The role of joint-associated autoantigen-specific immune responses in rheumatoid arthritis

James Locke BSc (Hons)

Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Institute of Cellular Medicine

May 2012

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic inflammation at multiple joints. Because of the association of the condition with certain alleles of the *DRB1* gene which encodes the β-chains of human leukocyte antigen (HLA)-DR molecules, it has been postulated that auto-reactive $CD4^+$ T-cells - specific for joint-associated autoantigens presented in the context of these RA-associated HLA-DR molecules – are involved in the initiation or perpetuation of the disease process. In addition, citrullination of autoantigen-derived peptides may enhance their binding affinity to RA-associated HLA-DR molecules, implicating citrullinated autoantigens in RA pathogenesis. Since RA primarily affects the joints, proteins of articular origin and those expressed in the joints such as human cartilage glycoprotein 39 (HCgp39), type II collagen (CII), aggrecan, α-enolase, fibrinogen and vimentin are candidate autoantigens in RA. However, it has been difficult to consistently detect T-cell responses to these candidate RA autoantigens in RA patients. In this project, I hypothesised that T-cell responses to candidate autoantigens are heightened in RA patients compared to healthy subjects and that the T-cell responses of RA patients are pro-inflammatory while those of healthy subjects are not. I first optimised an experimental system for detecting autoantigen-specific T-cell responses and applied the protocol to measure proliferative and cytokine production responses of peripheral blood mononuclear cells (PBMC) from RA patients, healthy subjects and disease controls to unmodified and citrullinated whole protein and peptide forms of the candidate RA autoantigens mentioned above. Responses to the candidate autoantigens were readily detectable in RA patients, healthy subjects and disease controls. Furthermore, I found no evidence of increased immunogenicity of citrullinated peptides versus their unmodified counterparts. Thus the study failed to provide evidence that T-cell responses to either unmodified or citrullinated autoantigens are important in RA pathogenesis.

Acknowledgements

Firstly I would like to thank my supervisors Doctor Wan-Fai Ng and Professor John Isaacs for their sound advice, encouragement and many helpful suggestions throughout my PhD. I would also like to express my gratitude to Doctor Alexei von Delwig for sharing both his research and expertise. Many thanks also to Professors Jaap Van Laar and John Robinson, Julie Diboll and the rest of the staff and students in the musculoskeletal research group. I am very grateful to Doctors Amit Patel (former PhD student of Professor Drew Rowan) and Caroline Wilson (former PhD student of Doctor Andrew Knight) for their kind gifts of human type II collagen and bovine aggrecan respectively.

My thanks are also extended to Professor Patrick Venables of Imperial College for giving me the opportunity to collaborate on his group's research into immune responses to citrullinated enolase and for kindly providing samples of the relevant peptides. Many thanks also to Doctor Natalia Wegner, Professor Venables' former PhD student, for her assistance with setting up the experiments described in section 5.2.5.

I would also like to thank Arthritis Research UK for funding this PhD.

Last but not least I would like to express my gratitude to my parents for their support and understanding whilst writing this thesis.

List of abbreviations

ACPA = anti-citrullinated peptide antibodies

ADA = adalimumab

AICD = activation-induced cell death

AIRE = autoimmune regulator

AMLR = autologous mixed lymphocyte reaction

APECED = autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome

APC = allophycocyanin

AS = autologous serum

BSA = bovine serum albumin

CII = type II collagen

Ca = calcium

CCP = cyclic citrullinated peptide (in RA patient clinical details tables, refers to 'cyclic citrullinated peptide antibodies')

CD = cluster of differentiation

CFSE = carboxyfluorescein succinimidyl ester

CIA = collagen-induced arthritis

(Cit) = citrulline

Cit = citrullinated

COX = cyclooxygenase

cpm = counts per minute

CRP = C-reactive protein

CS = chondroitin sulphate

CTLA-4 = cytotoxic T lymphocyte-associated antigen 4

DeglyAgg = deglycosylated aggrecan

dH_2O = de-ionised water

DisC = disease controls

DMARDs = disease-modifying anti-rheumatic drugs

DMSO = dimethyl sulphoxide

DNA = deoxyribonucleic acid

DPBS = Dulbecco's phosphate buffered saline

ECL = electrochemiluminescence

EDTA = ethylenedinitrilotetraacetic acid

ELISA = enzyme-linked immunosorbent assay

ESR = erythrocyte sedimentation rate

ETR = etanercept

F = female

FACS = fluorescence-activated cell sorting

FBS = foetal bovine serum

Fc = fragment, crystallisable

FITC = fluorescein isothiocyanate

FoxP3 = fork-head box P3

FSC = forward scatter

GAD = glutamic acid decarboxylase

GlyAgg = glycosylated aggrecan

GM-CSF = granulocyte-macrophage colony-stimulating factor

GPI = glucose-6-phosphate isomerase

HABS = human AB serum

HACPG = human adult cartilage proteoglycan

HBSS = Hank's balanced salt solution

HCgp39 = human cartilage glycoprotein 39

HCQ = hydroxychloroquine

HFCPG = human foetal cartilage proteoglycan

HLA = human leukocyte antigen

H_2O_2 = hydrogen peroxide

HRP = horse radish peroxidase

HS = healthy subjects

H_2SO_4 = sulphuric acid

3HTdR = ^{3}H-thymidine

IBD = inflammatory bowel disease

IFN-γ = interferon gamma

IL = interleukin

IPEX = immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome

IQR = inter quartile range

JIA = juvenile idiopathic arthritis

KS = keratin sulphate

LAL = limulus amoebocyte lysate

LEF = leflunomide

LPS = lipopolysaccharide

M = male

MCTD = mixed connective tissue disease

Mg = magnesium

MHC = major histocompatibility complex

Mo-DC = monocyte-derived dendritic cells

MPL = monophosphoryl lipid A

MTX = methotrexate

NaCl = sodium chloride

$NaHCO_3$ = sodium bicarbonate

$NaH_2PO_4H_2O$ = sodium dihydrogen phosphate monohydrate

Na_2HPO_4 = disodium hydrogen phosphate

$Na_2HPO_42H_2O$ = disodium hydrogen phosphate dihydrate

NaN_3 = sodium azide

ND = no data

Neg = negative

NSAIDs = non-steroidal anti-inflammatory drugs

OA = osteoarthritis

OPD = *o*-phenylenediamine dihydrochloride

OPG = osteoprotegerin

PAD = peptidyl arginine deiminase

PBMC = peripheral blood mononuclear cells

PBS = phosphate buffered saline

PD-1 = programmed cell death 1

PE = phycoerythrin

PFA = paraformaldehyde

PG = proteoglycan

PHA = phytohaemagglutinin

PMB = polymyxin B

PPD = purified protein derivative (tuberculin)

PsA = psoriatic arthritis

PTPN22 = protein tyrosine phosphatase non-receptor 22

RA = rheumatoid arthritis

RANK = receptor activator of nuclear factor kappa-B

RANKL = receptor activator of nuclear factor kappa-B ligand

RF = rheumatoid factor

RTX = rituximab

SAPHO = synovitis, acne, pustulosis, hyperostosis, osteitis

SCID = severe combined immunodeficiency

SE = shared epitope

SEM = standard error of mean

SFMC = synovial fluid mononuclear cells

SI = stimulation index

SLE = systemic lupus erythematosus

SSC = side scatter

SSZ = sulfasalazine

TCAR = T-cell antigen receptor

TNF-α = tumour necrosis factor alpha

Table of contents

List of tables

Chapter 1

Chapter 2

Chapter 4

Chapter 5

Chapter 6

List of figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 1. Introduction

1.1. Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease characterised by chronic inflammation of multiple joints and affecting around 1% of the western world's adult population (Firestein, 2003). Although RA can develop at any time, disease onset most commonly occurs between thirty and fifty years of age and the condition is three times more prevalent in females than males (Isaacs & Moreland, 2011). The disease predominantly manifests itself in the diarthroidal or synovial joints of the hands, wrists and feet, with joints often being affected in a symmetrical fashion. The inflammation, which occurs in the synovium of an affected joint (hence synovitis), involves the infiltration of various immune cells and causes symptoms of pain, swelling and stiffness which may also be accompanied by feelings of malaise, depression and fatigue (Isaacs & Moreland, 2011). Production of inflammatory mediators such as TNF-α is thought to have a key role in disease pathogenesis. Persistent synovitis results in the destruction of articular cartilage and the underlying bone which in turn can lead to deformation and the eventual loss of function of the affected joints (Isaacs & Moreland, 2011). Extra-articular or systemic manifestations of the disease affecting the heart, lungs, eyes, skin and blood can also occur in RA (Isaacs & Moreland, 2011).

1.1.1. Aetiology of rheumatoid arthritis

While the aetiology of RA remains unclear, certain genetic factors are well known to contribute to the condition. For example, the associations with RA of certain polymorphisms within the *DRB1*, *PTPN22*, *CTLA4* and *PADI4* genes are well established and other loci involved in immune responses such as the IL-1 genes and the TNF-α gene have also been implicated. Thus, while a number of genes seem to be involved in RA susceptibility, those predisposing to RA can differ between patients, and so RA is said to be a polygenic and genetically heterogeneous disease. However, various environmental factors are thought to compliment the genetic contribution to the aetiology of RA by acting as triggers for disease onset in the genetically predisposed. Immune responses to a transient infection could trigger chronic inflammation if they cross-react with autologous structures therefore infectious agents are possible candidates for environmental triggers. Alternatively, because RA is three times more prevalent in females than males, certain sex hormones may be involved in triggering disease onset. In addition, smoking increases the risk of developing seropositive RA in

individuals with disease-associated *DRB1* alleles (Padyukov *et al*, 2004; Klareskog *et al*, 2006). Indeed, smoking is a proven environmental factor that increases the risk of developing anti-CCP-positive RA (Kallberg *et al*, 2007).

1.1.2. Treatment of rheumatoid arthritis

As yet there is no cure for RA and once severe bone damage has occurred it is irreversible. Therefore, the current aim of RA treatment is to control the pathogenic inflammation so that symptoms can be alleviated and damage to articular cartilage and bone can be minimised. Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate the symptoms of pain, swelling and stiffness by reducing inflammation and can thus restore function to affected joints in the short term. NSAIDs work by inhibiting the enzyme cyclooxygenase (COX) which exists in two isoforms and is responsible for the production of prostaglandins which are involved in inflammation. Non-selective NSAIDs such as aspirin, diclofenac, ibuprofen, ketoprofen, naproxen and indometacin target both COX-1 and COX-2 and often cause gastrointestinal side-effects such as heartburn, indigestion and stomach upsets. In contrast, COX-2-specific NSAIDs such as celecoxib and etoricoxib provide the same therapeutic benefits as non-selective NSAIDs but have fewer side-effects on the gastrointestinal system. However, NSAIDs can cause other side-effects such as rashes, headaches, wheeziness and fluid retention, and their use may increase the likelihood of strokes and heart attacks. NSAIDs exert their therapeutic effect within a few hours and single doses can remain effective for anything from a few hours to a whole day but they ultimately have no long-term effect on the disease. Corticosteroids, administered intra-articularly, intra-venously, intra-muscularly or orally, act very quickly to potently suppress inflammation and thereby provide relief of pain and swelling in the joint and they also improve fatigue symptoms. While corticosteroids can have quite severe side-effects, there is evidence to suggest that they can reduce the rate of progression of RA if they are administered early on in the disease (Kirwan, 1995).

Disease-modifying anti-rheumatic drugs (DMARDs), as their name suggests, treat the disease and not the symptoms, although they do alleviate pain, swelling and stiffness over a protracted period of time by slowing the progress of the disease. Intramuscular gold injections, hydroxychloroquine (HCQ), sulfasalazine (SSZ) and the immunosuppressants azathioprine, cyclosporine, leflunomide (LEF) and methotrexate (MTX) are fairly well established in their use and are known as conventional DMARDs.

More recently there has been a lot of interest in newer types of DMARDs known as biological therapies that target specific proteins and cells involved in inflammation and joint damage. For example, adalimumab (ADA), etanercept (ETA) and infliximab target TNF-α, a pro-inflammatory cytokine involved in RA pathogenesis, while tocilizumab targets the IL-6 receptor, thus neutralising the action of IL-6, another pro-inflammatory cytokine involved in disease pathogenesis. Abatacept and rituximab (RTX) target T-cells and B-cells respectively, which both have roles in the pathogenesis of RA. All DMARDs take a long time to exert their beneficial effects therefore they do not provide immediate relief from the symptoms of RA. Therefore they are often used in combination with NSAIDs and/or corticosteroids and indeed combinations of DMARDs are also prescribed when single drugs can not effectively control the disease. In the past, RA was treated with NSAIDs, rest and corticosteroid injections and DMARDs were often used as a last resort. This is no longer the case as recent research has shown that administration of DMARDs in the early stages of disease can have a much more drastic effect on slowing progression of the disease. However, the search for novel targets of RA treatments continues.

