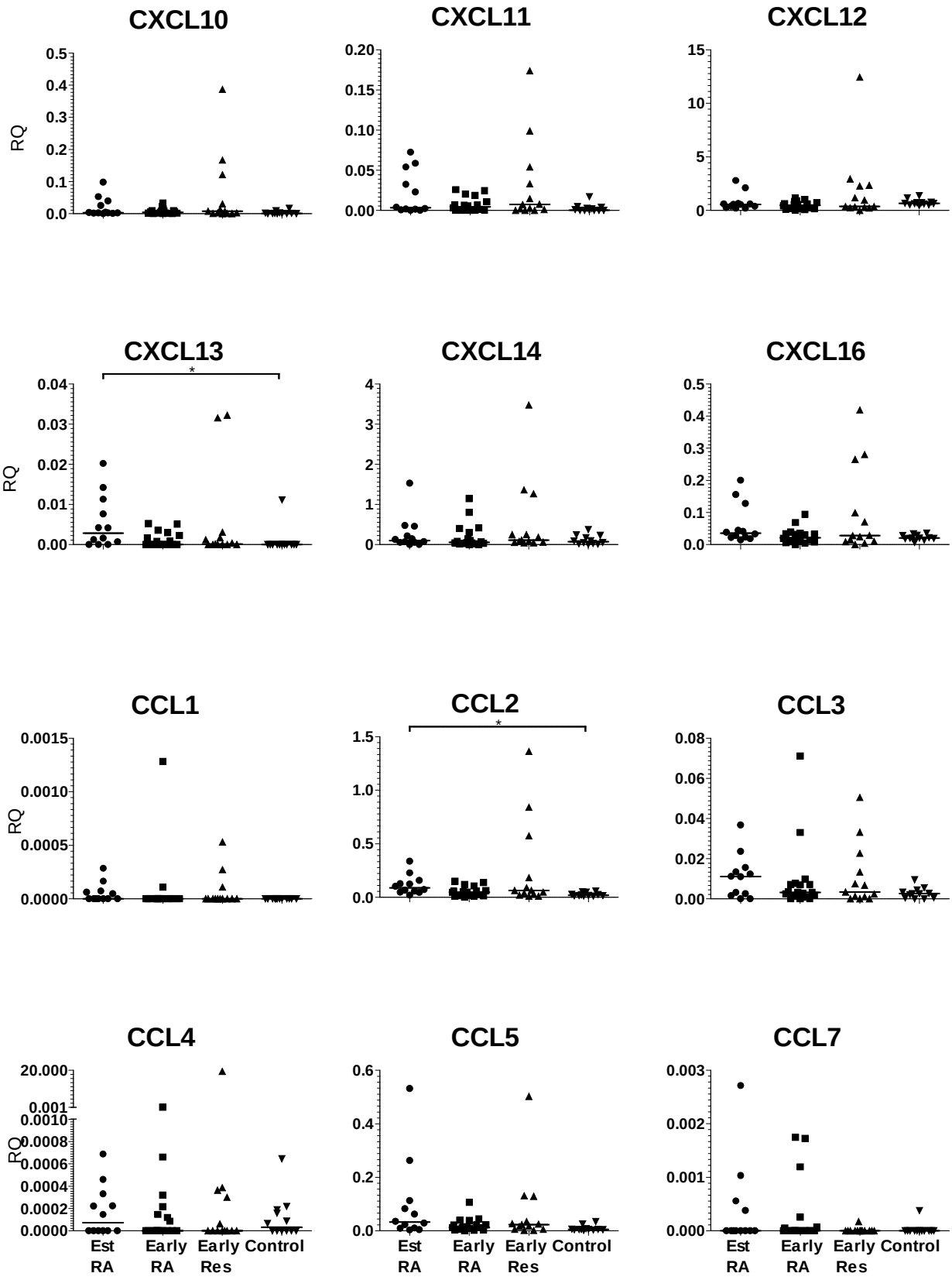
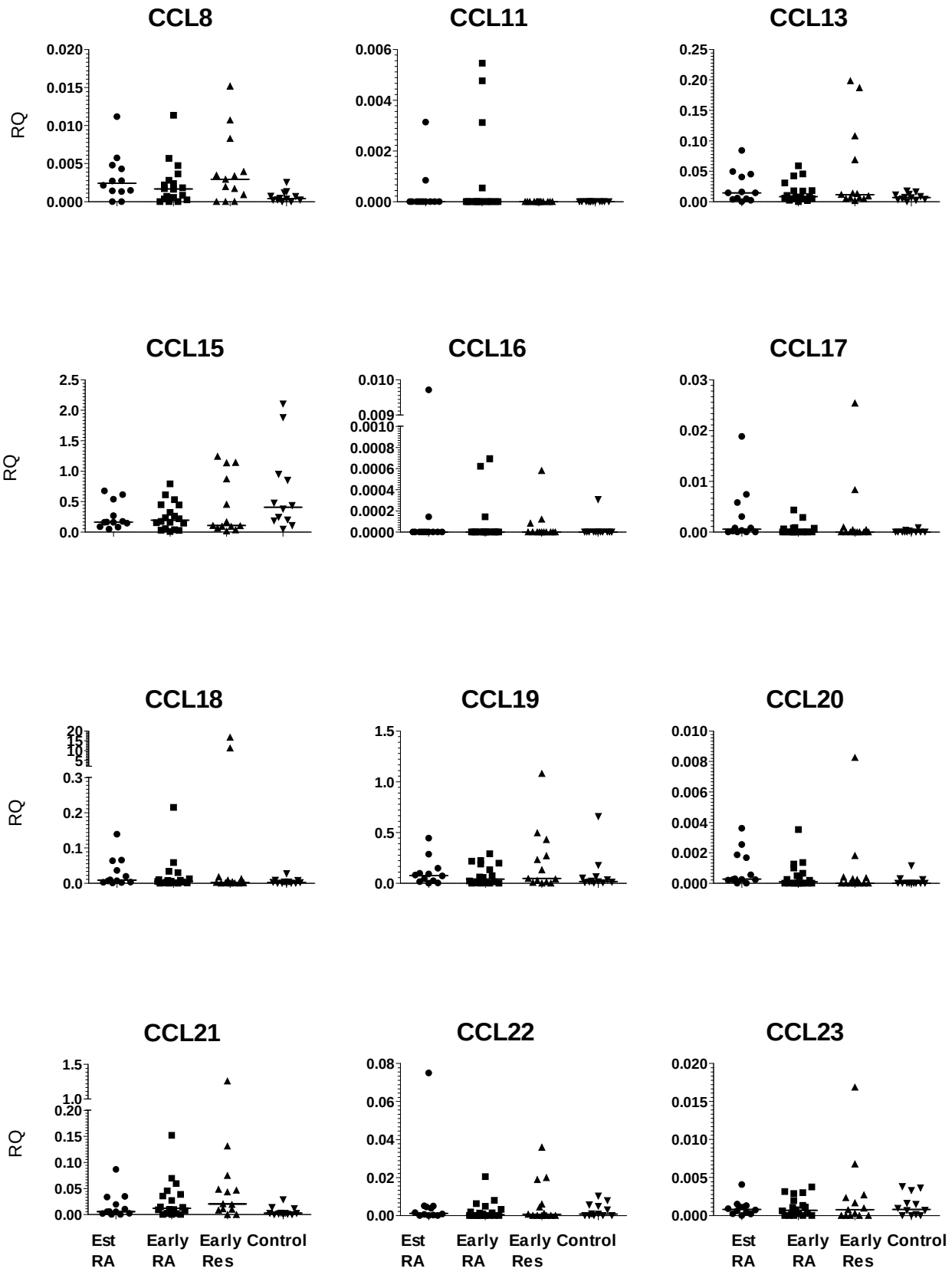
IL11
IL12A
IL12B
IL14
IL15
IL16
IL18
IL21
IL23A
IL26
RQ
Est RA
Early RA
Early Res
Control
*
*


IL27
IL32
IL33
LIF
OSM
LTA
TNF
LTB
OX40L
CD40L
FASL
CD70
RQ
Est RA
Early RA
Early Res
Control


CD30L
4-1BB-L
TRAIL
RANKL
TWEAK
APRIL
BAFF
LIGHT
TL1A
GITRL
FASLG
TGFB1
RQ
Est RA
Early RA
Early Res
Control


TGFB2
TGFB3
MIF
CXCL1
CXCL2
CXCL3
CXCL4
CXCL5
CXCL6
CXCL7
CXCL8
CXCL9
RQ
Est RA
Early RA
Early Res
Control