1.2. Synovial joints

Joints are the structures formed where the extremities of two bones come together and they can be cartilaginous, fibrous or synovial in nature. Synovial or diarthroidal joints are the most common joints in the body, and also the most moveable. The two bones involved in a synovial joint are surrounded by a fibrous capsule of tough ligaments which provides support to the structure and forms the synovial cavity that distinguishes synovial joints from cartilaginous and fibrous joints. The joining ends of each of the bones are covered with articular hyaline cartilage, and the interior of the joint capsule is lined by a synovial membrane or synovium which also covers any bone within the capsule that is not covered with cartilage. The synovium secretes a viscous, lubricating and nutrient-rich liquid known as synovial fluid into the joint cavity. The synovial fluid provides nutrients to the articular cartilage and keeps the surfaces lubricated with its hyaluronic acid content so they can glide over each other.

1.2.1. Articular cartilage

Articular cartilage is an avascular but well-hydrated structure of chondrocytes embedded in a matrix of collagen, proteoglycans and other matrix proteins such as fibronectin which are all secreted by the chondrocytes themselves. However, there is

actually only a low density of chondrocytes in articular cartilage and it is the collagens and proteoglycans that give the structure its tensile strength and compressibility. Type II collagen accounts for around 90% of the collagen present in articular cartilage and, like all collagens, it displays a triple helical structure which lends the protein great tensile strength. The most predominant proteoglycan component of articular cartilage is aggrecan and by virtue of its anionic nature it is very effective at trapping water so that the aggrecan can be subjected to deformation and compressive stress without being damaged. Interestingly, the surface layers of articular cartilage contain substantially more collagen than proteoglycan but proteoglycan content increases the nearer the cartilage is to the bone. Thus the surface layers of articular cartilage have a high degree of tensile strength while the deeper layers have more ability to be deformed and compressed. Because articular cartilage is avascular, it derives its nutrients from the synovial fluid.

1.2.2. The synovium in health and disease

The synovial membrane is composed of both a lining layer, known as the intima, which borders the synovial cavity, and an underlying, sub-lining layer known as the sub-intima, which contacts the walls of the joint capsule. The intima is a cellular structure composed of macrophage-like, bone marrow-derived type A synoviocytes and fibroblast-like type B synoviocytes which are arranged in loosely-packed layers. The synoviocytes within the intima are held together by a matrix composed predominantly of proteoglycans and glycosaminoglycans such as hyaluronic acid. The sub-intima is a body of connective tissue which contains blood vessels, lymphatics and nerve endings which are surrounded by a matrix composed of lipids, collagen fibrils and fibrous tissue. In a healthy synovial joint, the intima consists of only one or two layers of synoviocytes and the sub-intima is largely devoid of cells.

However, RA causes substantial changes in the morphology of the synovium. For example, the sub-intima becomes riddled with networks of new blood vessels which facilitate the recruitment of T-cells, macrophages, dendritic cells and B-cells. Within the intima, an increased influx of type A synoviocytes from the bone marrow and the *in-situ* proliferation of type B synoviocytes cause this layer to dramatically increase in thickness (Isaacs & Moreland, 2011). This causes increase in cell numbers (hyperplasia) and there is also an increase in cell size (hypertrophy). Furthermore, the cells within the RA synovium become activated and produce matrix metalloproteinases such as

collagenase and aggrecanase and other tissue-degrading enzymes which accumulate in SF and attack exposed articular cartilage. Activated macrophages produce TNF-α and IL-1β, and stimulate chondrocytes to produce proteolytic enzymes that degrade cartilage matrix. As the disease progresses, the hyperplastic synovium may evolve into a migratory pannus which encroaches on the articular cartilage (Isaacs & Moreland, 2011). Activated macrophages and fibroblasts within the pannus produce matrix metalloproteinases such as collagenase and aggrecanase which destroy articular cartilage. In addition the volume of synovial fluid within affected joints increases and ultimately articular bone is damaged. Bone erosion in RA is thought to be caused by osteoclasts, and the generation of such cells (osteoclastogenesis) and their subsequent activation requires the cytokine known as receptor activator of nuclear factor kappa-B ligand (RANKL) (Boyle *et al*, 2003). RANKL exerts its osteoclastogenic and osteoclast-activating effects through binding with its receptor, receptor activator of nuclear factor kappa-B (RANK), which is expressed on myeloid precursor cells and osteoclasts (Li, J. *et al*, 2000). Osteoprotegerin (OPG) is a soluble receptor of RANKL which regulates osteoclast generation and function - by preventing RANKL from interacting with RANK - and thus osteoclast-mediated bone erosion (Simonet *et al*, 1997). Interestingly, RANKL is expressed by synovial fibroblasts and infiltrating T-cells in the rheumatoid joint where sites of bone erosion are associated with higher levels of RANKL expression as opposed to OPG expression (Pettit *et al*, 2006). Synovial B-cells have recently been identified as a major cellular source of RANKL in RA (Yeo *et al*, 2011).

1.3. The role of T-cells in rheumatoid arthritis

As mentioned, the pathogenesis of RA involves infiltrations of lymphocytes into the synovial membranes of affected joints. It has been shown that 70-85% of the lymphocytes infiltrating the synovial membranes of RA patients are T-cells (van Boxel & Paget, 1975). The majority of these infiltrating T-cells express markers of activation such as CD69, which is involved in activation of macrophages, HLA-DR and VLA-1 yet only a small proportion express the IL-2 receptor CD25 and indeed levels of IL-2 in RA synovium are low (Iannone *et al*, 1994). It was initially thought that IFN-γ produced by these T-cells was responsible for the activation of synovial monocytes, many of which have an activated phenotype in the synovium (Firestein & Zvaifler, 1987). Indeed, macrophage-derived pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α are highly abundant in the RA synovium, especially at the cartilage-pannus

junction where joint damage occurs (Chu *et al*, 1991 and Chu *et al*, 1992). However, the levels of IFN-γ in the synovial fluid and tissue of RA patients are very low therefore activation of synovial monocytes may be independent of IFN-γ (Firestein & Zvaifler, 1987). In addition, the low levels of IL-2 present in the rheumatoid synovium created some speculation as to how the synovial T-cells had activated phenotype (Iannone *et al*, 1994). This initially suggested that T-cells are not very important in pathogenesis. IL-15 produced by macrophages has been found to be chemoattractant for T-cells and it also induces T-cell proliferation acting like IL-2 (McInnes *et al*, 1996). Furthermore, it has been shown that T-cells activated by IL-15 can induce macrophages to produce substantial levels of TNF-α and activate synoviocytes and osteoclast formation, all in a cell contact dependant manner that involves CD69, LFA-1 and ICAM-1 (McInnes *et al*, 1997). Thus T-cells appear important for driving pathogenesis. Indeed, the paucity of classical T-cell cytokines such as IL-2 and IFN-γ in the RA synovium can be explained by the predominant role of TH17 cells in RA pathogenesis (see section 1.3.3).

1.3.1. T-cell-implicating genetic factors in rheumatoid arthritis

A well established susceptibility locus in RA is *DRB1*, and several alleles of this gene are associated with the disease, namely *DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0409, *0410, *1001, *1402* and **1406* (Weyand *et al*, 1995 and Saruhan-Direskeneli, 1998). *DRB1* encodes the β-chains of HLA-DR MHC class II molecules (Svendsen *et al*, 2004) which are each composed of a polymorphic β-chain and an invariant α-chain, encoded by the gene *DRA1*. Thus RA-associated *DRB1* allomorphs pair with invariant HLA-DR α-chains to produce complete HLA-DR molecules of the DR1, DR4, DR10 and DR14 subtypes (Gorman *et al*, 2004). The β-chains encoded by the RA-associated *DRB1* alleles all contain a similar amino acid sequence of either QKRAA (**0401*), QRRAA (**0101, *0102, *0404, *0405, *0408*) or RRRAA (**1001*) at positions 70-74 of the third hypervariable region (Saruhan-Direskeneli, 1998). Analysis of this so-called 'shared epitope' (SE) has shown that the amino acids at positions 70 and 71 have positively-charged side chains (Hammer *et al*, 1995). *DRB1* alleles such as *DRB1*0402* which encode β-chains with negative or neutral charges at positions 70 and 71 have been reported to protect against RA therefore the presence of positively-charged amino acids at positions 70 and 71 of HLA-DR β-chains may predispose to RA (Reviron *et al*, 2001). Interestingly, positions 67-74 of the β-chain border the peptide-binding groove of the resulting HLA-DR molecule (Svendsen *et al*, 2004). Therefore, the charges of the side chains on these amino acids will influence

which peptides can bind the MHC molecule and indeed which T-cells the MHC molecule can present peptides to (Hammer *et al*, 1995). The association of RA with certain HLA-DR molecules suggests that MHC class II-restricted $CD4^+$ T-cells are important in the pathogenesis of the disease (Svendsen *et al*, 2004). One hypothesis, termed the 'shared epitope' hypothesis, is that disease-associated HLA-DR molecules bind peptides of articular autoantigens and present them to antigen-specific $CD4^+$ T-cells which in turn initiate or perpetuate the inflammatory process that characterises RA (Gregersen *et al*, 1987). Indeed, it has been shown recently that SE-positive MHC class II molecules bind certain citrullinated peptides with high affinity (Hill *et al*, 2003). Alternatively, SE-positive HLA-DR molecules may select a repertoire of T-cells that include ones specific for self antigens (Kotzin *et al*, 2000).

Further evidence implicating T-cells in the pathogenesis of RA is the association of the disease with the 1859 C→T polymorphism of the protein tyrosine phosphatase non-receptor 22 gene, *PTPN22* (Bowes & Barton, 2008), and the +49G polymorphism of the cytotoxic T lymphocyte-associated antigen-4 gene, *CTLA-4* (Lei *et al*, 2005). *PTPN22* encodes a 110kD, lymphoid-specific intracellular protein tyrosine phosphatase (PTP) known as Lyp (Orozco *et al*, 2005). The mouse homologue of Lyp, PEP, is known to function as a down-regulator of T-cell receptor signalling when associated with Csk (Begovich *et al*, 2004). Several studies have shown that the *PTPN22* 1858T allele which arises from the 1859 C→T polymorphism is significantly more prevalent in RA patients than in healthy subjects (Begovich *et al*, 2004; Orozco *et al*, 2005; Hinks *et al*, 2005 and Michou *et al*, 2007) and an increased occurrence of this allele has also been observed in other autoimmune diseases such as type I diabetes mellitus, systemic lupus erythematosus (SLE) and juvenile idiopathic arthritis (JIA) (Bottini *et al*, 2004; Orozco *et al*, 2005 and Hinks *et al*, 2005). Interestingly, the *PTPN22* 1858 C→T polymorphism has been shown to prevent Lyp from binding Csk therefore it is postulated that it prevents down-regulation of T-cell activation (Bottini *et al*, 2004). Alternatively, it has been reported that this mutation leads to increased phosphatase activity and more potent suppression of T-cell receptor signalling (Begovich *et al*, 2004). It has been postulated that this dampening of T-cell signalling allows the survival of autoreactive thymocytes during the negative selection process of T-cell development and allows their subsequent development into mature T-cells capable of causing autoimmune responses once in the periphery (Bottini *et al*, 2004). CTLA-4 is a co-stimulatory receptor expressed on activated T-cells and, like CD28, it binds B7 co-stimulatory molecules expressed on

antigen-presenting cells. However, while signalling through ligated CD28 provides a T-cell with an activating signal, signalling through ligated CTLA-4 provides an inhibitory signal. An A/G polymorphism exists at nucleotide 49 in exon 1 of the *CTLA-4* gene, and the G allele of this polymorphism has been shown to be more prevalent in RA and other autoimmune diseases than in healthy subjects in Asian and Far Eastern populations (Pratt *et al*, 2009). It is postulated that the +49G polymorphism of CTLA-4 is involved in pathogenesis of RA because it prevents the inhibition of T-cell activation (Pratt *et al*, 2009).

1.3.2. The role of regulatory T-cells in rheumatoid arthritis

Rodent studies originally identified a population of $CD4^+$ $CD25^+$ T-cells which once activated by recognition of their specific antigen can suppress the activation and proliferation of other T-cells *in vitro*. These cells, which account for 10% of peripheral $CD4^+$ T-cells in healthy rodents, play an important role in the maintenance of self-tolerance and the prevention of autoimmunity (Sakaguchi *et al*, 2001). Indeed mice deficient in $CD4^+$ $CD25^+$ T-cells are susceptible to autoimmune diseases which can be prevented by adoptive transfer of $CD4^+$ $CD25^+$ T-cells from healthy, syngeneic mice (Asano *et al*, 1996 and Suri-Payer *et al*, 1998). Furthermore, depletion of such T-cells from mice with experimentally-induced collagen-induced arthritis (CIA), a mouse model of RA exacerbates the disease (Morgan *et al*, 2003). A similar population of $CD4^+$ $CD25^+$ T-cells has also been identified in humans, although it is less clear what the role of these regulatory T-cells is in human autoimmunity (Ng *et al*, 2001; Baecher-Allan *et al*, 2001 and Annunziato *et al*, 2002). In RA patients $CD4^+$ $CD25^+$ T-cells are enriched in synovial fluid compared to peripheral blood and can suppress proliferation of T-cells in vitro (Cao *et al*, 2003; Mottonen *et al*, 2005 and Lawson *et al*, 2006). $CD4^+$ $CD25^+$ T-cells from RA patients can not suppress production of pro-inflammatory cytokines from activated T-cells and monocytes suggesting defective functioning (Ehrenstein *et al*, 2004). Further evidence for the defective functioning of $CD4^+$ $CD25^+$ regulatory T-cells in RA comes from the study carried out by van Bilsen *et al* which analysed the responses of T-cells from RA patients and healthy controls to the candidate RA autoantigen human cartilage glycoprotein 39 (HCgp39). PBMC from healthy controls responded to HCgp39 with production of IL-10 while those from RA patients responded with production of IFN-γ. The HCgp39-specific T-cells from the healthy controls had phenotypes very similar to those of $CD4^+$ $CD25^+$ regulatory T-cells and appeared to have a regulatory function after stimulation with HCgp39, having the ability

to suppress both proliferation of and production of IFN-γ by conventional $CD4^+$ T-cells specific for other antigens. However, HCgp39-specific T-cells from RA patients lacked this regulatory/suppressive function (van Bilsen *et al*, 2004).

1.3.3. The role of TH17 cells in rheumatoid arthritis

It has recently been shown that a certain subset of $CD4^+$ T-cells known as TH17 cells appear to be of importance in the pathogenesis of animal models which mimic RA (Nakae *et al* #1, 2003; Nakae *et al* #2, 2003 and Hirota *et al* #1, 2007). TH17 cells, which secrete IL-17 but not IFN-γ and IL-4, are dependant on IL-23 for development and IL-23-deficient mice have been shown to be resistant to CIA, a mouse model of RA, as well as other experimentally-induced autoimmune diseases (Langrish *et al*, 2005). There are substantial levels in the synovial tissue and fluid of RA patients and it can activate osteoclasts, therefore it may be involved in the pathogenesis of the disease and joint destruction (Kotake *et al*, 1999). IL-17 induces the production of IL-1β and TNF-α by macrophages and the production of IL-6 and IL-8 by synovial fibroblasts (Jovanovic *et al*, 1998 and Fossiez *et al*, 1996) and therefore induces production of pro-inflammatory cytokines involved in RA pathogenesis. IL-1, IL-6 and TNF-α stimulate synoviocytes, cause inflammation and activate matrix metalloproteinases (Panayi, 2005). In addition to inducing production of these pro-inflammatory cytokines, IL-17 also induces metalloproteinases directly (Chabaud *et al*, 2001). A dominant role for TH17 cells in RA pathogenesis (van Hamburg *et al*, 2011) would explain the low levels of the Th1 cytokine IFN-γ in RA synovium. Indeed, IL-17 producing $CD4^+$ T-cells have been reported in RA patients in greater proportions than in healthy controls (Shen *et al*, 2009 and Colin *et al*, 2010). Interestingly, TH17 cells express CCR6, and synoviocytes from inflamed joints produce CCL20, the ligand for CCR6. Additionally, levels of synovial CCL20 correlate with levels of synovial IL-17, and CCL20 production in inflamed joints attracts Th17 cells (Hirota *et al* #2, 2007). High levels of IL-15 in synovial fluid of RA patients and the levels of IL-17 in synovial fluid of RA patients correlate very well. IL-15 induces PBMC to produce IL-17 and production can be reduced by immunosuppressive cyclosporine A (Ziolkowska *et al*, 2000).

1.3.4. The efficacy of treatments targeting T-cells

The benefits of T-cell-targeting therapies in RA have been reviewed (Isaacs, 2008). The conventional DMARDs cyclosporine, leflunomide and methotrexate all interfere with T-cell function. Cyclosporine inhibits production of IL-2 which is essential for T-cell

activation and proliferation and reduces symptoms of RA and disease progression. It was introduced into RA management in the 1990s although it is relatively toxic to the kidneys so is now only used occasionally, and when other DMARDs fail to control disease. Leflunomide inhibits proliferation of activated T-cells and slows progression of RA, high dosages are associated with gastrointestinal toxicity and blood counts, liver function and blood pressure need to be monitored during treatment. Methotrexate also inhibits proliferation of activated T-cells but only for a short while after each administration therefore it is unclear whether its efficacy relates to this. It is thought that methotrexate may exert its effect by causing release of anti-inflammatory adenosine as polymorphisms in certain genes involved in the adenosine pathway are associated with differing clinical responses to treatment with the drug (Wessels *et al*, 2006). While monoclonal antibodies targeting T-cell surface antigens could reverse autoimmunity in animal models, the use of monoclonal antibodies targeting T-cell antigens CD4, CD52 and CD28 (TGN1412) to treat RA in humans was less successful, these agents only providing transient clinical benefit (Strand *et al*, 2007). Indeed, the monoclonal antibody known as TGN1412 which targets the T cell antigen CD28 caused severe side-effects in healthy controls in its first phase 1 clinical trial (Suntharalingham *et al*, 2006 and Stebbings *et al*, 2007). However, the efficacy of the co-stimulatory signal inhibitor abatacept suggests that T-cells are important in the pathogenesis of RA.

For a T-cell that has recognised a peptide-MHC complex to gain the ability to proliferate, produce cytokines and activate other immune cells, it requires an additional co-stimulatory signal which is received when CD28 on the T-cell binds CD80 and CD86 on an antigen-presenting cell. Blocking of this co-stimulatory signal has been shown to slow disease progression in animal models of RA and the autoimmune disease, lupus (Finck *et al*, 1994 and Webb *et al*, 1996). Abatacept, also known as CTLA4Ig, is a soluble recombinant fusion protein which consists of the external domain of human CTLA4 fused to the Fc portion of human IgG1. The CTLA4 portion of abatacept (CTLA4Ig) enables the protein to out-compete CD28 for binding to CD80 and CD86 on antigen-presenting cells, thereby preventing delivery of a co-stimulatory signal to T-cells, thus preventing activation of T-cells. It is a licensed, efficacious treatment for RA. Disease activity/signs/symptoms in RA patients not responding to methotrexate were significantly reduced after CTLA4Ig treatment and physical function/quality of life improved (Kremer *et al*, 2003; Kremer *et al*, 2005; Kremer *et al*, 2006 and Kremer *et al*, 2008). Significant clinical and functional improvements were also observed in RA patients with disease activity not responding to anti-TNF treatment

and it has proved a safe therapy (Genovese *et al*, 2005 and Genovese *et al*, 2008). However, it has been reported that while the use of abatacept in combination with conventional DMARDs like methotrexate is safe, combining it with biologic DMARDs can cause serious adverse effects (Weinblatt *et al*, 2006).

1.3.5. T-cell tolerance and autoimmune disease

During T-cell development in the thymus, the random rearrangement of T-cell antigen receptor (TCAR) genes generates an extraordinarily diverse repertoire of TCARs. Many thymocytes express TCARs that are incapable of recognising self peptide-MHC complexes and are consequently of no use to the immune system, while those expressing TCARs with low affinity for self peptide-MHC complexes are positively selected and allowed to differentiate further into $CD4^+$ or $CD8^+$ T-cells (Hogquist *et al*, 2005). However, thymocytes expressing TCARs highly reactive with self peptide-MHC complexes are also created. If these auto-reactive thymocytes are allowed to differentiate unchecked they could mount autoimmune reactions once in the periphery which may lead to autoimmune diseases, therefore the differentiation of such thymocytes must be strictly controlled so that self tolerance is established within the mature T-cell population. The induction of T-cell tolerance in the thymus is achieved in part by the process of negative selection, in which thymocytes expressing highly self-reactive TCARs are selected against, mainly by elimination (clonal deletion) but also by 'neutralisation', achieved via both anergy induction and receptor editing (Palmer, 2003; Hammerling *et al*, 1991 and McGargill *et al*, 2000). Another mechanism inducing central T-cell tolerance is the selection of auto-reactive thymocytes for differentiation into specific subsets of regulatory T-cells, such as the fork-head box P3 (FoxP3)-expressing $CD4^+$ $CD25^+$ population mentioned in 1.3.2 (Sakaguchi, 2005). The observation that mutations in the autoimmune regulator gene *AIRE* which cause a defect in clonal deletion lead to the development of autoimmune polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome (APECED) and the association of immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) with mutations in the *FOXP3* gene which impede the development of $CD4^+$ $CD25^+$ regulatory T-cells demonstrate the importance of mechanisms of T-cell central tolerance in maintaining self tolerance (Villasenor *et al*, 2005 and Sakaguchi, 2005).

However, thymocytes expressing TCARs with low affinity for self peptide-MHC complexes as well as those with TCARs capable of recognising tissue-specific antigens

not expressed in the thymus evade the mechanisms of T-cell central tolerance. Therefore, for self tolerance to be maintained, mechanisms for inducing T-cell tolerance in the periphery are also necessary (Mueller, 2010). Firstly, autoreactive T-cells may remain ignorant of any self-antigens which are expressed in compartments not readily accessible to T-cells (Walker & Abbas, 2002). Secondly, when T-cells encounter peptide-MHC complexes in conjunction with inhibitory signalling through CTLA-4 or the programmed cell death 1 (PD-1) molecule, they become anergic (Walunas *et al*, 1996; Greenwald *et al*, 2001 and Freeman *et al*, 2000). In addition, even when autoreactive T-cells become activated, phenotype skewing may enable tolerance to be maintained by altering the ensuing T-cell responses in such a way that pathogenic effects are minimised (Walker & Abbas, 2002). Furthermore, repetitive TCAR stimulation, which could possibly occur when autoreactive T-cells encounter a ubiquitous self-antigen, can trigger activation-induced cell death (AICD), a peripheral form of clonal deletion mediated by Fas and Fas Ligand interactions (Walker & Abbas, 2002). Additionally, regulatory immune cells such as $CD4^+$ $CD25^+$ regulatory T-cells may control the actions of autoreactive T-cells in the periphery (Asano *et al*, 1996). The existence of an additional peripheral tolerance mechanism known as T-cell receptor revision in which the autoreactive TCARs of peripheral T-cells may be replaced with new ones produced by extrathymic TCAR gene rearrangements has also been discussed (Hale & Fink, 2010). The fact that autoreactive T-cells can be found in the peripheries of healthy subjects suggests that peripheral tolerance mechanisms are also essential for maintaining self tolerance, and that autoimmune diseases can develop when these mechanisms fail (Walker & Abbas, 2002).

The K/BxN and SKG mouse models are two examples of genetically manipulated spontaneous arthritis which demonstrate how the failure of tolerance inducing mechanisms can lead to the development of autoimmune arthritis (Asquith *et al*, 2009). The K/BxN strain of mice develop an erosive arthritis similar to human RA which is dependant on self-reactive $CD4^+$ T-cells but caused by autoantibodies (Kouskoff *et al*, 1996). $CD4^+$ T-cells from K/BxN mice are capable of recognising a peptide derived from the self-protein glucose-6-phosphate isomerase (GPI) which is expressed in the joints but because some of these T-cells escape negative selection, the mice have only partial tolerance to GPI (Korganow *et al*, 1999). The $CD4^+$ T-cells recognising GPI-derived peptide provide help to GPI-specific B-cells to produce GPI-specific IgG autoantibodies that in turn form immune complexes with GPI in joints which, through

activating complement and interacting with Fc (Fragment, crystallisable) receptors, cause the release of the inflammatory mediators IL-1 and TNF-α from mast cells and neutrophils (Ditzel, 2004). The SKG strain of mice develop a $CD4^+$ T-cell-mediated arthritis very similar to human RA which is caused by a mutation in the gene encoding the T-cell signalling molecule ZAP-70 (Sakaguchi *et al*, 2003). The mutation alters TCAR signal transduction and affects the development and differentiation of T-cells by interfering with the positive and negative selection processes and the generation of $CD4^+$ $CD25^+$ regulatory T-cells. The consequence of this is that SKG mice develop self-reactive, potentially arthritogenic T-cells and regulatory T-cells with a reduced ability to suppress self-reactive T-cells (Sakaguchi *et al*, 2006). However, development of arthritis in SKG mice requires an environmental stimulus in the form of exposure to microbial components which may provide danger signals which facilitate the activation of self-reactive, arthritogenic $CD4^+$ T-cells (Sakaguchi *et al*, 2006 and Asquith *et al*, 2009).