CXCL10
CXCL11
CXCL12
CXCL13
CXCL14
CXCL16
CCL1
CCL2
CCL3
CCL4
CCL5
CCL7
RQ
*
Est RA
Early RA
Early Res
Control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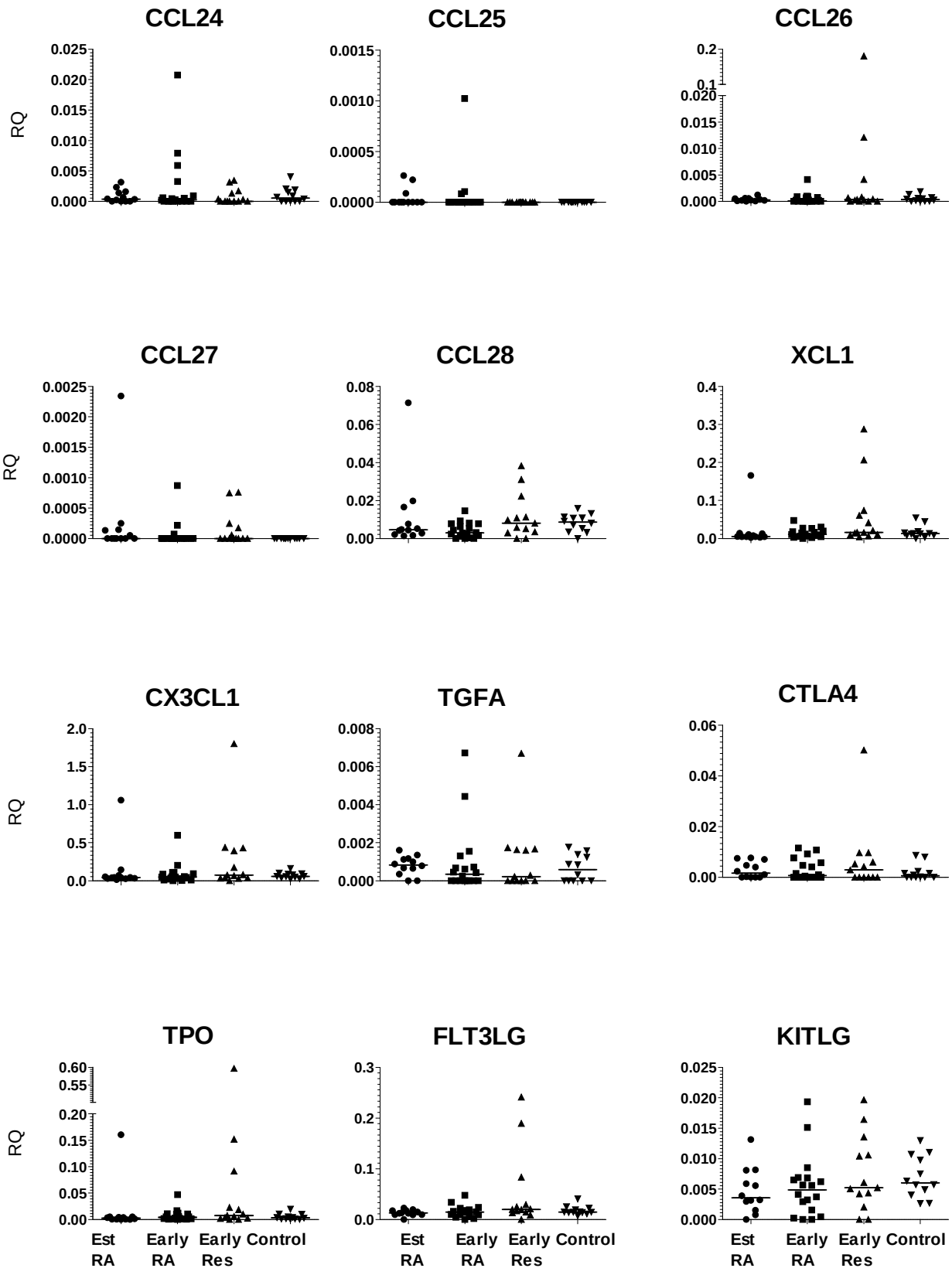
CCL11
CCL13
CCL15
CCL16
CCL17
CCL18
CCL19
CCL20
CCL21
CCL22
CCL23
RQ
Est RA
Early RA
Early Res
Control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CCL25
CCL26
CCL27
CCL28
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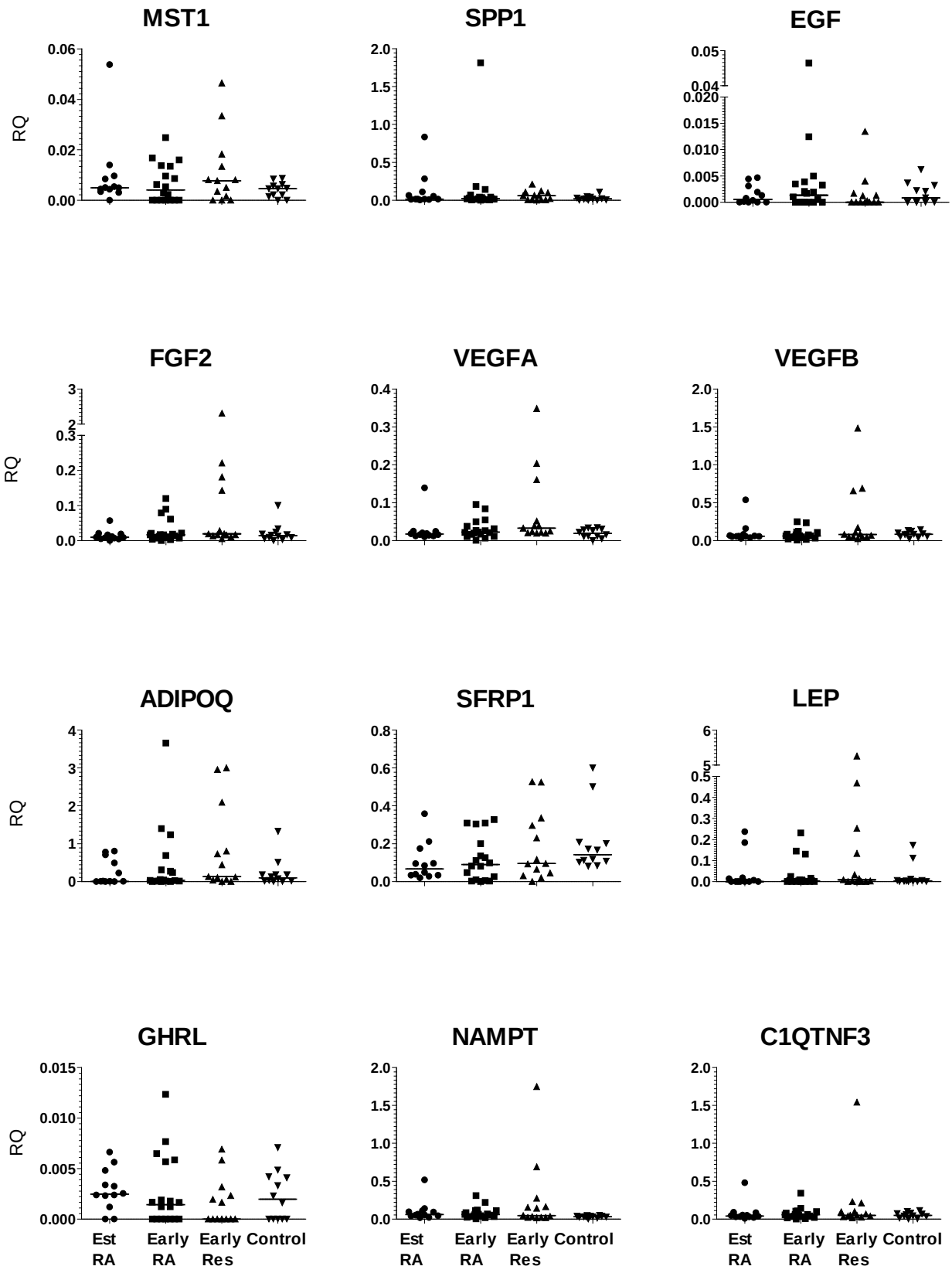
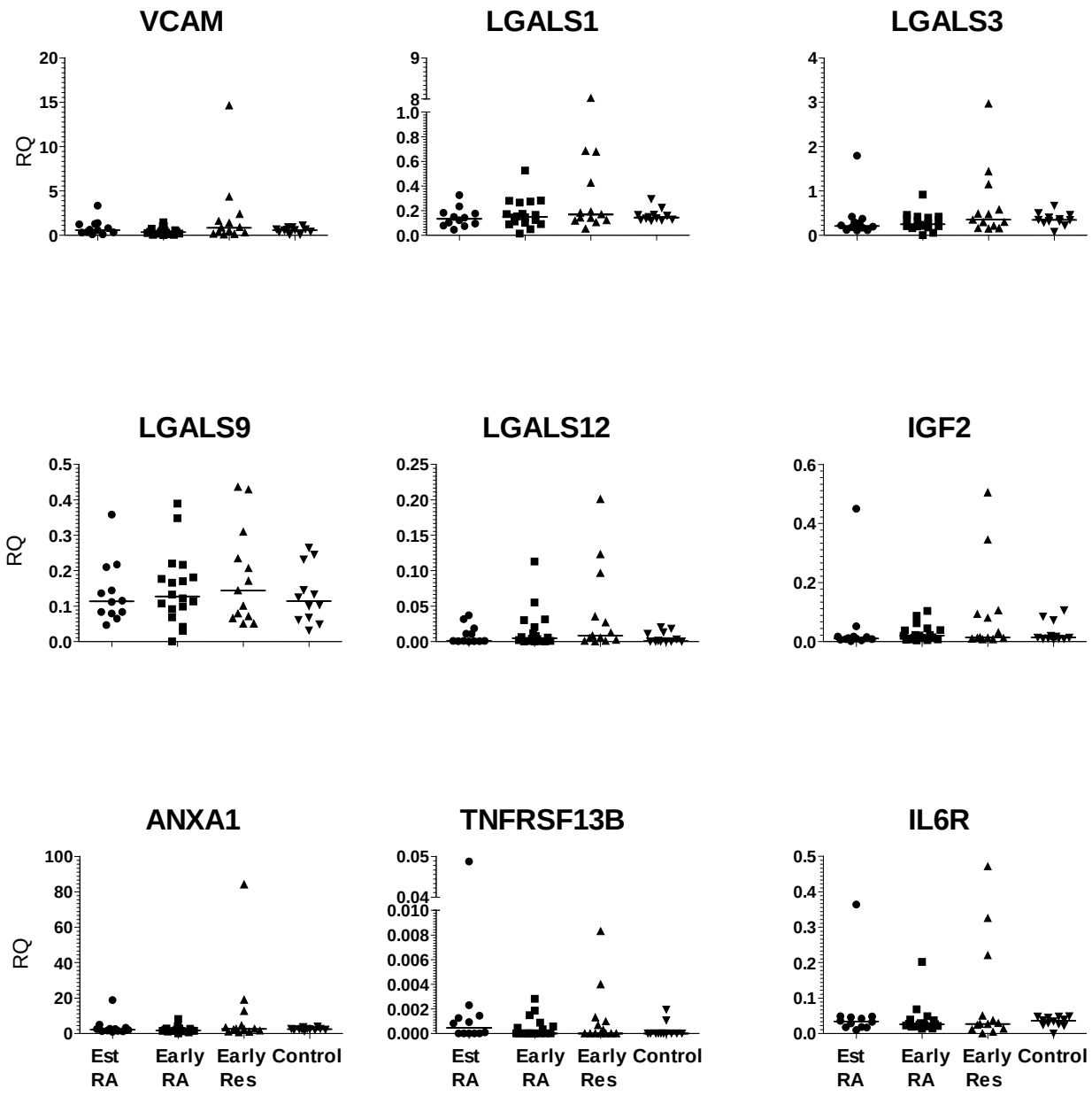
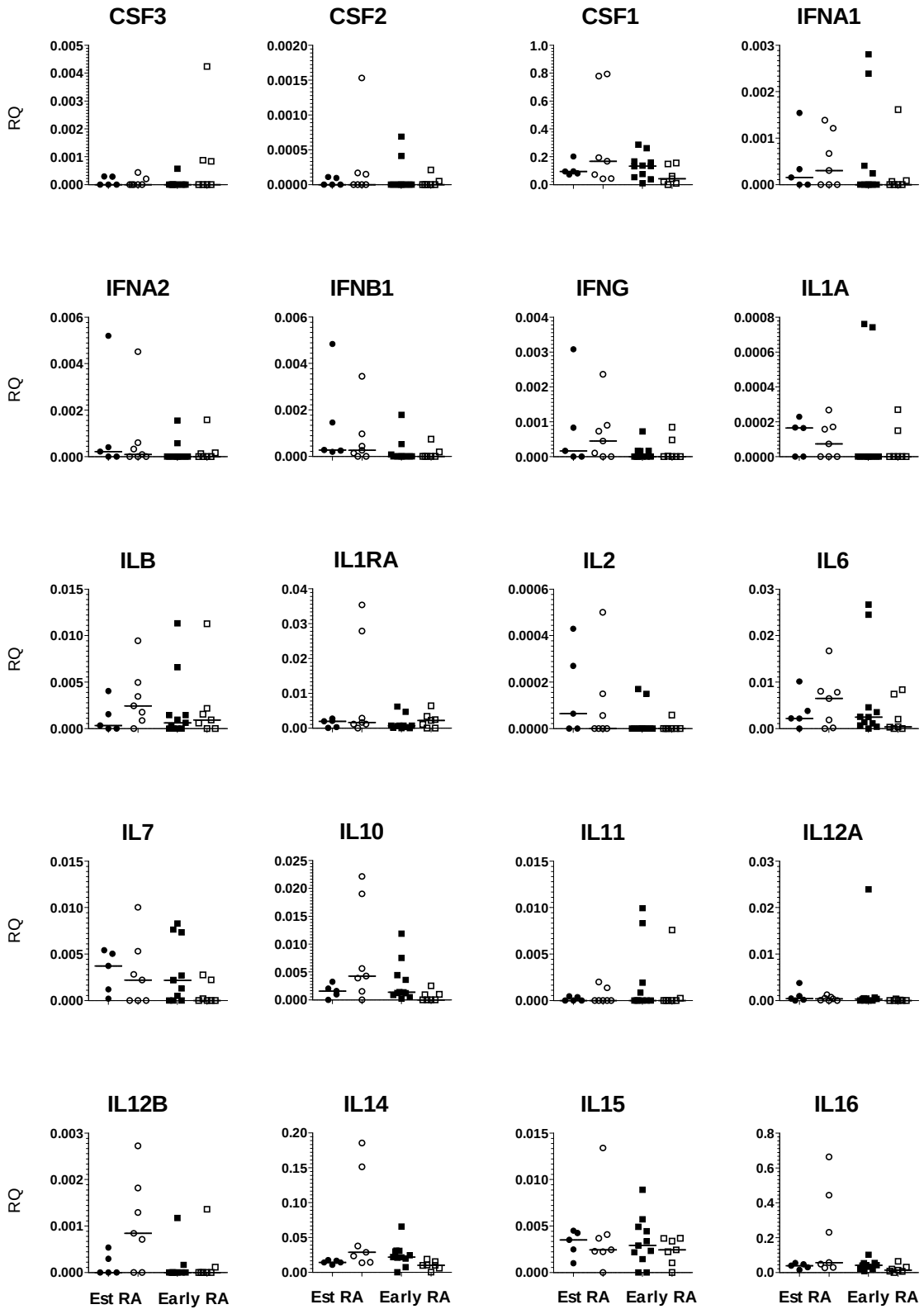
CX3CL1
TGFA
CTLA4
TPO
FLT3LG
KITLG
RQ
Est RA
Early RA
Early Res
Control

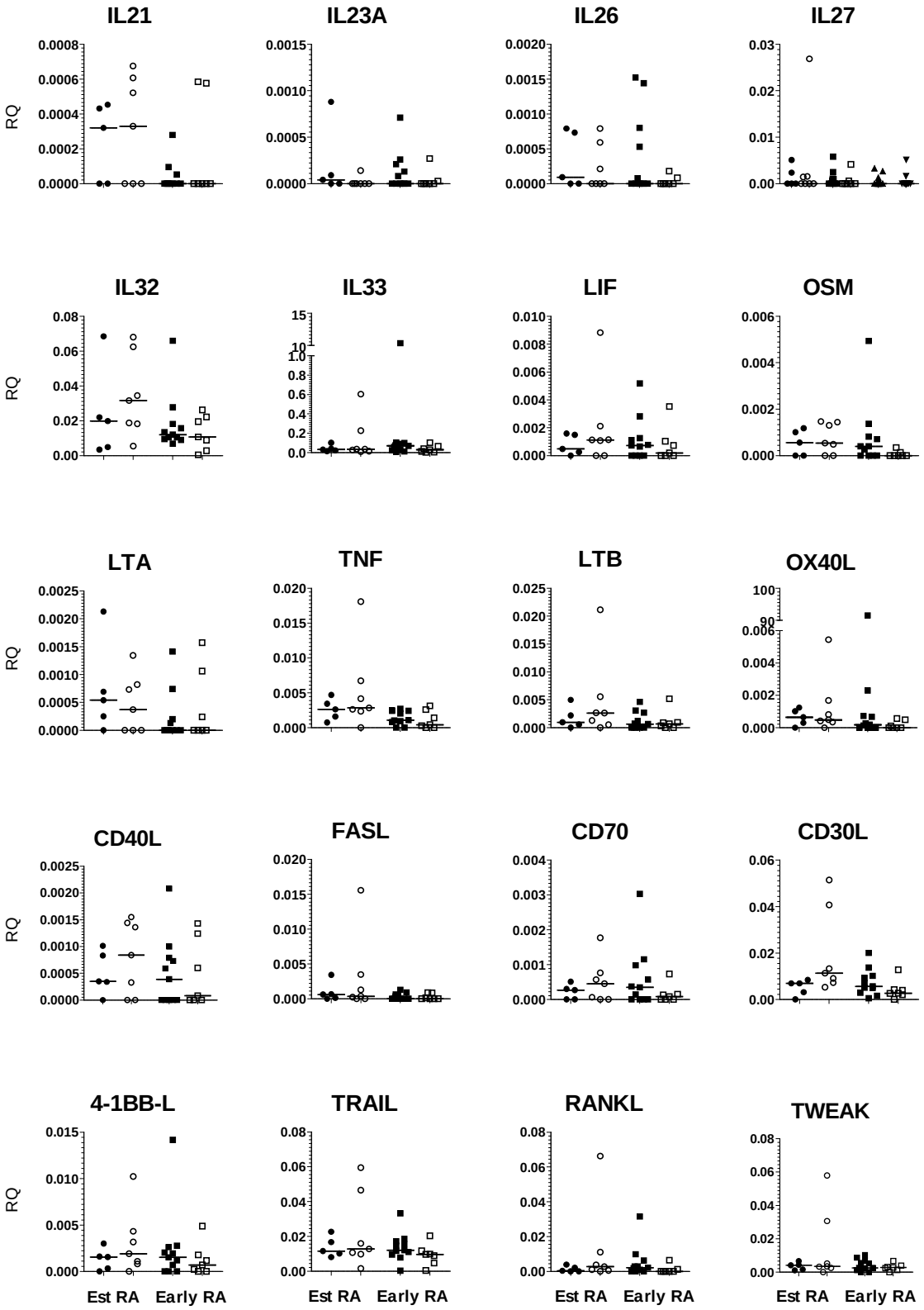

MST1
SPP1
EGF
FGF2
VEGFA
VEGFB
ADIPOQ
SFRP1
LEP
GHRL
NAMPT
C1QTNF3
RQ
Est RA
Early RA
Early Res
Control



Established RA (circles), early RA (squares), early resolving synovitis (upward triangles) and controls (downward triangles). Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons; p*<0.05.