1.4. The role of B-cells in rheumatoid arthritis

Along with T-cells, macrophages and dendritic cells, B-cells contribute to a proportion of the inflammatory infiltrate observed in the synovial membranes of RA joints. While the synovial membranes of some RA patients show diffuse infiltrations where there is no organisation of the infiltrating cells (diffuse synovitis), in other RA patients the infiltrating T-cells and B-cells aggregate into highly organised, follicle-like structures (follicular synovitis) which surround follicular dendritic cells (Weyand & Goronzy, 2003 and Bugatti *et al*, 2007). While B-cells account for only a small proportion of the lymphocyte infiltrate, they do however appear to of be of great importance in disease pathogenesis, as demonstrated in a study by Takemura *et al* (2001). Using severe combined immunodeficiency (SCID) mice transplanted with inflamed synovial tissue from RA patients, they demonstrated that B-cells are essential for the activation of T-cells in the RA synovium and the subsequent production of pro-inflammatory cytokines. Because the mice lacked their own T-cells and B-cells, they were unable to reject the grafts of synovial tissue which were observed to produce substantial amounts of IFN-γ, IL-1β and TNF-α. However, when the mice were treated with anti-CD20 monoclonal antibodies that depleted the $CD20^+$ B-cells in the transplanted synovial tissue, the production of IFN-γ, IL-1β and TNF-α decreased dramatically. In addition, B-cells have been shown to be essential for the development of type II collagen-induced arthritis, a mouse model of rheumatoid arthritis (Svensson *et al*, 1998). A likely role for B-cells in

the pathogenesis of RA is as antigen-presenting cells, presenting self antigens to T-cells and providing a co-stimulatory signal so that they can be activated to proliferate and activate other immune cells via cytokine production or cell-cell interactions. B-cells may also be a source of pro-inflammatory cytokines themselves. Further evidence implicating the importance of B-cells in the pathogenesis of RA is the presence of autoantibodies in the sera of RA patients and the efficacy of B-cell depleting therapies in the treatment of the disease.

1.4.1. Autoantibodies in rheumatoid arthritis

Rheumatoid factors (RF), autoantibodies specific for the Fc portion of IgG, can be detected in the sera of 60-80% of RA patients with established disease (Goldbach-Mansky *et al*, 2000 and Steiner *et al*, 2002). Because of the high sensitivity of these autoantibodies for established RA, their presence in serum is one of the American College of Rheumatology revised classification criteria for defining disease (Goldbach-Mansky *et al*, 2000 and Steiner *et al*, 2002). Indeed, autoantibodies have an even higher 'weighting' in the more recently updated RA classification criteria (Aletaha *et al*, 2010). Low titres of RF are also detectable in the sera of around 55% of patients with early arthritis therefore it may be a useful marker for making an early diagnosis of RA (Steiner, 2006). However, low titres of RF are only around 75% specific for RA, as they are detectable in the sera of substantial proportions of patients with other rheumatic diseases such as Sjǒgren's syndrome (62%), scleroderma (44%), SLE (27%) and osteoarthritis (OA, 25%), and even in 10-15% of healthy controls (Steiner *et al*, 2002; Mewar & Wilson, 2006 and Steiner, 2006). High titre RF is a more specific marker of established RA, although this is detected in only 46% of RA patients (Steiner, 2006). Interestingly, serum levels of RF correlate well with disease activity in RA, with higher titres being associated with more severe joint damage (Knijff-Dutmer *et al*, 2002). This suggests that RF may have a role in the pathogenesis of the disease. Indeed, it is postulated that RF form immune complexes which fix complement and that the resulting chemotactic factors that are produced recruit inflammatory cells to the affected joint which contribute to the articular destruction (Firestein, 2003).

Anti-citrullinated peptide antibodies (ACPA), autoantibodies specific for citrullinated epitopes found within proteins such as α-enolase, fibrin, filaggrin and vimentin, are detectable in the sera of up to 75% of patients with established RA (Steiner, 2006). Furthermore, ACPA are 95-98% specific for RA and are also detectable in the sera of 41% of early arthritis patients therefore they are the most specific serological marker of

RA to date and are valuable for making a correct diagnosis during the very early stages of disease (Steiner, 2006). The presence of citrulline in the epitopes of the self proteins appears to be of great importance for their ability to be bound by ACPA (Schellekens *et al*, 1998). Indeed, a synthetic, cyclic citrullinated peptide (CCP) is now used to determine the presence of ACPA in sera of early arthritis patients in order to allow early diagnosis of RA (Schellekens *et al*, 2000). The presence of ACPA is associated with more joint destruction (Vries-Bouwstra *et al*, 2008). This suggests that ACPA, like RF, may have a role in joint damage in RA. Interestingly, both ACPA and RF can be detected several years before the onset of RA (Rantapää-Dahlqvist *et al*, 2003).

Anti-A2/RA33 autoantibodies specific for heterogenous nuclear ribonucleoprotein A2 (hnRNP-A2/RA33) are detectable in the sera of around 35% of RA patients (Hassfeld *et al*, 1995). However, they are also detectable in the sera of around 20% of SLE patients and up to 60% of mixed connective tissue disease (MCTD) patients (Hassfeld *et al*, 1995 and Skriner *et al*, 1997), thus are not greatly specific for RA. Like RF and ACPA, they can be detected in the early stages of disease but in contrast their presence does not appear to predict the severity of the disease (Goldbach-Mansky *et al*, 2000). However, it has been reported that hnRNP-A2/RA33 is over expressed in the synovial tissue of RA patients but not in that of OA patients (Fritsch *et al*, 2002). Furthermore, while humoral immunity to the protein is detectable in a substantial proportion of RA patients, it is rarely detected in OA patients (Steiner *et al*, 2002). Therefore anti-A2/RA33 autoantibodies may have a role in the pathogenesis of RA.

Anti-BiP autoantibodies specific for the chaperone/stress protein immunoglobulin heavy-chain binding protein (BiP) are also detectable in the sera of RA patients and are reported to be fairly specific for the disease (Bläss *et al*, 1995; Bläss *et al*, 2001 and Bodman-Smith *et al*, 2004). Interestingly, BiP is over expressed in the synovial tissue of RA patients compared to those of OA patients or healthy controls (Bläss *et al*, 2001), thus autoimmunity directed at this protein may have a role in the pathogenesis of RA.

1.4.2. The efficacy of treatments targeting B-cells

CD20 is a B-cell-specific antigen expressed by B-cell precursors and both resting and activated mature B-cells, but not by stem cells or plasma cells (Silverman & Weisman, 2003). Rituximab is a chimeric monoclonal antibody composed of the variable regions of a mouse antibody and the constant regions of a human antibody which is specific for human CD20. (Silverman & Weisman, 2003), and has been shown to be very effective at transiently depleting B-cells in vivo for up to 6 months (Shaw *et al*, 2003). An early

study by Edwards and Cambridge demonstrated the efficacy of rituximab as a treatment for RA, providing further evidence that B-cells are important in the pathogenesis of the disease (Edwards and Cambridge, 2001), and subsequent studies have supported this. RA patients not responding to treatment with methotrexate and cyclosporin A showed clinical improvements when treated with rituximab (De Vita *et al*, 2002). RA patients treated with rituximab showed clinical improvements and their serum levels of autoantibodies were significantly reduced (Cambridge *et al*, 2003). Treatment with the combination of rituximab and methotrexate improves symptoms of RA patients not responding to treatment with methotrexate alone (Edwards *et al*, 2004). The combination has also been shown to have positive effects on disease activity in RA patients not responding to anti-TNF treatments (Cohen *et al*, 2006). Further studies into the use of rituximab as a therapy for RA refractory to anti-TNF treatments have suggested that treatment with the former may be more effective than treatment with alternative anti-TNF agents (Finckh *et al*, 2007).

1.5. Candidate autoantigens in rheumatoid arthritis

As mentioned, it has been suggested that auto-reactive $CD4^+$ T-cells mediate the pathological process in RA. As the disease is characterised by inflammation at the joints, proteins of articular origin are popular candidates for autoantigens involved in disease pathogenesis. However, because the sera of RA patients often contain antibodies specific for certain systemically-expressed antigens, these extra-articular antigens are also considered as possible targets for auto-reactive $CD4^+$ T-cells.

1.5.1. Human cartilage glycoprotein 39 (HCgp39)

HCgp39, a 383 amino acid protein also known as YKL-40, is the main secreted product of both synoviocytes and articular chondrocytes from OA and RA patients (Nyirkos & Golds, 1990 and Hakala *et al*, 1993). HCgp39-encoding mRNA has been identified in abundance in both the synovial fluid and articular cartilage of RA patients yet the protein does not appear to be expressed in healthy articular cartilage (Hakala *et al*, 1993). Indeed, there is evidence to suggest that it is inflammation and tissue damage that induces the secretion of HCgp39, and high serum levels of the protein are associated with joint disease (Nyirkos & Golds, 1990; Hakala *et al*, 1993, and Johansen *et al*, 1993). Interestingly, while increased serum levels of HCgp39 can be detected in patients with RA, OA, SLE and inflammatory bowel disease (IBD), the highest serum levels of HCgp39 are detected in RA patients and in RA patients increased HCgp39

production appears to correlate with the degree of joint destruction and disease activity (Vos *et al* #1, 2000 and Volck *et al*, 2001). All this suggests that HCgp39 may have some role in the joint destruction seen in RA and a common postulation is that HCgp39 is an autoantigen that is presented to antigen-specific T-cells by RA-associated HLA-DR molecules. Indeed, there is a wealth of evidence suggesting that this may be the case.

Verheijden *et al* have shown that immunisation of BALB/c mice with HCgp39 in Freund's incomplete adjuvant induces an arthritis manifested in the paws in which swelling is observed at symmetrical joints. The incidence of this HCgp39-induced arthritis was 100% and while doses of HCgp39 as small as 1µg were capable of inducing the disease, more severe symptoms were observed in mice immunised with higher doses of the protein. Histological analysis of affected ankle joints of the arthritic mice showed that paw swelling was a result of inflammation which involved infiltration of mononuclear cells and proliferation of synovial fibroblasts. The group postulated that the arthritis was a result of HCgp39-specific T-cells being activated/induced by immunisation and then cross-reacting with peptides derived from mouse gp39 and thereby initiating autoimmune responses against this endogenous protein. Interestingly, the HCgp39-induced arthritis reported in this study was not as severe as proteoglycan-induced and collagen-induced arthritis in BALB/c and DBA/1 mice respectively. The study also showed that intranasal administration of HCgp39 prior to immunisation with the protein appears to induce tolerance in HCgp39-specific T-cells. Indeed, 50% of mice that were HCgp39-tolerised in this way prior to immunisation did not develop HCgp39-induced arthritis (Verheijden *et al*, 1997).