8.3.2 Cytokine expression in ACPA-positive and ACPA-positive early RA and established RA

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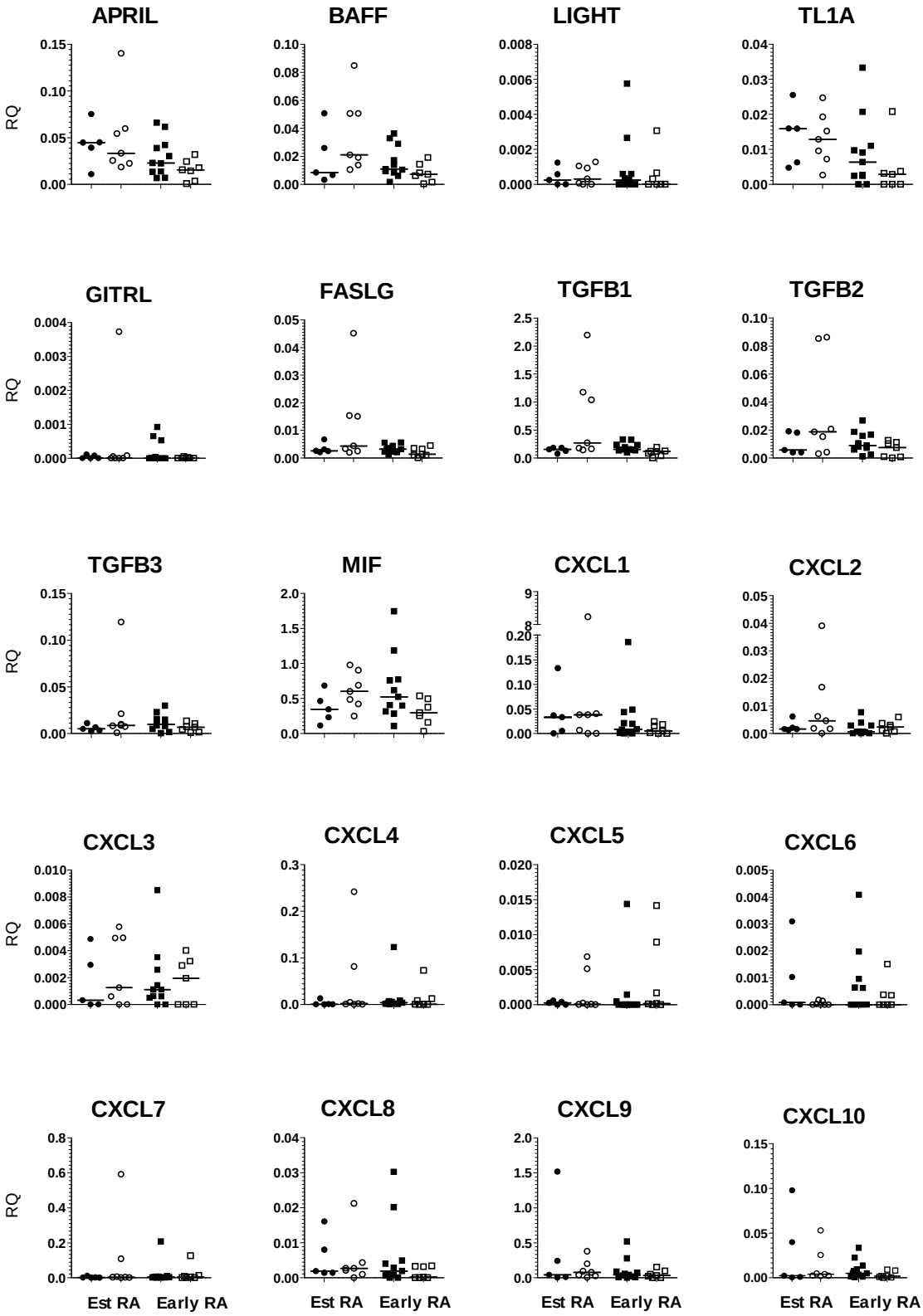
IL26
IL27
IL32
IL33
LIF
OSM
LTA
TNF
LTB
OX40L
CD40L
FASL
CD70
CD30L
4-1BB-L
TRAIL
RANKL
TWEAK
RQ
Est RA
Early RA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BAFF
LIGHT
TL1A
GITRL
FASLG
TGFB1
TGFB2
TGFB3
MIF
CXCL1
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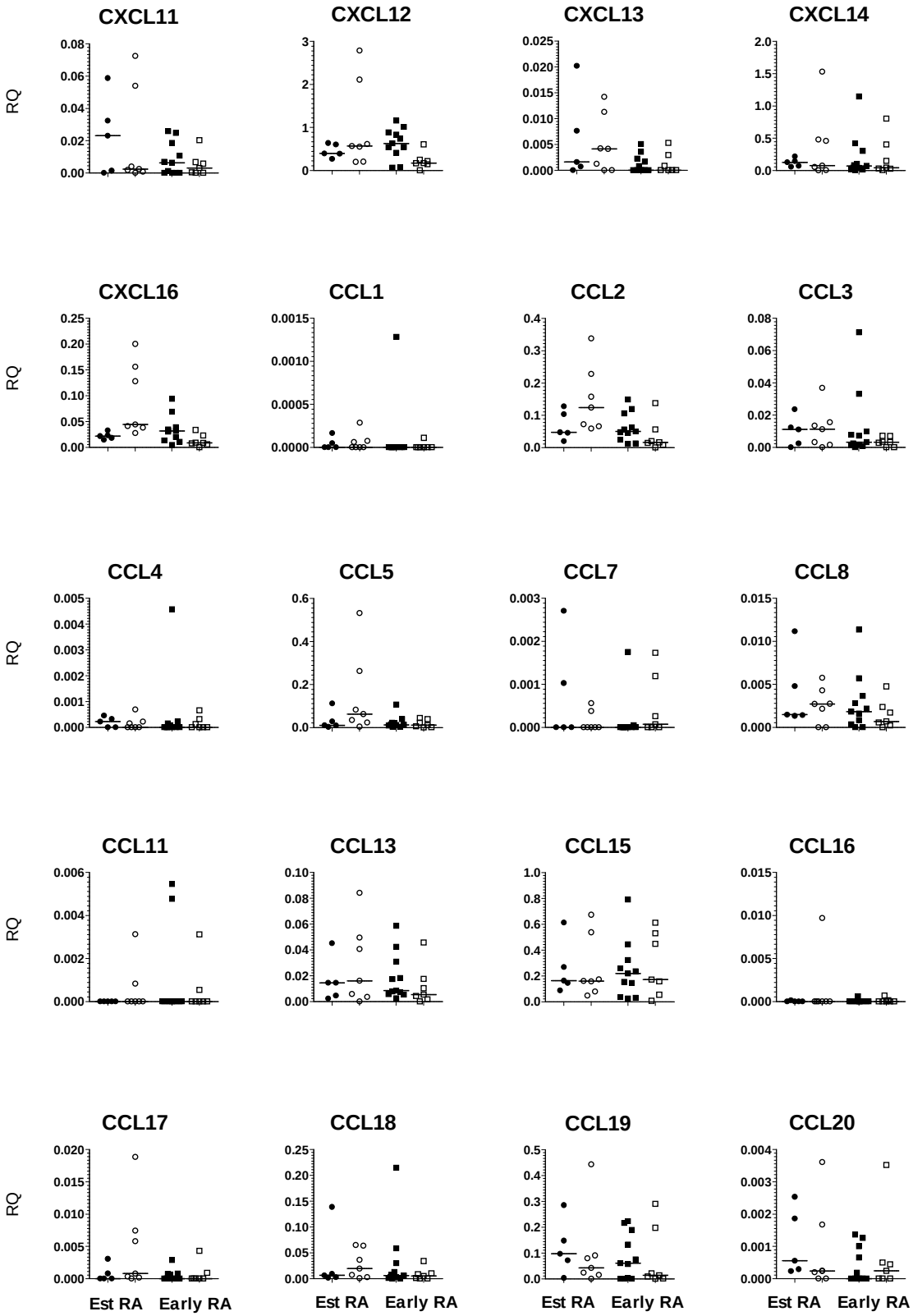
CXCL5
CXCL6
CXCL7
CXCL8
CXCL9
CXCL10
RQ
Est RA
Early RA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CXCL12
CXCL13
CXCL14
CXCL16
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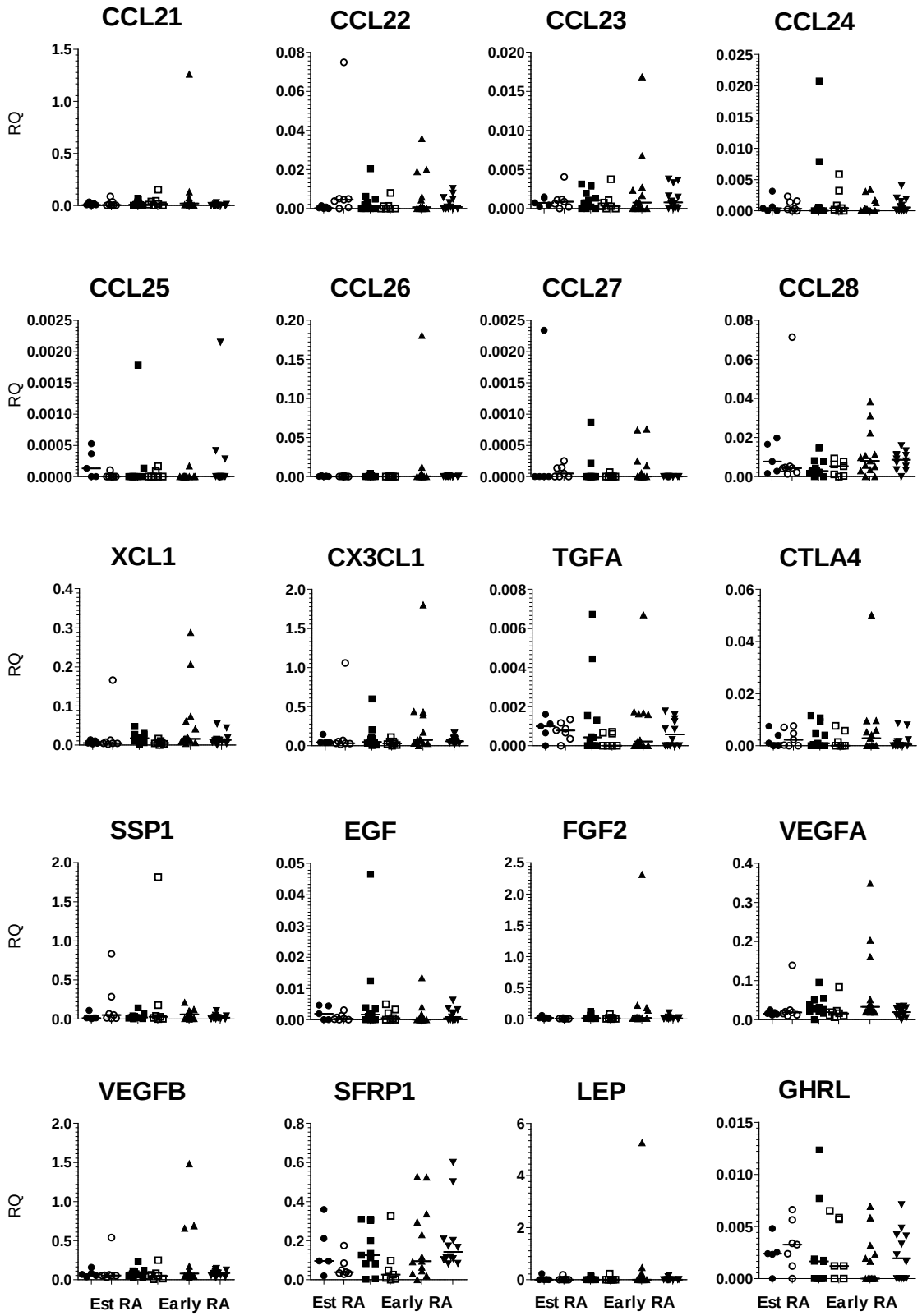
CCL2
CCL3
CCL4
CCL5
CCL7
CCL8
CCL11
CCL13
CCL15
CCL16
CCL17
CCL18
CCL19
CCL20
RQ
Est RA
Early RA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CCL22
CCL23
CCL24
CCL25
CCL26
CCL27
CCL28
XCL1
CX3CL1
TGFA
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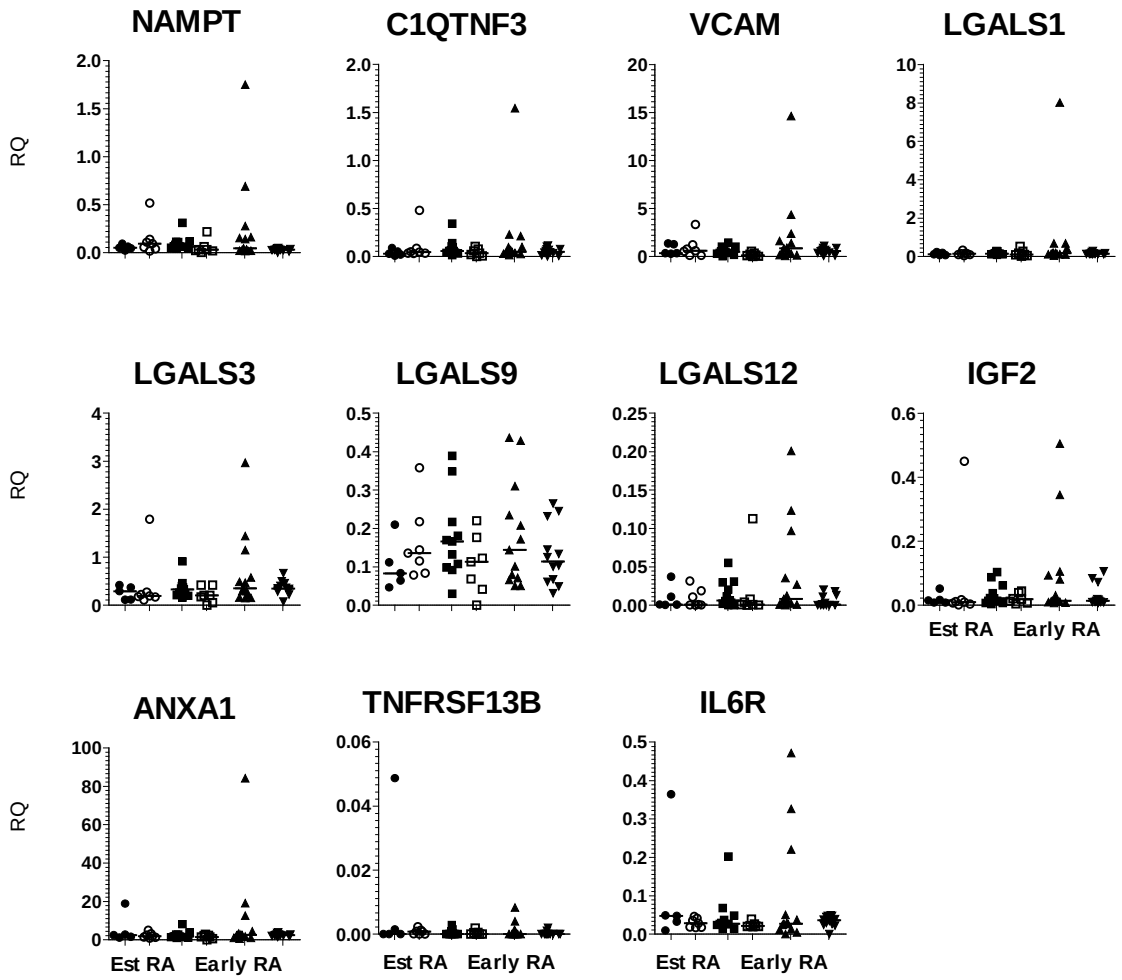
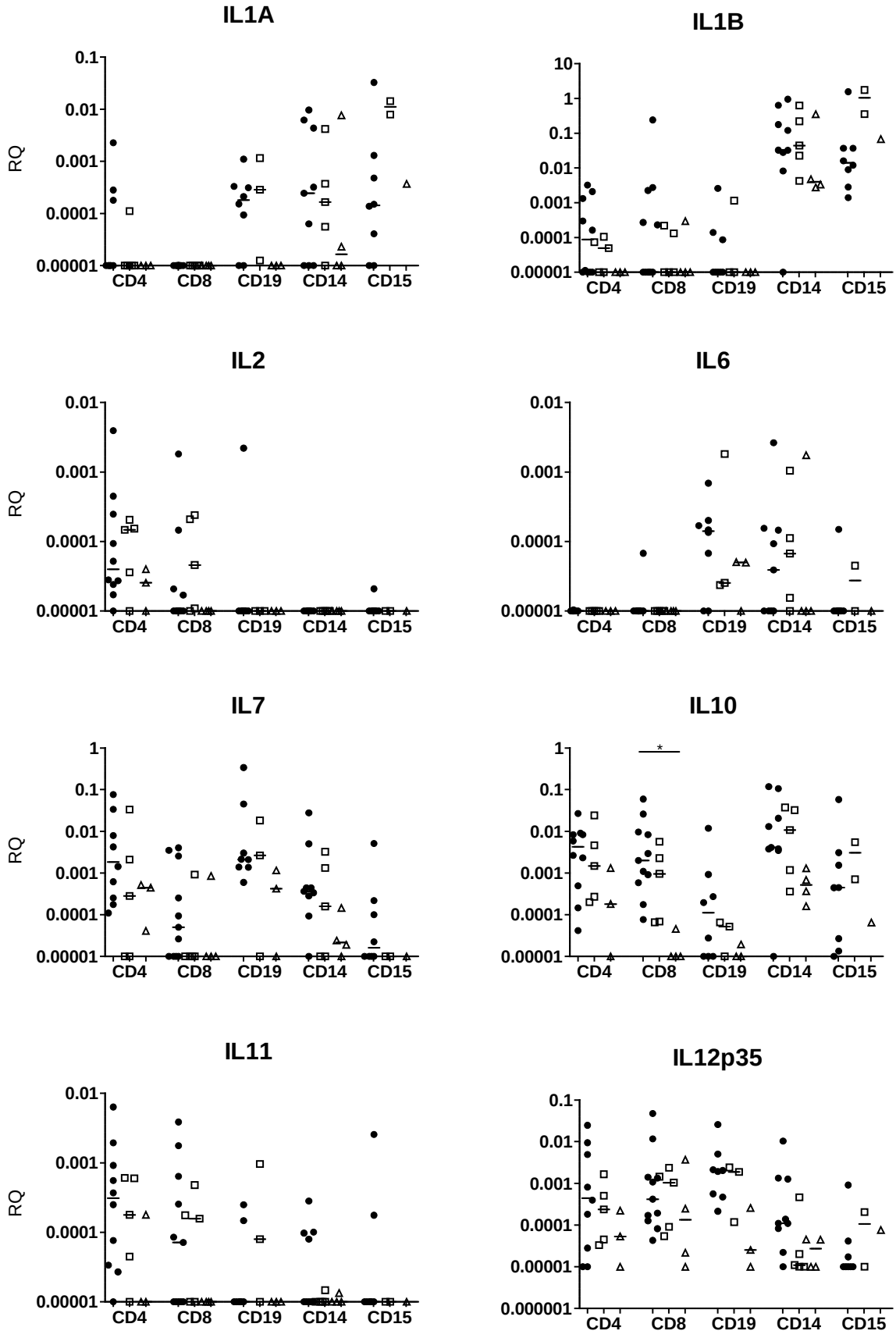
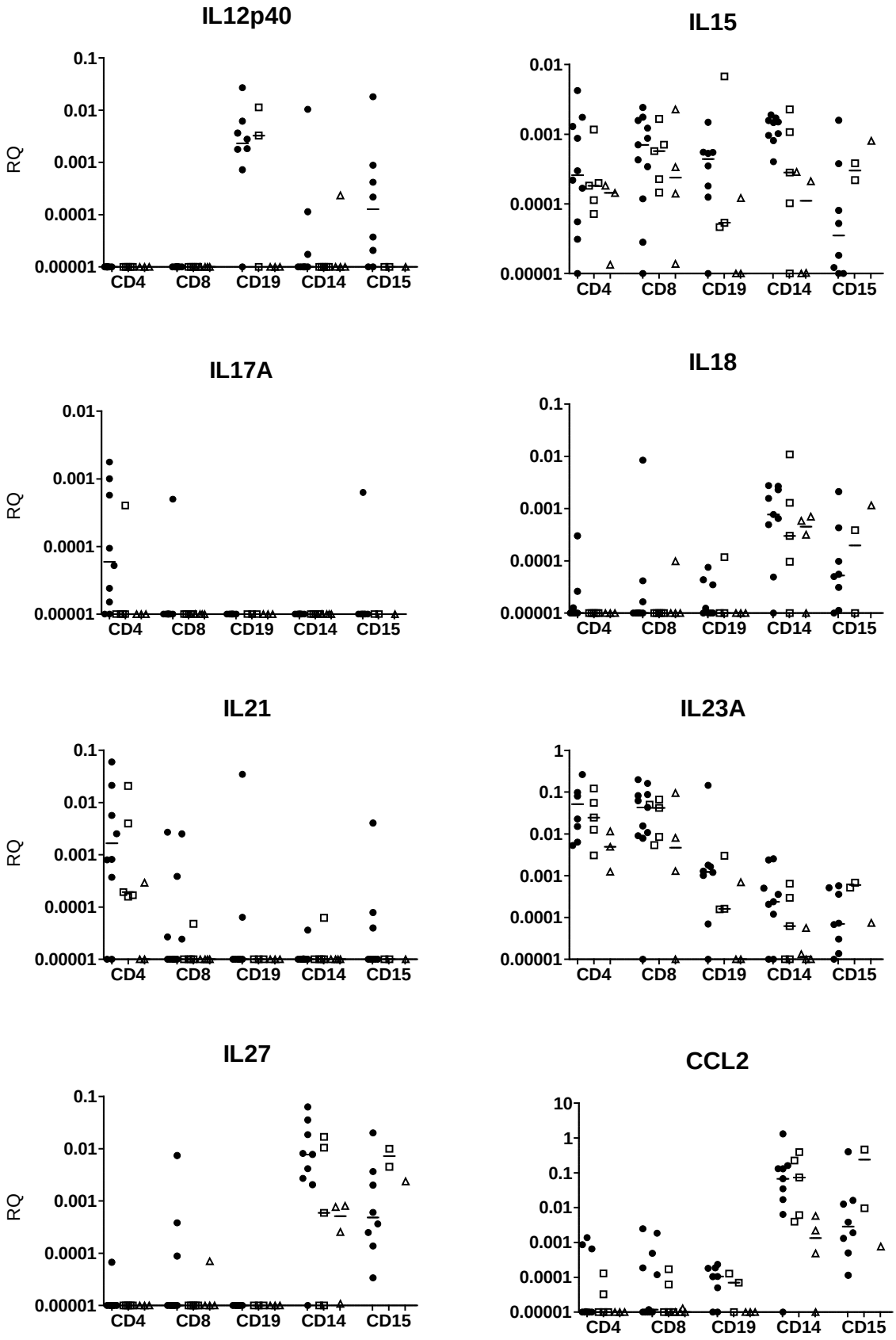
SSP1
EGF
FGF2
VEGFA
VEGFB
SFRP1
LEP
GHRL
RQ
Est RA
Early RA