Certain HCgp39-derived peptides have been shown to bind RA-associated HLA-DR molecules with high affinity and induce HLA-DR-restricted peripheral blood T-cell responses in RA patients but not in healthy controls (Verheijden *et al*, 1997). In addition, HCgp39-derived peptides containing T-cell epitopes identified in HCgp39-immunised DR4(DRB1*0401) transgenic mice have been shown to induce proliferation of peripheral blood T-cells in individuals expressing RA-associated HLA-DR4 molecules. The immunodominant epitope of HCgp39 identified in the HCgp39-immunised DR4(DRB1*0401) transgenic mice was residues 263-275 and this was also the predominant epitope recognised by DR4 individuals (Cope *et al*, 1999). Peripheral blood T-cell responses to HCgp39-derived peptides have also been identified in

apparently healthy individuals, yet they are not detected to the same degree as in RA patients (Verheijden *et al*, 1997 and Vos *et al* #2, 2000). Furthermore, while peripheral blood T-cell responses to HCgp39-derived peptides have also been detected in patients with OA, SLE and IBD, to a greater degree than in healthy controls, only in RA do T-cell responses to HCgp39-derived peptides correlate with disease activity (Vos *et al* #2, 2000). One of the immunodominant T-cell epitopes of HCgp39 identified in DR4(DRB1*0401) transgenic mice immunised with the protein was a sequence residing in residues 263-275 (Cope *et al*, 1999). Interestingly, Tsark *et al* showed that when $HCgp39_{263-275}$-specific T-cells from $HCgp39_{263-275}$-immunised DR4(DRB1*0401) transgenic mice were cultured with native HCgp39 in the presence of human DR4+ mo-DC and macrophages, T-cell responses were detected (Tsark *et al*, 2002). This suggested that human antigen-presenting cells are capable of processing and presenting HCgp39 and that the previously identified immunogenic epitopes of the protein can be generated *in vivo*. Indeed, in a study by van Bilsen *et al*, human PBMC were shown to respond to native HCgp39. These HCgp39-specific PBMC responses were identified in both RA patients and healthy controls yet whereas RA patient PBMC responded to the protein with production of both IL-10 and IFN-γ, PBMC from healthy subjects responded with production of IL-10 alone (van Bilsen *et al*, 2004).

1.5.2. Type II collagen (CII)

CII is the most abundant protein in articular cartilage. Several studies have reported the presence of anti-CII antibodies in the sera of RA patients (Cook *et al*, 1996 and Terato *et al*, 1990). Indeed, it has been reported that the presence of CII-specific antibodies in RA sera is associated with active disease and more severe symptoms (Cook *et al*, 1996). Furthermore, the arthritogenic nature of CII has been demonstrated in several animal models (Trentham *et al*, 1977; Wooley *et al*, 1981 and Yoo *et al*, 1988).

Two separate studies by Rosloniec *et al* using DR1(DRB1*0101) transgenic and DR4(DRB1*0401) transgenic B10.M mice (Rosloniec *et al*, 1997 and Rosloniec *et al* 1998) have defined a relationship between the expression of RA-associated HLA-DR molecules and immune response to CII by showing that expression of these RA-associated HLA-DR molecules can allow development of CII-induced CIA due to their ability to present CII-derived peptides. The group showed that B10.M mice, normally resistant to CIA, could be made susceptible to CII-induced CIA through transgenic expression of DR1(DRB1*0101) or DR4(DRB1*0401) molecules. The incidence of

CII-induced CIA in the DR1(DRB1*0101) transgenic mice was between 88% and 100%, and arthritic mice showed T-cell responses to both the immunising human CII as well as the endogenous mouse CII. Additionally, T-cell responses to human CII were much stronger in the transgenic mice than in wild type B10.M mice that had been immunised but were resistant to arthritis. Furthermore, the arthritic mice produced high levels of anti-CII antibodies while the non-arthritic wild type B10.M mice only produced low levels of anti-CII antibodies. The arthritic mice also produced antibodies that recognised mouse CII while the non-arthritic mice did not. The immunodominant epitope of CII identified in both the DR1(DRB1*0101) transgenic and DR4(DRB1*0401) transgenic B10.M mice was residues 263-270 and CII-derived peptides containing this epitope induced strong, DR1(DRB1*0101)-restricted responses in T-cells from arthritic mice (Rosloniec *et al*, 1997).

Several studies have reported CII-specific T-cell responses in RA. Londei *et al* (1989) have reported the persistence of CII-reactive T-cells in the arthritic synovial membrane of an individual RA patient over a three year period. Furthermore, another study detected reactivity to CII or a peptide composed of residues 255-274 of the protein in the synovial fluid mononuclear cells (SFMC) of 62% of RA patients (Kim *et al*, 1999). CII-reactive cells have also been reported in the peripheral blood of RA patients (Snowden *et al*, 1997; Kim *et al*, 1999 and Park *et al*, 2001). Indeed, two studies showed that significantly higher proportions of RA patients than OA patients or healthy controls show CII-specific PBMC reactivity and that the magnitudes of CII-specific responses detected in the RA patients were significantly greater than those detected in either of the other groups (Kim *et al*, 1999 and Park *et al*, 2001). However, Snowden *et al* (1997) reported that while 50% of RA patients with anti-CII antibodies in their sera showed positive CII-induced PBMC responses, only 5% of the RA patients lacking detectable humoral immunity to CII showed CII-specific PBMC reactivity, as opposed to 36% of healthy controls. Interestingly, T-cell responses to CII have been reported as being more prevalent and significantly higher in magnitude in the SFMC of RA patients compared to PBMC (Kim *et al*, 1999).

1.5.3. Aggrecan

Aggrecan is the predominant proteoglycan component of articular cartilage and exists in this tissue in large, multi-molecular aggregates of individual aggrecan monomers that are each attached to hyaluronan in the extra cellular matrix via non-covalent bonds

(Kiani *et al*, 2002). Individual aggrecan molecules are each composed of two N-terminal globular domains (G1 and G2) linked by an interglobular domain, which are separated from a third, C-terminal globular domain (G3) by a large region of glycosaminoglycan (GAG) side chains (Kiani *et al*, 2002). The GAG region is composed of a domain of around 60, 5-15kDa keratin sulphate chains (KS domain) and two further domains consisting of a total of around 100, 20kDa chondroitin sulphate chains (CS-1 and CS-2), and also contains a number of O- and N-linked oligosaccharides (Kiani *et al*, 2002). Some keratin sulphate chains are also present in the G1 and G2 domains as well as in the interglobular domain (Guerassimov *et al*, 1998). These trap water due to their anionic nature, and give cartilage its compressibility (Kiani *et al*, 2002). Both the whole molecule of aggrecan as well as the G1 domain alone have been shown to be arthritogenic in animal models (Glant *et al*, 1987 and Zhang *et al*, 1998).

Proteoglycan-induced arthritis (PGIA) is an RA-like condition that can be induced in BALB/c mice and in certain sub-strains of C3H mice by immunisation with cartilage proteoglycan in complete Freund's adjuvant (Glant *et al*, 1987). This animal model of RA has given great insight into the potential pathogenic mechanisms involved in human RA. It is thought that PGIA develops as a result of immune responses being generated to the immunising proteoglycan cross-reacting with mouse cartilage proteoglycan and attacking structures containing the antigen, such as joints (Glant *et al*, 1987). This is supported by the fact that PGIA can be adoptively transferred to irradiated, non-immunised BALB/c mice by injecting them with lymphocytes from arthritic mice (Mikecz *et al*, 1990). However, if the lymphocytes are depleted of either T-cells or B-cells prior to injection, they cannot induce arthritis therefore both T- and B-cells are needed for development of arthritis (Mikecz *et al*, 1990). Development of PGIA is related to production of antibodies specific for proteoglycan which suggests that $CD4^+$ (helper) T-cells are important (Glant *et al*, 1987). Indeed, it has been shown that if PG-immunised BALB/c mice are depleted of $CD4^+$ T-cells, they do not develop PGIA and show greatly reduced anti-PG antibody responses compared to arthritic mice (Banerjee *et al*, 1992). Early studies using KS-free human foetal cartilage proteoglycan (HFCPG) to induce arthritis in BALB/c mice showed that the removal of the CS chains from the proteoglycan drastically increased its arthritogenicity (Leroux *et al*, 1992). However, human adult cartilage proteoglycan (HACPG), which is rich in KS chains, does not become more arthritogenic after removal of CS chains therefore glycosylation of

proteoglycan may influence arthritogenicity (Leroux *et al*, 1992). Studies using proteoglycan-specific T-cell lines and hybridomas generated from mice with PGIA have suggested that the immunodominant epitopes of aggrecan reside in the G1 domain and indeed, injection of the G1 domain alone can induce arthritis in BALB/c mice, but only after it is depleted of KS (Leroux *et al*, 1992 and Leroux *et al*, 1996). Leroux *et al* showed that MHC class II-restricted G1-specific $CD4^+$ T-cell hybridomas showed enhanced responses to KS-depleted G1 as opposed to native G1. This is because KS-depleted G1 can be taken up, processed and presented by antigen-presenting cells more successfully than native G1 (Leroux *et al*, 1996). G1-specific T-cell hybridomas generated from PG-immunised BALB/c mice were found to respond to two G1-derived peptides, $aggrecan_{70\text{-}84}$ and $aggrecan_{150\text{-}169}$, when presented by syngeneic antigen-presenting cells. Therefore these sequences contain immunodominant epitopes which can be naturally processed and presented by antigen-presenting cells. When T-cells specific for $aggrecan_{70\text{-}84}$ were injected into mice they developed arthritis. T-cells specific for G1 or $aggrecan_{70\text{-}84}$ respond much better to KS-depleted G1 than native G1. Enhanced antibody responses were also detected in mice immunised with KS-depleted G1 compared to mice immunised with native G1 (Zhang *et al*, 1998). Therefore, it is likely that the presence of KS chains on G1 impedes its ability to induce arthritis by inhibiting T- and B-cell responses to epitopes contained within G1. Interestingly, the T-cells responding to $aggrecan_{70\text{-}84}$ showed reactivity to HFCPG, HACPG, bovine foetal cartilage proteoglycan (BFCPG), bovine G1 and bovine link protein, which is similar in structure to G1 (Leroux *et al*, 1992).

Peripheral blood T-cells from a greater proportion of RA patients than healthy controls show proliferative responses to DeglyAgg which is free of both KS and CS. Peripheral blood T-cells from some RA patients responding to DeglyAgg also proliferated in response to peptides from CS1 domain (Goodstone *et al*, 1996). PBMC from RA patients but not healthy controls show G1-specific reactivity, and immunogenicity of G1 in RA patients but not healthy controls is greatly enhanced by the removal of the keratin sulphate chains (Guerassimov *et al*, 1998). G1-reactive T-cells have been identified in both the peripheral blood and synovium of RA patients although they are more frequent in the synovium (Li, N.L. *et al*, 2000). G1-reactive T-cells have also been identified in peripheral blood of healthy controls but are much less frequent than in peripheral blood of RA patients (Li, N.L. *et al*, 2000 and de Jong *et al*, 2010). It has been reported that peripheral blood $CD4^+$ T-cells from substantially greater proportions

of RA patients than of healthy controls show G1-induced IFN-γ and TNF-α production (Zou *et al*, 2003). PBMC from RA patients produce more pro-inflammatory cytokines and chemokines in response to G1-derived peptides than healthy controls (de Jong *et al*, 2010). A G1-derived peptide, albeit in citrullinated form, has been shown to induce proliferation of PBMC from RA patients but not healthy controls and the responses to the peptide were characterised by high levels of IL-17 production and the appearance of IL-17^+ $CD4^+$ T-cells (von Delwig *et al*, 2010). Boots *et al* have identified eleven aggrecan-derived peptides that bind DR4(DRB1*0401) molecules with high affinity and one of these eleven also binds both DR4(DRB1*0404) and DR1(DRB1*0101) molecules with high affinity. Furthermore, five of the peptides were identified as T-cell epitopes in humans although responses to these peptides were more prevalent in healthy controls than in RA patients (Boots *et al*, 1997).

1.5.4. Antigens recognised by antibodies in rheumatoid arthritis sera

The sera of RA patients often contain antibodies specific for citrulline-containing epitopes which can be generated from certain self proteins including α-enolase, filaggrin, fibrinogen and vimentin.

While antibodies reacting with placenta-derived α-enolase have been identified in the sera of 25% of RA patients, serum reactivity to the recombinant form of the protein was less frequently observed (Saulot *et al*, 2002). Therefore it was suggested that the antibodies in the RA sera were recognising a post-translationally modified form of the protein. In a study by Kinloch *et al*, unmodified and citrullinated RA synovial cell lysates were probed with the sera of RA patients and a 47 kDa protein from the citrullinated lysate, identified as citrullinated α-enolase, was shown to be a target for antibodies in RA sera. The group went on to show that while the sera of 46% of RA patients showed reactivity to the citrullinated form of α-enolase, only 13% showed serum reactivity to the unmodified form of the protein. Sera from 15% of healthy controls showed reactivity to both forms of the protein. In addition, they confirmed the status as a joint-associated autoantigen by demonstrating that α-enolase was expressed in samples of inflamed synovial tissue from 100% of RA patients and provided evidence suggesting that the citrullinated form of the protein is present in the joints (Kinloch *et al*, 2005). More recently, the immunodominant epitope of citrullinated α-enolase recognised by antibodies reacting to the protein has been identified as

citrullinated enolase$_{5-21}$ and has been shown to react with sera of 37% of RA yet only 3% of DC and 2% of HC (Lundberg *et al*, 2008).

Anti-perinuclear factor and anti-keratin antibodies, autoantibodies both highly specific for RA, recognise citrullinated epitopes from within a 40 kDa protein known as filaggrin and have thus been collectively named anti-filaggrin antibodies (Simon *et al*, 1993; Sebbag *et al*, 1995 and Girbal-Neuhauser *et al*, 1999). In RA patients, concentrations of anti-filaggrin antibodies are greater in the synovial membrane compared to in the synovial fluid or serum (Masson-Bessiere *et al*, 2000). Furthermore, filaggrin-specific T cell responses have been reported in some RA patients, with both the unmodified and citrullinated forms of the protein eliciting responses (Fritsch *et al*, 2002). However, as filaggrin is not expressed in the joint, it is unclear how responses to this protein could lead to joint damage (Kinloch *et al*, 2005). Interestingly, citrullinated fibrinogen is expressed in the joints of RA patients and it is recognised by anti-filaggrin antibodies (Masson-Bessiere *et al*, 2001). In addition, Auger *et al* have reported that the sera of both shared epitope-negative and shared epitope-positive RA patients contain antibodies specific for citrullinated fibrinogen. Furthermore, they demonstrated that various RA-associated as well as non-associated HLA-DR molecules bind peptides derived from both the α- and β-chains of fibrinogen, and that both unmodified and citrullinated forms of the peptides bound equally well. The group also showed that peripheral blood T-cells from RA patients responded to a greater number of both unmodified and citrullinated fibrinogen-derived peptides than did peripheral blood T-cells from healthy controls (Auger *et al*, 2005). Furthermore, citrullinated fibrinogen has been shown to be arthritogenic as 35% of DR4(DRB1*0401) transgenic mice immunised with the protein developed arthritis, and citrulline-specific, DR4-restricted T-cell responses were detected in arthritic mice (Hill *et al*, 2008).

Various studies have reported the presence of anti-Sa antibodies in the sera of RA patients and the mean sensitivity of such antibodies for the disease is around 37% (Hueber *et al*, 1999; El-Gabalawy & Wilkins, 2004 and Vossenaar *et al*, 2004). However, the sensitivity of anti-Sa antibodies for RA varies with disease stage as the antibodies are more prevalent in patients with established disease than those with early disease (Hueber *et al*, 1999 and El-Gabalawy & Wilkins, 2004). Anti-Sa antibodies have been shown to be very rare in the sera of patients with other rheumatic diseases and were reported to be 98% specific for RA (Hueber *et al*, 1999). Anti-SA antibodies

recognise a 50 kDa protein found in human placenta and spleen and the synovial tissue of RA patients (Despres *et al*, 1994). The protein recognised by anti-Sa antibodies has been identified as vimentin and it is only recognised by anti-Sa antibodies when citrullinated (Vossenaar *et al*, 2004). A peptide derived from citrullinated vimentin was shown to induce DR-restricted T-cell responses in mice transgenic for the RA-associated DR4(DRB1*0401) molecule (Hill *et al*, 2003).

1.6. The role of citrullination in rheumatoid arthritis

As a non-standard amino acid, citrulline is unavailable for incorporation into proteins during translation, yet it can be introduced into proteins or peptides by way of a post-translational modification known as citrullination (Vossenaar, Zendman & van Venrooij, 2004). The citrullination reaction is catalysed by Ca^{2+}-dependant peptidyl arginine deiminase (PAD) enzymes which deiminate protein or peptide-incorporated arginine, thus converting it to citrulline (Vossenaar & van Venrooij, 2004). Of the five known isotypes of PAD that have been identified in mammals, PAD2 and PAD4 are expressed by monocytes and macrophages in the synovial fluid (Vossenaar, Zendman & van Venrooij, 2004 and van Gaalen *et al*, 2005). Interestingly, antibodies specific for citrulline-containing epitopes of joint-associated autoantigens such as α-enolase, fibrinogen, filaggrin and vimentin are the most disease-specific autoantibodies detected in RA and are present in the sera of 60-70% of RA patients (van Gaalen *et al*, 2005). Furthermore, a certain haplotype of the PAD4-encoding *PADI4* gene, which appears to confer enhanced stability of *PADI4* mRNA, is known to be associated with RA in some ethnic groups (Vossenaar, Zendman & van Venrooij, 2004). Therefore it is possible that citrullination is an important post-translational modification in RA.

When a protein is citrullinated there is a very slight change in its molecular weight and the positive charges associated with its arginine residues are substituted for the neutral charges of citrulline (van Venrooij & Pruijn, 2000). It is postulated that the citrullination-induced change in the charge of a protein may alter the way that it interacts with other proteins, either by abolishing existing charge interactions or enabling new ones to form (van Venrooij & Pruijn, 2000). Thus it is possible that the citrullination of peptides may allow them to bind to MHC molecules that in their unmodified form they were unable to bind, and thereby become visible to the immune system. Interestingly, residues 70-74 of an HLA-DR β-chain contributing to a complete HLA-DR molecule form the P4 peptide binding pocket of the molecule's peptide

binding groove (Hill *et al*, 2003). Furthermore, the shared epitope sequences found at residues 70-74 in RA-associated HLA-DR β-chains possess overall positive charges by virtue of the positively-charged side chains on residues 70 and 71 (Hill *et al*, 2003). Therefore, shared epitope-containing HLA-DR molecules are proposed to favour peptides that have negative or neutral charges in the positions that anchor in the P4 binding pocket (van Gaalen *et al*, 2005). Thus, as citrullination involves the conversion of positively-charged arginine to neutrally-charged citrulline, then it is possible that this post translational modification may alter the antigenicity and immunogenicity of self peptides in shared epitope-positive individuals and thereby initiate autoimmune responses (van Gaalen *et al*, 2005). Possession of the RA-associated *PADI4* haplotype may be associated with increased expression of PAD4 which could in turn lead to increased citrullination of self proteins. Alternatively, smoking may also lead to increased citrullination of self proteins. Peptides derived from such citrullinated self proteins may then be preferentially bound by SE-positive HLA-DR molecules and may thus be presented to citrulline-specific T-cells which become activated and provide help for citrulline-specific B-cells so that they can produce ACPA (van Gaalen *et al*, 2005). Indeed, the production of anti-citrulline antibodies has been found to be strongly associated with the possession of shared epitope-containing HLA-DR molecules (van Gaalen *et al*, 2004) and citrulline-specific B-cells would require help from citrulline-specific $CD4^+$ T-cells in order to produce these antibodies. This suggests that shared epitope-positive HLA-DR molecules may indeed present citrullinated self peptides to antigen-specific $CD4^+$ T-cells. In addition, several studies have shown how citrullination can enhance the antigenicity and immunogenicity of candidate autoantigen-derived peptides in shared epitope-positive individuals. Using a vimentin-derived peptide that was predicted to bind DR4(DRB1*0401) molecules with one of its arginine residue anchored in the P4 binding pocket, Hill *et al* (2003) showed that the conversion of this arginine residue to citrulline greatly increased the affinity of the peptide for shared epitope-positive but not shared epitope-negative HLA-DR molecules. Furthermore, the citrullinated form of the peptide but not the unmodified form was shown to elicit DR-restricted T-cell responses in DR4(DRB1*0401) transgenic mice (Hill *et al*, 2003). Further studies by Hill *et al* (2008) showed that immunisation of DR4(DRB1*0401) transgenic mice with citrullinated but not unmodified fibrinogen induced an inflammatory arthritis in the mice in which citrulline-specific T-cell response were observed. In addition, the citrullinated form but not the unmodified form of a fibrinogen-derived peptide predicted to bind DR4(DRB1*0401) molecules with one

of its arginine residues anchored in the P4 pocket was shown to elicit DR-restricted T-cell responses in the mice when citrullinated but not when unmodified (Hill *et al*, 2008). There is also evidence suggesting that the citrullination of two filaggrin-derived peptides alters the way in which they interact with MHC molecules as a number of human subjects responded to one form but not the other (Fritsch *et al*, 2002). More direct evidence for a role of citrullination in the pathogenesis of RA comes from the study of von Delwig *et al* (2010). Using unmodified and citrullinated forms of aggrecan$_{84\text{-}103}$, the peptide identified as the immunodominant T-cell epitope in mice with proteoglycan-induced arthritis, the group identified citrulline-specific T-cell responses in RA patients but not healthy controls. While PBMC from healthy subjects showed no reactivity to either unmodified or citrullinated aggrecan$_{84\text{-}103}$, the citrullinated form of the peptide but not the unmodified form induced proliferative PBMC responses in 60% of tested RA patients. The response to citrullinated aggrecan$_{84\text{-}103}$ was characterised by high levels of IL-17 production and an induction of IL-17^{+} CD4^{+} T-cells (von Delwig *et al*, 2010).

Recently, a new antibody system recognising proteins altered by another post-translational modification has been demonstrated in RA by Shi *et al* (2011). The group identified antibodies specific for carbamylated peptides which contain homocitrulline in over 45% of RA patients. These so-called anti-CarP antibodies are distinct from ACPA and appear to be predictive of a more severe form of ACPA-negative RA.

1.7. The importance of measuring T-cell responses to candidate autoantigens

At present, the only parameters of RA that are measured in clinics are joint damage, measured via X-ray, and the presence of markers of inflammation which are both end results of the disease process. Modern RA therapies are aimed at modifying the disease process at an early stage and the induction of tolerance to autoantigens thought to be involved in disease pathogenesis is a major goal. Thus it is important to be able to measure candidate RA autoantigen-specific T-cell responses.

1.8. Aims of project

1.8.1. Specific aims of the project

- To optimise an experimental system for the detection of candidate RA autoantigen-specific T-cell responses.

- To compare, both quantitatively and qualitatively, joint-associated, candidate RA autoantigen-specific T-cell responses in RA patients, healthy subjects and disease controls.
- To investigate whether citrullination of candidate RA autoantigens alters their immunogenicity in RA patients versus healthy subjects/disease controls.

1.9. Hypotheses

- Candidate RA autoantigen-specific T-cell responses are significantly more prevalent in RA patients than in healthy subjects.
- Candidate RA autoantigen-specific T-cell responses detected in RA patients are qualitatively different from those detected in healthy subjects, the former being pro-inflammatory in nature and the latter being immunosuppressive.
- Immune responses to citrullinated candidate RA autoantigens are detected more frequently than immune responses to unmodified candidate RA autoantigens, but only in RA patients and not in healthy subjects.
- The immune responses elicited by citrullinated candidate RA autoantigens are qualitatively different from those elicited by their unmodified counterparts, the former being more pro-inflammatory in nature and the latter being immunosuppressive.

	Description of methods	Prevalence of positive responses / Observations
Verheijden *et al*, 1997	PBMC from 18 RA patients and 11 HS were cultured in 10% autologous plasma-supplemented medium with HCgp39-derived peptides ($_{103-116}$, $_{259-271}$, $_{263-275}$ or $_{326-338}$ at 100, 25 or 10µg/ml). Proliferation in replicate wells ($1.5x10^5$ cells/well) was assessed after 7 days via 3HTdR incorporation. SI values of 2 or greater were considered to represent positive responses.	HCgp39-induced proliferative responses were detected in 56% of RA patients and 27% of HS. $_{103-116}$, $_{263-275}$ and $_{326-338}$ induced proliferative responses in RA patients alone. $_{263-275}$ was the most immunogenic peptide, inducing proliferative responses in over 40% of RA patients.
Cope *et al*, 1999	PBMC from 8 SE $^{+}$ve RA patients, 6 SE $^{+}$ve HS and 9 SE $^{-}$ve HS were cultured in 15% autologous serum-supplemented medium with HCgp39-derived peptides ($_{40-55}$, $_{100-115}$, $_{197-211}$, $_{256-271}$, $_{262-277}$, $_{322-337}$ or $_{334-349}$ at 10µg/ml). Proliferation in replicate wells ($2x10^5$ cells/well) was assessed after 6 days via 3HTdR incorporation. SI values greater than 2 were considered to represent positive responses.	Proliferative responses to the HCgp39-derived peptides were detected in SE $^{+}$ve individuals from both the RA patient and HS groups, but not in SE $^{-}$ve subjects.
Vos *et al* #2, 2000	PBMC from 50 RA patients and 49 HS were cultured in 10% heat-inactivated autologous plasma-supplemented medium with HCgp39-derived peptides ($_{75-87}$, $_{103-116}$, $_{259-271}$, $_{263-275}$ or $_{362-338}$ at 10µg/ml). Proliferation in replicate wells ($1.5x10^5$ cells/well) was assessed after 7 days via 3HTdR incorporation. SI values of 2 or greater were considered to represent positive responses.	Proliferative responses to $HCgp39_{259-271}$ were detected in 36% of RA patients and 8% of HS. $_{75-87}$, $_{263-275}$ and $_{326-338}$ induced proliferative responses in RA patients alone. $_{103-116}$ induced proliferative responses in both RA patients and HS.
Zou *et al*, 2003	Peripheral blood from 22 RA patients and 20 HS was stimulated with aggrecan G1 domain, HCgp39 or CII (20µg/ml). After 6 hours, production of IL-10, IL-4, TNF-α and IFN-γ by $CD4^+$ T-cells was determined via intracellular cytokine staining.	55% of RA patients showed production of substantial levels of TNF-α and IFN-γ in response to aggrecan G1 domain, compared to 10% of HS. HCgp39 and CII failed to induce production of substantial levels of TNF-α of IFN-γ in both groups.