ACPA-negative patients (closed symbols) and ACPA-positive patients (open symbols) with established RA (circles) or early RA (squares). Expression quantified relative to GAPDH. Mann-Whitney unpaired t-test; p*<0.05.

8.3.3 Cytokine mRNA expression in populations sorted from synovial fluid of patients with resolving synovitis, early RA and established RA

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IL27
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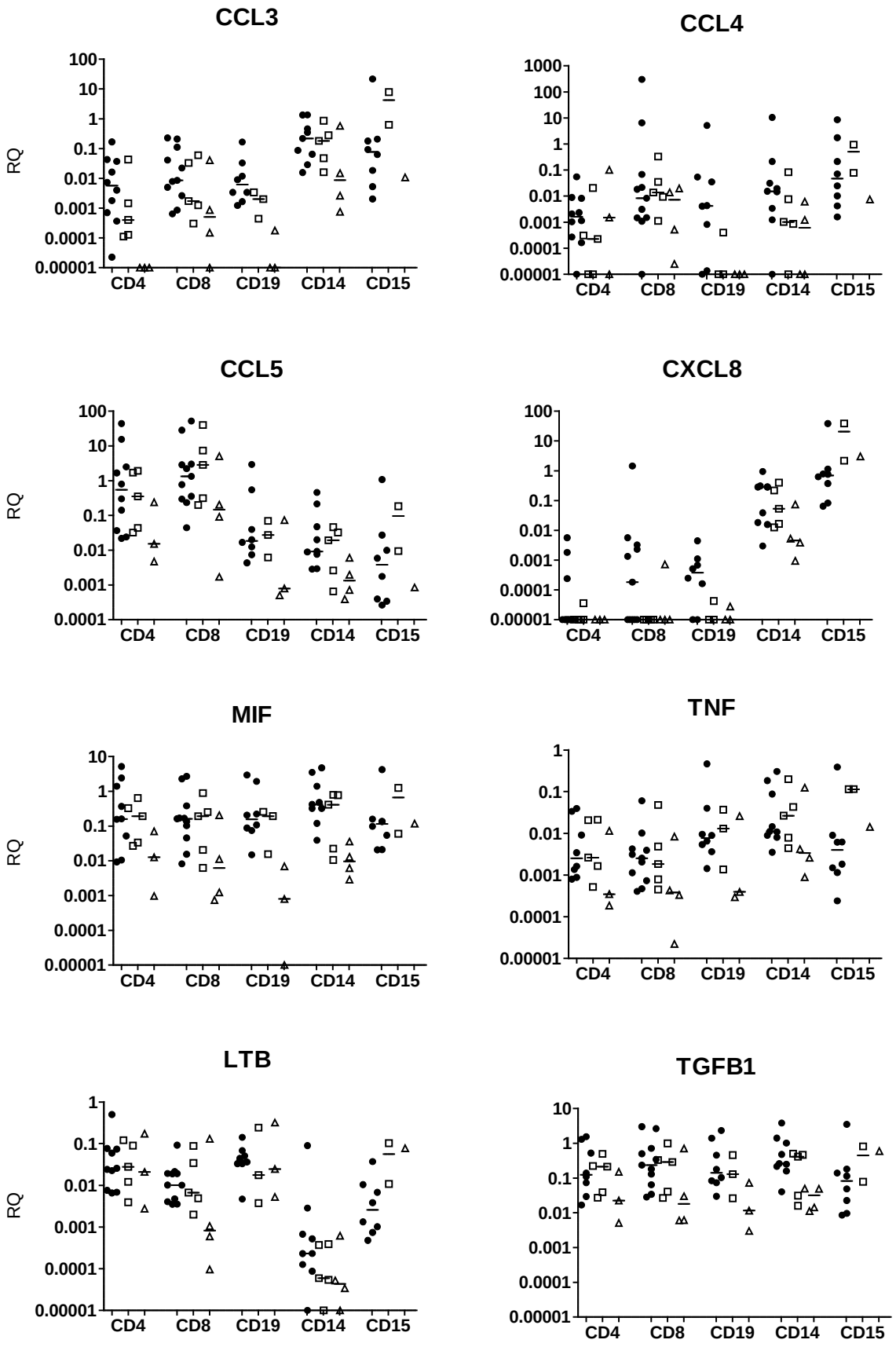
RQ
CD4
CD8
CD19
CD14
CD15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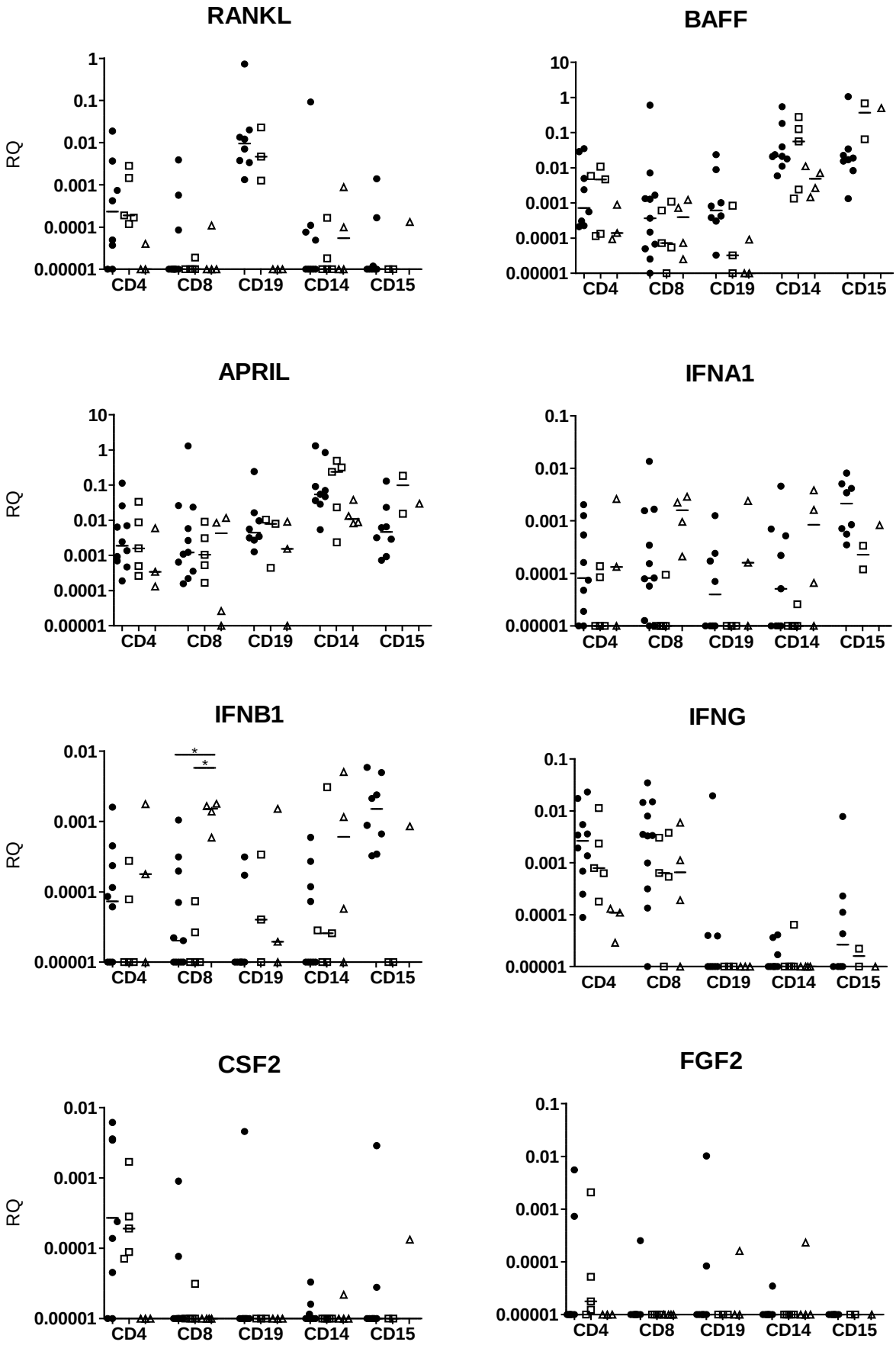
TGFB1
RQ
CD4
CD8
CD19
CD14
CD15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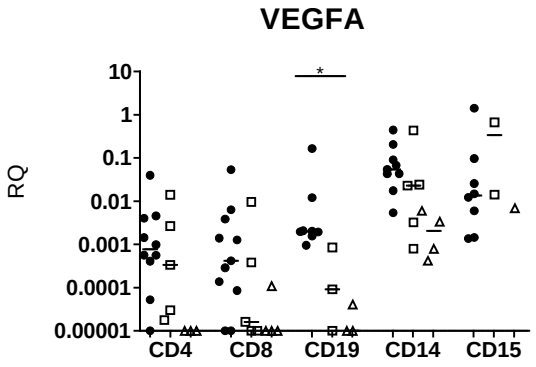
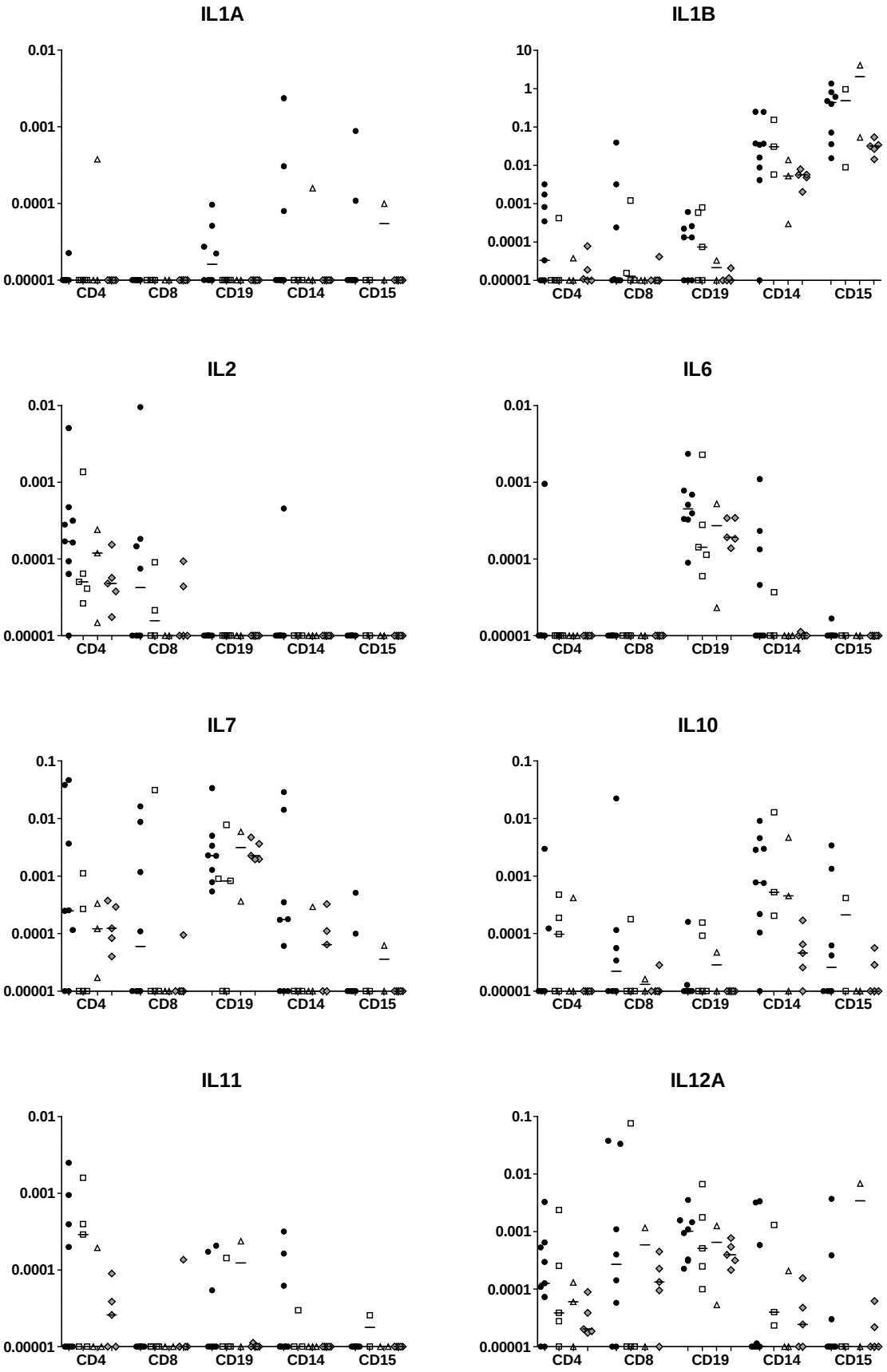
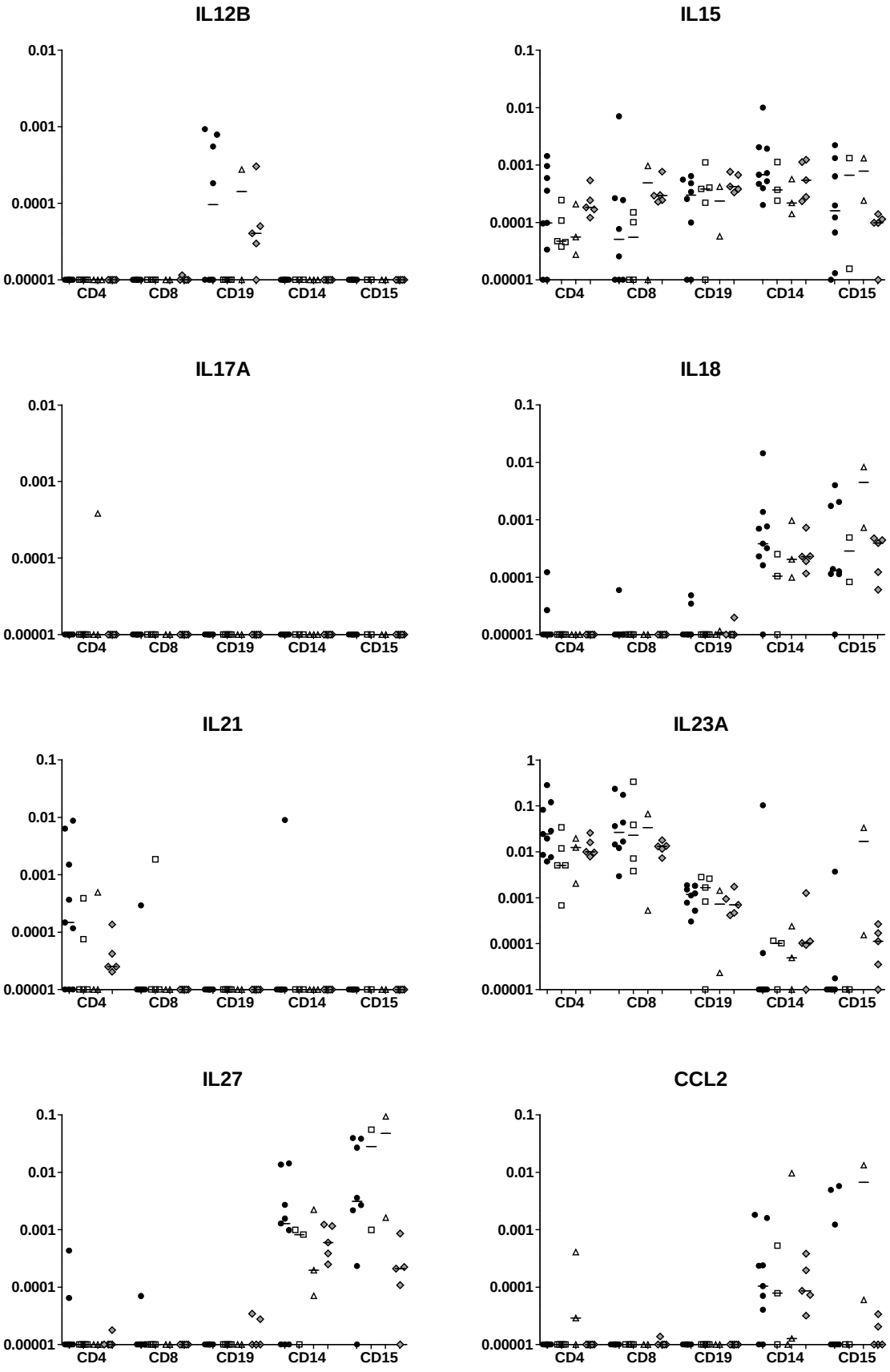
BAFF
APRIL
IFNA1
IFNB1
IFNG
CSF2
FGF2
RQ
1
0.1
0.01
0.001
0.0001
0.00001
10
CD4
CD8
CD19
CD14
CD15
*
*



Established RA (circles), early RA (squares), early resolving synovitis (upward triangles). CD3+CD45RO+CD4+ T cells (CD4), CD3+CD45RO+CD8+ T cells (CD8), CD19+ B cells (CD19), CD14+ macrophages (CD14) and CD15+ neutrophils (CD15) sorted from synovial fluid. Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons; *$p<0.01$.

8.3.4 Cytokine mRNA expression in peripheral blood cell populations of patients with resolving synovitis, early RA, established RA, and healthy controls.