Table 1.1. Summary of previous studies investigating human cartilage glycoprotein 39 (HCgp39)-induced responses. Zou *et al*, 2003 also investigated responses to type II collagen (CII) and aggrecan. Abbreviations; PBMC = peripheral blood mononuclear cells, SE = shared epitope, RA = rheumatoid arthritis, HS = healthy subjects, 3HTdR = ^{3}H-thymidine, SI = stimulation index.

	Description of methods	Prevalence of positive responses / Observations
Snowden *et al*, 1997	PBMC from 56 anti-CII antibody $^{-}$ve RA patients, 10 anti-CII antibody $^{+}$ve RA patients and 28 HS were cultured in 10% human AB serum-supplemented medium with CII (50µg/ml). Proliferation in replicate wells ($5x10^5$ cells/well) was assessed after 4, 6 and 8 days via 3HTdR incorporation. SI values greater than 2 were considered to represent positive responses.	CII-induced proliferative responses were observed in 5% of the anti-CII antibody $^{-}$ve RA patients, 50% of the anti-CII antibody $^{+}$ve RA patients and 36% of the HS. The kinetics of the responses observed in RA patients were suggestive of recall responses while those observed in HS were suggestive of primary responses.
Kim *et al*, 1999	PBMC from 106 RA patients and 34 HS were cultured in 10% foetal bovine serum-supplemented medium with bovine CII (40µg/ml) and/or a CII-derived peptide ($_{255-274}$ at 10µg/ml). Proliferation in replicate wells ($1x10^5$ $CD3^+$ cells & $1x10^5$ irradiated $CD3^-$ cells/well) was assessed after 5 days via 3HTdR incorporation. SI values of 2 or greater were considered to represent positive responses.	Proliferative responses to bovine CII were detected in 35% of RA patients and 3% of HS. Proliferative responses to $CII_{255-274}$ were detected in 38% of RA patients and 12% of HS.
Park *et al*, 2001	PBMC and SFMC from 40 RA patients and PBMC from 39 HS were cultured in 10% FBS-supplemented medium with bovine CII (40µg/ml). Proliferation in replicate wells ($1x10^5$ $CD3^+$ cells & $1x10^5$ irradiated $CD3^-$ cells/well) was assessed after 5 days via 3HTdR incorporation. SI values of 2 or greater were considered to represent positive responses.	CII-induced proliferative responses were detected in the peripheral blood of 33% of RA patients and 3% of HS. CII-induced proliferative responses were increased in synovial fluid versus peripheral blood in RA patients (62% versus 33%).

Table 1.2. Summary of previous studies investigating type II collagen (CII)-induced responses. Abbreviations; PBMC = peripheral blood mononuclear cells, RA = rheumatoid arthritis, HS = healthy subjects, 3HTdR = ^{3}H-thymidine, SI = stimulation index, SFMC = synovial fluid mononuclear cells.

	Description of methods	Prevalence of positive responses / Observations
Goodstone *et al*, 1996	PBMC from 48 RA patients and 32 HS were cultured in 10% heat-inactivated autologous serum-supplemented medium with deglycosylated human aggrecan (10µg/ml). Proliferation in replicate wells (1 or $2x10^5$ cells/well) was assessed after 5 days via 3HTdR incorporation. SI values greater than 1.5 were considered to represent positive responses.	Proliferative responses to deglycosylated aggrecan were observed in 69% of RA patients and 37% of HS. SI values of deglycosylated aggrecan-induced proliferation detected in RA patients were significantly greater than those detected in HS.
Boots *et al*, 1997	PBMC from 9 SE^+ve RA patients and 7 SE^+ve HS were cultured in 10% human AB serum-supplemented medium with a selection of human aggrecan-derived peptides ($_{107-119}$, $_{201-213}$, $_{299-311}$, $_{623-635}$, $_{739-751}$, $_{743-756}$, $_{954-966}$, $_{1777-1789}$ or $_{1805-1819}$ at 50, 5 or 0.5µg/ml). Proliferation in replicate wells ($1.5-2x10^5$ cells/well) was assessed after 7 days via 3HTdR incorporation. SI values of 3.5 or greater were considered to represent positive responses.	Proliferative responses to the aggrecan-derived peptides were observed in both the RA patients and HS. The magnitudes of the aggrecan-derived peptide-induced responses and the numbers of aggrecan-derived peptide responders were decreased in RA patients versus HS (this was not due to RA patients being hyporesponsive).
Guerassimov *et al*, 1998	PBMC from 43 RA patients and 13 HS were cultured in 10% heat-inactivated human AB serum-supplemented medium with either native or keratin sulphate-depleted bovine aggrecan G1 domain (25µg/ml). Proliferation in replicate wells ($2-4x10^5$ cells/well) was assessed after 5 days via 3HTdR incorporation. SI values greater than 2 were considered to represent positive responses.	Proliferation in response to native G1 domain was detected in 16% of RA patients but was absent in HS. Proliferative responses to keratin sulphate-depleted G1 domain were detected in 51% of RA patients and 8% of HS. SI values of keratin sulphate-depleted G1 domain-induced proliferation were significantly greater in RA patients than in HS.
de Jong *et al*, 2010	PBMC from 60 RA patients and 32 HS were cultured in 10% human AB serum-supplemented medium with aggrecan-derived peptides (50µg/ml). Proliferation in replicate wells ($2x10^5$ cells/well) was assessed after 4 days via 3HTdR incorporation. SI values greater than 1.8 were considered to represent positive responses. Levels of IL-1, IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ in culture supernatants harvested after 3 days were determined via electrochemiluminescence multiplex assay.	Proliferative responses to several aggrecan-derived peptides were increased in the RA patients compared to the HS. Certain aggrecan-derived peptides induced higher levels of IL-1, IL-2, IL-6, IL-10 and TNF-α production in the RA patients than in the HS.

Table 1.3. Summary of previous studies investigating aggrecan-induced responses. Abbreviations; PBMC = peripheral blood mononuclear cells, RA = rheumatoid arthritis, HS = healthy subjects, 3HTdR = ^{3}H-thymidine, SI = stimulation index, SE = shared epitope.

	Description of methods	Prevalence of positive responses / Observations
Fritsch *et al*, 2002	PBMC from 19 RA patients and 20 HS were cultured in serum-free medium with native and citrullinated forms of filaggrin-derived peptides ($_{2776-2793}$ or $_{3005-3022}$ at 2.5µg/ml). Proliferation in replicate wells ($1x10^5$ cells/well) was assessed after 5 days via 3HTdR incorporation. SI values of 2 or greater were considered to represent positive responses.	Proliferative responses to both native and citrullinated forms of the peptides were observed in both RA patients and HS.
Auger *et al*, 2005	PBMC from 12 RA patients and 10 HS were cultured in 10% autologous serum at $1x10^6$/ml with native and citrullinated forms of peptides derived from the α- and β-chains of fibrinogen. Proliferation was assessed after 7 days via bromodeoxyuridine incorporation. Optical density readings of 2x background or greater were considered to represent positive responses.	Proliferation in response to both native and citrullinated forms of the fibrinogen-derived peptides was more common in RA patients than in HS. 18 of the α-chain-derived peptides and 27 of the β-chain-derived peptides induced responses in RA patients compared to 2 of the α-chain-derived peptides and 5 of the β-chain-derived peptides in HS.
von Delwig *et al*, 2010	PBMC from 28 RA patients and 18 HS were cultured in 5% human AB serum-supplemented medium with either native or N terminal citrullinated forms of an aggrecan-derived peptide (84-103 at 10µg/ml). Proliferation in replicate wells ($2x10^5$ cells/well) was assessed after 9 days via 3HTdR incorporation. SI values greater than 2 were considered to represent positive responses.	Proliferative responses to N terminal citrullinated aggrecan$_{84-103}$ were observed in over 60% of RA patients but were absent in HS. Non-citrullinated aggrecan$_{84-103}$ failed to induce proliferative response in both the RA patients and the HS.
Law *et al*, 2012	PBMC from 21 RA patients and 6 SE $^+$ve HS were cultured in 10% healthy serum-, autologous serum- or allogeneic RA donor serum-supplemented medium with either native or citrullinated forms of vimentin-, CII-, fibrinogen- and aggrecan-derived peptides at 3 or 30µg/ml. Proliferation in replicate wells ($2x10^5$ cells/well) was assessed after 5 days via 3HTdR incorporation. Levels of IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ in culture supernatants harvested after 5 days were assessed via cytometric bead array.	SI values of peptide-induced proliferation were very low (i.e. between 1 and 2) in both the RA patients and the HS. In the RA patients but not the HS, the SI values of citrullinated aggrecan-derived peptide-induced proliferation were significantly greater than those of native aggrecan-derived peptide-induced proliferation. The RA patients produced IL-6, IL-10, IL-17, TNF-α and IFN-γ in response to the peptides while the HS produced IL-6, IL-17 and TNF-α.

Table 1.4. Summary of previous studies investigating responses to citrullinated RA autoantigen-derived peptides. Abbreviations; PBMC = peripheral blood mononuclear cells, RA = rheumatoid arthritis, HS = healthy subjects, 3HTdR = ^{3}H-thymidine, SI = stimulation index, SE = shared epitope, CII = type II collagen.

Chapter 2. Materials and methods

2.1. Study subjects

Subjects used in this study were healthy volunteers recruited from within the Musculoskeletal Research Group of the University of Newcastle upon Tyne, and patients attending rheumatology clinics at the Freeman Hospital, Newcastle upon Tyne. RA patients were identified in accordance with the American College of Rheumatology revised classification criteria for RA (1987), while patients with other autoimmune inflammatory rheumatic diagnoses served as disease controls. Tables 2.1-2.7 show the clinical details of RA patients and disease controls used in the different sections of the study. Peripheral venous blood samples were collected from subjects after informed consent. Research described in this thesis was approved by the Newcastle and North Tyneside Research Ethics Committee.

2.2. Reagents and plastics

2.2.1. Reagents for cell culture, separation and storage

The following reagents were used:

- Calcium (Ca)- and magnesium (Mg)-free Dulbecco's Phosphate-Buffered Saline (DPBS) and Ca- and Mg-free Hank's Balanced Salt Solution (HBSS) containing Phenol Red (Biowhittaker, Lonza, Verviers, Belgium).
- Lymphoprep (Axis Shield, Oslo, Norway).
- Dimethyl sulphoxide (DMSO) and RPMI 1640 containing sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Gillingham, UK).
- Foetal bovine serum (FBS) and pooled human AB serum (HABS) (PAA Laboratories, Pasching, Austria).
- CellGro DC medium (CellGenix, Freiburg, Germany).

Table 2.8 shows the details of other reagents used for cell culture.

2.2.2. Cell culture stimuli

Tables 2.9 and 2.10 show the details of the various mitogens, antigens and peptides used as cell stimuli. Phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) were supplied as lyophilised powders and were reconstituted with RPMI 1640 to form stock solutions. All peptides were supplied as lyophilised powders and were reconstituted with sterile distilled water. DMSO was used as a solvent for certain peptides but at

concentrations no greater than 1%. For all stimuli, RPMI 1640 was used as a diluent to obtain the desired working dilutions.

2.2.3. Reagents for flow cytometry

The following reagents were used for flow cytometric analysis:
Bovine serum albumin (BSA), sodium azide (NaN_3) and paraformaldehyde (PFA) were all obtained from Sigma-Aldrich. Details of the fluorochrome-conjugated antibodies used are shown in Table 2.11.