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0.0001
0.00001
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CD8
CD19
CD14
CD15
IL15
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0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IL17A
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0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IL18
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IL21
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0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IL23A
1
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IL27
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0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
CCL2
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0.01
0.001
0.0001
0.00001
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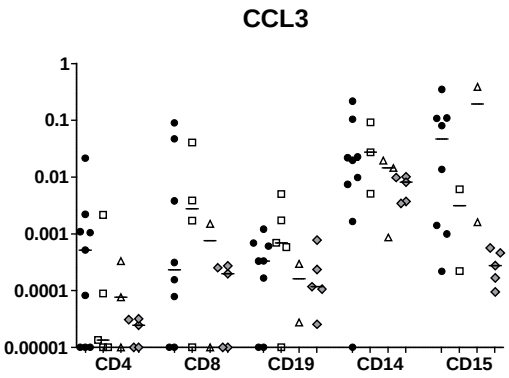
CD19
CD14
CD15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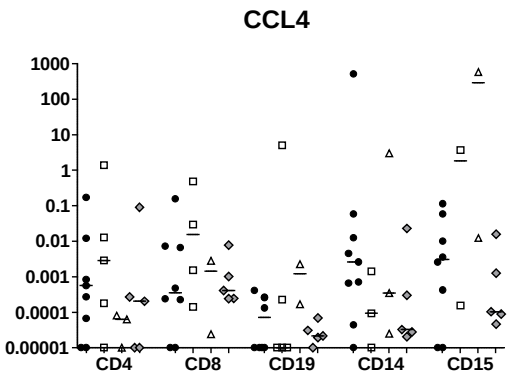
CD15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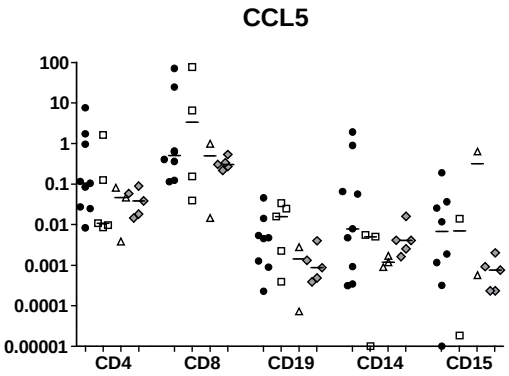
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0.001
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0.00001
CD4
CD8
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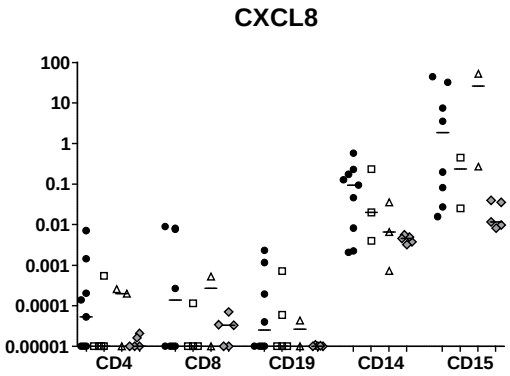
CD14
CD15


CXCL8
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10
1
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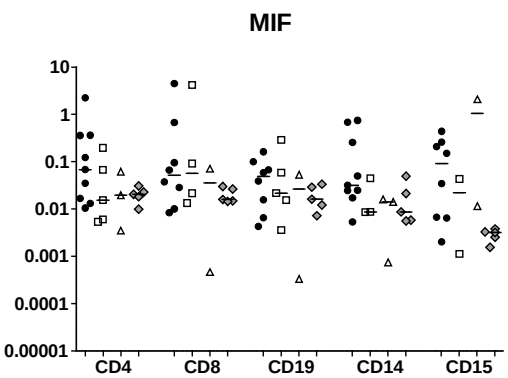
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15


MIF
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0.1
0.01
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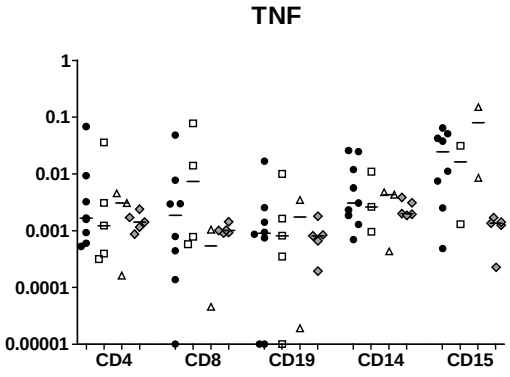
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15


TNF
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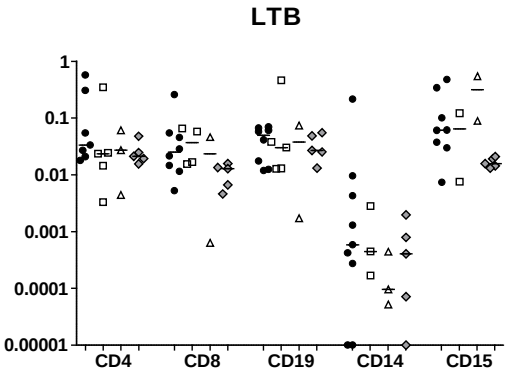
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15


LTB
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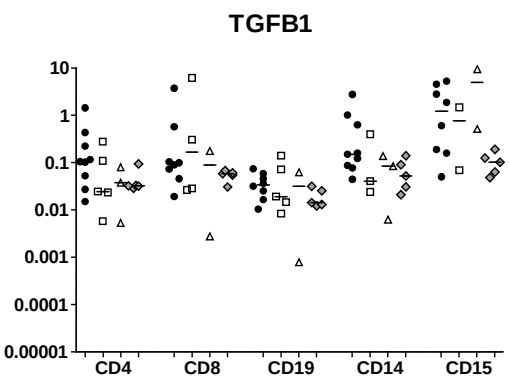
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15


TGFB1
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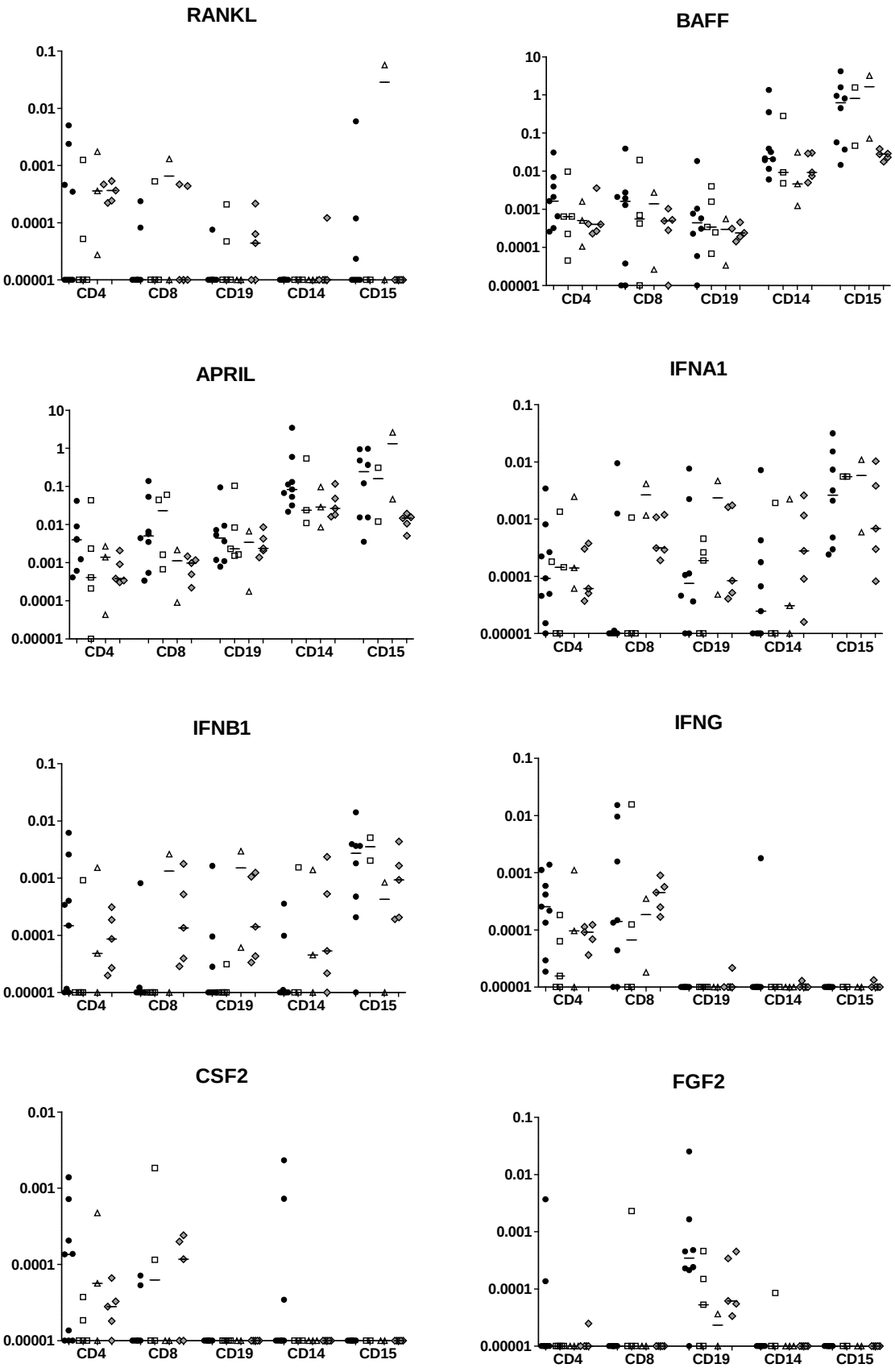
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15


RANKL
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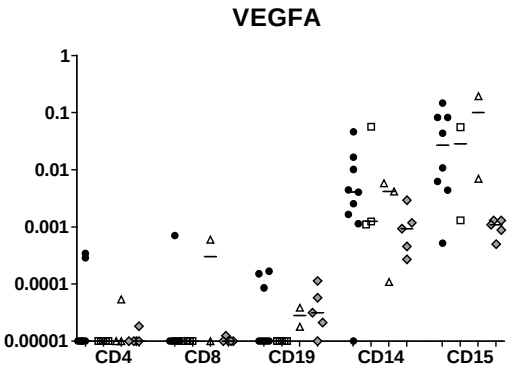
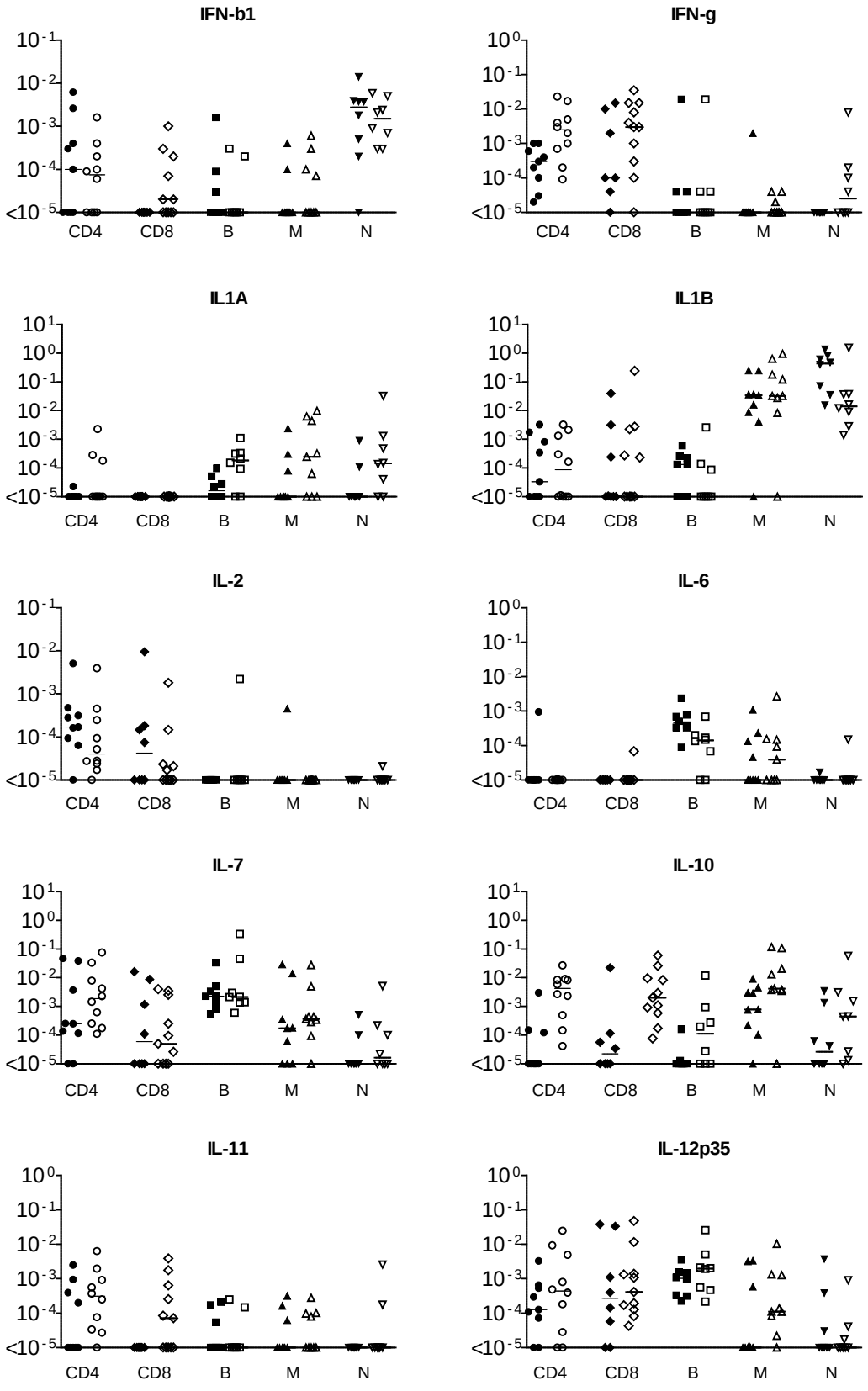
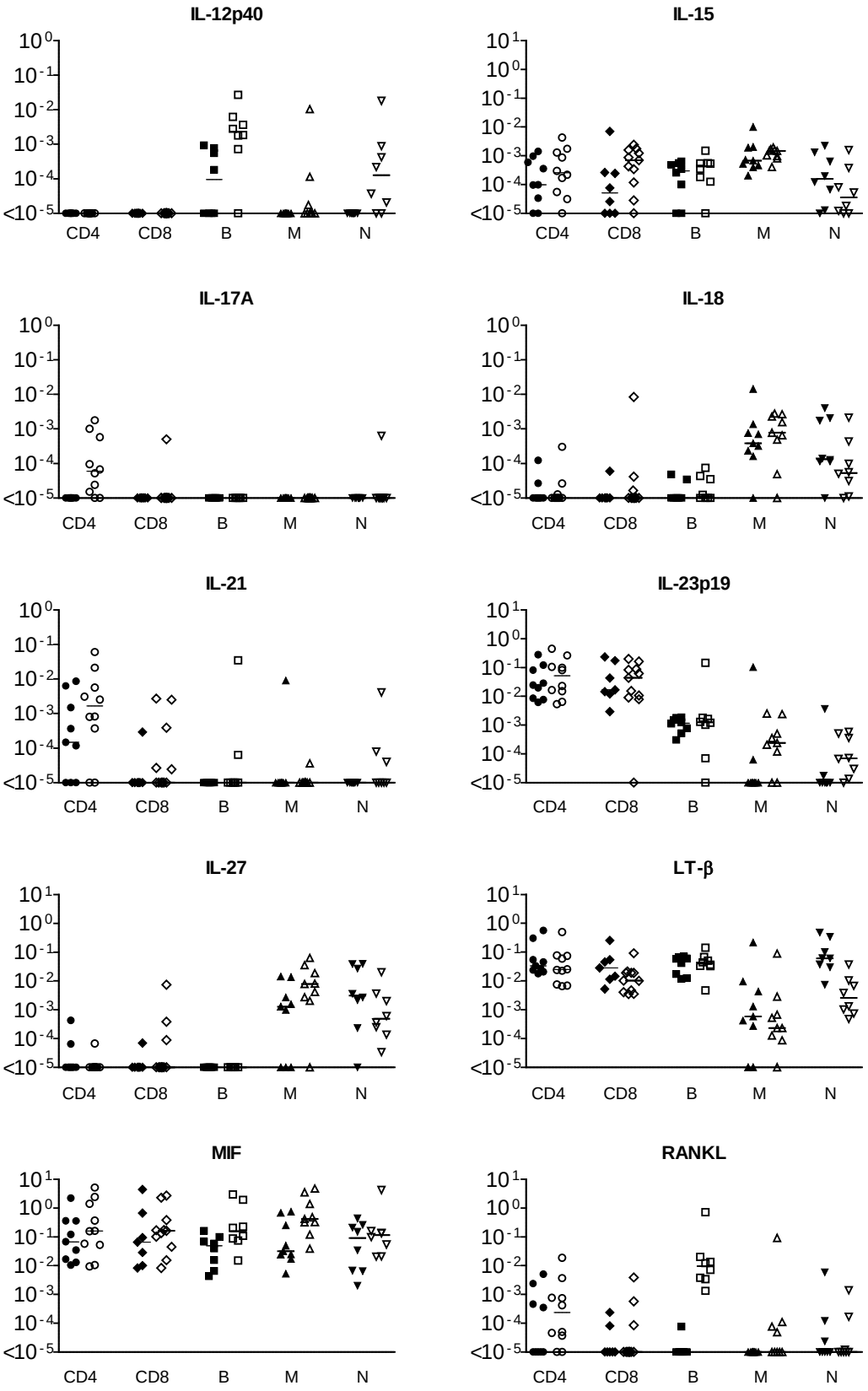
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
BAFF
10
1
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
APRIL
10
1
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IFNA1
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IFNB1
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
IFNG
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
CSF2
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15
FGF2
0.1
0.01
0.001
0.0001
0.00001
CD4
CD8
CD19
CD14
CD15

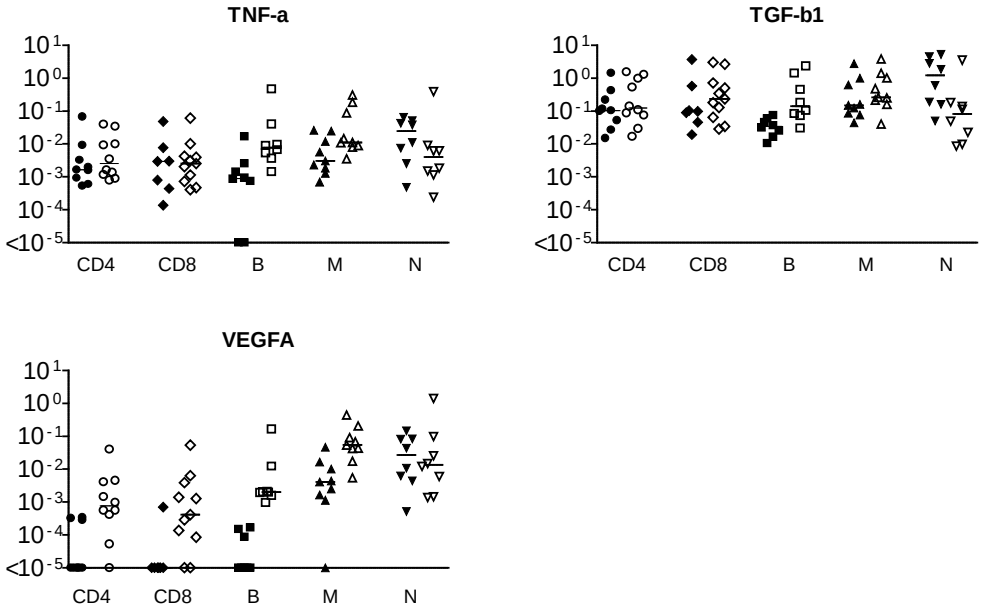


Established RA (circles), early RA (squares), early resolving synovitis (upward triangles) and healthy donors (diamonds) .CD3+CD45RO+CD4+ T cells, CD3+CD45RO+CD8+ T cells, CD19+ B cells, CD14+ macrophages and CD15+ neutrophils were sorted from peripheral blood . Expression was quantified relative to 18S. Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons; none of tests performed had a level of significance of $p<0.01$.

8.3.5 Cytokine mRNA expression in populations sorted from synovial fluid and peripheral blood of patients established RA.




IL-12p40
IL-15
IL-17A
IL-18
IL-21
IL-23p19
IL-27
LT-β
MIF
RANKL
CD4
CD8
B
M
N



CD3+CD45RO+CD4+ T cells (CD4), CD3+CD45RO+CD8+ T cells (CD8), CD19+ B cells (B), CD14+ macrophages (M), CD15+ neutrophils (N).

Table 8.8 Kruskal-Wallis and Dunn's post-test analysis of cytokine expression in synovial fluid cell populations. Cytokine expression was compared across CD4 T cells (CD4), CD8 T cells (CD8), B cells (CD19), macrophages (CD14) and neutrophils (CD15) using the Kruskal-Wallis test (K-W); the P value is shown. Paired analysis between cell populations was performed using the Dunn's post-test. ns = non-significant; *P<0.05, **P<0.001; ***p<0.0001.

	K-W	Dunn's post-test									
	P value	CD4 vs CD8	CD4 vs CD19	CD4 vs CD14	CD4 vs CD15	CD8 vs CD19	CD8 vs CD14	CD8 vs CD15	CD19 vs CD14	CD19 vs CD15	CD14 vs CD15
IL1A	0.0050	ns	ns	ns	ns	ns	*	*	ns	ns	ns
IL1B	0.0001	ns	ns	*	*	ns	*	*	**	**	ns
IL2	0.0003	ns	*	***	**	ns	ns	ns	ns	ns	ns
IL6	0.0018	ns	*	ns	ns	*	ns	ns	ns	ns	ns
IL7	0.0033	ns	ns	ns	*	ns	ns	ns	ns	*	ns
IL10	0.0212	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
IL11	0.0480	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL12A	0.0038	ns	ns	ns	ns	ns	ns	*	ns	**	ns
IL12B	<0.0001	ns	***	ns	*	***	ns	*	ns	ns	ns
IL15	0.0196	ns	ns	ns	ns	ns	ns	ns	ns	ns	**
IL17A	<0.0001	**	***	***	*	ns	ns	ns	ns	ns	ns
IL18	0.0010	ns	ns	**	ns	ns	**	ns	ns	ns	ns
IL21	0.0161	ns	ns	*	ns	ns	ns	ns	ns	ns	ns
IL23A	<0.0001	ns	ns	**	***	ns	*	**	ns	ns	ns
IL27	<0.0001	ns	ns	***	*	ns	**	ns	***	**	ns
CCL2	0.0002	ns	ns	**	ns	ns	**	ns	*	ns	ns
CCL3	0.0060	ns	ns	*	ns	ns	ns	ns	*	ns	ns
CCL4	0.1008	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CCL5	0.0002	ns	ns	ns	*	ns	*	***	ns	ns	ns
CXCL8	<0.0001	ns	ns	**	***	ns	ns	**	ns	**	ns
MIF	0.3684	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TNF	0.0227	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
LTB	0.0002	ns	ns	**	ns	ns	ns	ns	***	ns	ns
TGFb	0.2789	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
RANKL	0.0005	ns	ns	ns	ns	***	ns	ns	**	**	ns
BAFF	<0.0001	ns	ns	ns	**	ns	ns	**	**	***	ns
APRIL	0.0074	ns	ns	*	ns	ns	**	ns	ns	ns	ns
IFNa	0.0143	ns	ns	ns	ns	ns	ns	ns	ns	*	*
IFNg	0.0001	ns	*	**	ns	*	**	ns	ns	ns	ns
IFNb	0.0010	ns	ns	ns	*	ns	ns	**	ns	**	**
CSF2	0.0064	*	*	ns	ns	ns	ns	ns	ns	ns	ns
FGF2	0.5576	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
VEGFA	0.0002	ns	ns	**	ns	ns	***	ns	ns	ns	ns

8.4 Publications

Publications arising directly from this thesis or contributed to during this PhD programme:

Yeo L, Toellner KM, Salmon M, Filer A, Buckley CD, Raza K, Scheel-Toellner D. Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis. *Ann Rheum Dis.* 2011 Nov;70(11):2022-8

Hidalgo E, Essex SJ, Yeo L, Curnow SJ, Filer A, Cooper MS, Thomas AM, McGettrick HM, Salmon M, Buckley CD, Raza K, Scheel-Toellner D. The response of T cells to interleukin-6 is differentially regulated by the microenvironment of the rheumatoid synovial fluid and tissue. *Arthritis Rheum*. 2011 Nov;63(11):3284-93.

Boumans MJH, Thurlings RM, Yeo L, Scheel-Toellner D, Vos K, Gerlag D, Tak PP. Rituximab abrogates joint destruction in rheumatoid arthritis by inhibiting osteoclastogenesis. *Ann Rheum Dis.*2012 Jan;71(1):108-13