2.2.4. Reagents for measurement of cytokine production in culture supernatant

The following reagents were used for the measurement of cytokine production in cell culture supernatants:
(i) For Enzyme-linked Immunosorbent Assays (ELISA):
Disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4H_2O$), sodium chloride (NaCl), Citric acid and disodium hydrogen phosphate dihydrate ($Na_2HPO_42H_2O$) were all obtained from Fisher Scientific, Loughborough, UK. Tween-20, hydrogen peroxide (H_2O_2), *o*-phenylenediamine dihydrochloride (OPD) tablets (15mg substrate/tablet) and concentrated sulphuric acid (H_2SO_4) were all obtained from Sigma-Aldrich.
(ii) For Electrochemiluminescence (ECL) Multiplex:
All reagents and materials were supplied by Meso Scale Discovery, Gaithersberg, Maryland, USA.
Table 2.12 shows details of additional cytokine detection reagents while Table 2.13 shows details of cytokine detection antibodies.

2.2.5. Plastics

The following were obtained from Greiner Bio-One, Stonehouse, UK:

- EDTA-containing and gel clot activator-containing specimen tubes.
- 5ml capacity bijou tubes.
- 30ml capacity universal tubes.
- 15ml and 50ml capacity centrifuge tubes.
- 3ml Pasteur pipettes.

The following were obtained from Corning Costar, Cambridge, Massachusetts, USA:

- 5ml, 10ml and 25ml capacity serological pipettes.
- 1ml capacity cryovials.

- Round-bottom 96-well tissue culture plates.
- Flat-bottom 96-well tissue culture plates.
- 24-well tissue culture plates.
- 48-well tissue culture plates.
- Flat-bottom high-bind 96-well ELISA plates.

The following were obtained from BD Falcon, Oxford, UK:

- Fluorescence-activated cell sorting (FACS) tubes.
- 100µm cell strainers.

The following were obtained from Starlab, Milton Keynes, UK:

- Eppendorf tubes.
- Repeater pipette tips in various sizes.

0.2µm vacuum filter units were obtained from Millipore, Watford, UK.

2.3. Cell culture techniques

2.3.1. Media and conditions

RPMI 1640 supplemented with 2mM L-glutamine, Penicillin-Streptomycin solution (100 units/ml & 100µg/ml respectively), 10mM HEPES and 50µM 2-Mercaptoethanol was used as culture medium. In addition, autologous serum (AS) at 5% was added to the culture medium unless stated otherwise. Cells were cultured at 37ºC in a humidified atmosphere containing 95% air and 5% carbon dioxide.

2.3.2. Isolation of peripheral blood mononuclear cells (PBMC)

Ethylenedinitrilo tetraacetic acid (EDTA)-treated peripheral blood samples were diluted 1:1 (volume:volume) with DPBS and layered onto lymphoprep and centrifuged at 895g with slow acceleration and deceleration for 30 minutes at room temperature. PBMC were collected from the interface of the two liquid layers (lymphoprep at the bottom and the diluted plasma at the top), washed twice in DPBS and resuspended for counting in RPMI 1640 containing 1% FBS. Viable PBMC were counted using a Neubauer haemocytometer and non-viable cells were excluded based on positive staining with Trypan Blue (Sigma-Aldrich).

2.3.3. Serum isolation

Peripheral blood samples collected into gel-based clot activator-containing tubes were subjected to centrifugation at 1800g for 10 minutes at room temperature. At the end of the centrifugation, clot (bottom layer) and serum (top layer) were separated by the gel layer and the serum was collected. Serum samples were either used immediately as a culture medium supplement as described in 2.3.1 or were stored at -20ºC.

2.3.4. Immunomagnetic isolation of $CD14^+$ and $CD3^+$ PBMC

CD3 and CD14 MACS® MicroBeads, MACS® LS separation columns and a VarioMACS® separator magnet (all obtained from Miltenyi Biotec, Surrey, UK) were used for all cell subsets isolations. MACS® buffer solution was prepared by supplementing DPBS with 0.5% FBS and 2mM EDTA and was used ice-cold after filtration through a 0.2µm membrane. Isolation of $CD14^+$ and $CD3^+$ PBMC was carried out according to manufacturer's protocol. In brief, suspensions of $5x10^7$-$1x10^8$ PBMC in 50ml ice-cold 1% FBS/HBSS were filtered through a 100µm cell strainer. PBMC were then centrifuged at 400g for 5 minutes and resuspended in 800µl MACS® buffer solution. 100µl of human CD14 MicroBeads were added prior to 20 minutes incubation on ice with gentle resuspension at 5 minute intervals. Cells were then washed and resuspended in 500µl of MACS® buffer solution before being added to a MACS® column (pre-rinsed with 3ml MACS® buffer solution) mounted on a MACS® separator magnet. Cells bound to the CD14 MicroBeads ($CD14^+$ fraction) were retained in the column and flow-through cells ($CD14^-$ fraction) were collected. The column was washed thrice, removed from the separator magnet and placed over a collection tube. Column-bound $CD14^+$ cells were recovered by flushing the column with 7ml MACS® buffer solution under gentle pressure using a plastic plunger. $CD14^+$ PBMC were then centrifuged at 400g for 5 minutes, resuspended in MACS® buffer solution and kept on ice until use. The $CD14^-$ flow-through cells were washed in MACS® buffer solution and resuspended in 800µl MACS® buffer solution. 100µl human CD3 MicroBeads were then added and the cells were incubated on ice for 20 minutes. At the end of the incubation period, the cells were washed and resuspended in 500µl MACS® buffer solution. $CD3^+$ cells (cells bound to the CD3 MicroBeads) were then positively selected with a fresh, pre-rinsed MACS® column as described earlier for the isolation of $CD14^+$ cells. The number of viable $CD3^+$ PBMC was enumerated using Trypan Blue and a Neubauer haemocytometer as described earlier prior to cryopreservation at -80ºC for 7 days until use in co-culture experiments. Purities of $CD14^+$ and $CD3^+$ fractions were

assessed in 4 separate isolations and the mean percentage purities of these fractions (± SEM) were 96 (± 1) and 97 (± 2) respectively.

2.3.5. PBMC cryopreservation

Un-separated or $CD3^+$ PBMC were resuspended in an ice-cold freezing medium comprising 10% DMSO/90% FBS (volume:volume) at $2x10^7$ cells/ml. 1ml aliquots were transferred to ice-cold cryovials which were then placed in a Mr Frosty cryopreserver (Nalgene, Thermo Fisher Scientific, Loughborough, UK) at -80ºC to allow controlled lowering of temperature at approximately 1ºC/minute. Cryogenically preserved cells were kept at -80ºC for the duration of storage.

For use in experiments, frozen aliquots of cells were partially thawed by immersion in a water bath at 37ºC for 1 minute and diluted immediately with 20ml warm 1% FBS-supplemented RPMI 1640. Thawed PBMC were washed twice in 1% FBS-supplemented RPMI 1640 and viable PBMC (counted as described in 2.3.2) were resuspended in culture medium at $1x10^6$/ml. The mean percentage (± SEM) of viable cells recovered from cryopreserved aliquots of un-separated PBMC (*n*=6) was 49 (± 3). The mean percentage (± SEM) of viable cells recovered from cryopreserved aliquots of $CD3^+$ PBMC (*n*=9) was 64 (± 4).

2.3.6. Monocyte-derived dendritic cells (mo-DC)

Freshly-isolated $CD14^+$ PBMC were cultured in CellGro DC medium supplemented with Penicillin-Streptomycin solution (100 units/ml & 100µg/ml respectively) in 24-well plates ($0.5x10^6$ cells/well in a volume of 1ml) with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) each at 50ng/well. After 3 days, 450µl of supernatant were removed from each well and replaced with 500µl of Penicillin-Streptomycin solution-supplemented CellGro DC medium containing 100ng/ml IL-4 and GM-CSF. After 6 days, loading of mo-DC with antigen was achieved by addition of PPD (1µg/ml) or test antigens (see Table 2.9 for concentrations) to appropriate cultures of mo-DC. At the same time-point, maturation of mo-DC, where required, was achieved through addition of LPS (0.1µg/ml) to cultures of mo-DC. After 7 days, mo-DC were harvested. Briefly, plates of mo-DC were incubated on ice for 1 hour in order to loosen the cells from the plastic, then cells were harvested through gentle scraping and pipetting motion and transferred to centrifuge tubes. Each well was rinsed with a 1ml volume of ice-cold 1% FBS/HBSS which was then added to the mo-DC in the centrifuge tubes and cells were washed 4 times at 4ºC in 1% FBS/HBSS then

resuspended in 500µl culture medium. Cells were counted using a Neubauer haemocytometer and non-viable cells were excluded based on positive staining with Trypan Blue. Suspensions of mo-DC were made up to the required concentration by addition of culture medium.

2.3.7. Cell labelling with carboxyfluorescein succinimidyl ester (CFSE)

PBMC were washed once in 0.1% FBS-supplemented DPBS and resuspended in 0.1% FBS-supplemented DPBS at $1x10^7$ cells/ml. The cells were then incubated with an equal volume of 2µM CFSE in DPBS for 5 minutes at 37ºC. CFSE labelling was terminated by adding 5ml of ice-cold 10% FBS-supplemented RPMI 1640 and incubating the cells on ice for 5 minutes. The cells were than washed once in 10% FBS-supplemented RPMI 1640 and resuspended in culture medium at $1x10^6$/ml.

2.3.8. Assessment of cellular proliferation using ^{3}H-thymidine (3HTdR) incorporation

Several different approaches were used in this study. In the first (triplicate culture) approach, PBMC at $1x10^6$ cells/ml were cultured in triplicates in round-bottom 96-well plates ($2x10^5$ cells/well, 200µl) with or without PHA (5µg/ml), tuberculin purified protein derivative (PPD, 1µg/ml) or test antigens/peptides (see Table 2.9 for concentrations). In the second (bulk culture) approach, PBMC at $1x10^6$ cells/ml were cultured in 24-well plates ($2x10^6$ cells/well, 2ml) with or without PHA, PPD or test antigens as described earlier. At the start of the final 20 hours of the culture period, the cells were resuspended in the well with a pipette and transferred in triplicate to round-bottom 96-well plates ($2x10^5$ cells/well, 200µl). Remaining cells were discarded. In the third (T-cell:mo-DC co-culture) approach, antigen-loaded mo-DC at varying concentrations were co-cultured with $CD3^+$ PBMC at $1x10^6$ cells/ml in 5% AS-supplemented RPMI 1640 in triplicates in round-bottom 96-well plates (200µl/well). Mo-DC without antigen-loading were used as negative controls.

For all 3 approaches, 3HTdR (20µl/well, 11 kBq) was added 20 hours prior to the end of the culture period. Cells were harvested using a semi-automated harvester onto filter papers which trapped the deoxyribonucleic acid (DNA) of the lysed cells. 3HTdR incorporation into the DNA of proliferating cells was quantified using a Microbeta TriLux scintillation counter (Perkin Elmer, Emeryville, California, USA). All data are expressed as either counts per minute (cpm) readings or as stimulation index (SI) values defined as mean cpm reading of the test (mitogen/antigen/peptide-stimulated) culture wells divided by the mean cpm value of the negative control (unstimulated) culture

wells. SI values of 2 or greater were considered to represent positive proliferative responses. Peak SI values of proliferation highlighted in red in data tables represent positive responses.

2.3.9. Assessment of cellular proliferation using CFSE dilution

CFSE-labelled PBMC at $1x10^6$ cells/ml were cultured in 48-well plates ($1x10^6$ cells/well, 1ml) with or without PHA, PPD or test antigens. Unlabelled PBMC cultured in the same conditions were used as controls as well as for determination of the appropriate compensation settings for flow cytometric analysis. Culture plates were wrapped in aluminium foil to protect from light exposure. At the end of the culture period, cells were stained with appropriate fluorochrome-conjugated antibodies before being analysed with a flow cytometer as described in 2.4.

For staining with fluorochrome-conjugated antibodies, CFSE-labelled and unlabelled PBMC were transferred to FACS tubes, washed in FACS buffer (DPBS supplemented with BSA at 1% and NaN_3 at 0.05%), and resuspended in 80µl of FACS buffer. Fluorochrome-conjugated antibodies at pre-determined optimal dilutions (Table 2.11) were added and the cells were incubated on ice for 30 minutes. Cells were then washed twice in FACS buffer and resuspended in 200µl of FACS buffer. The cells were fixed by adding 200µl of a solution of 2% PFA in DPBS to minimise internalisation of bound antibodies and stored at 4ºC until analysis with a flow cytometer. All data are expressed as either percentages of $CFSE^{dim}$ cells or as SI values defined as the percentage of $CFSE^{dim}$ cells in the test culture well divided by the percentage of $CFSE^{dim}$ cells in the negative control culture well. Antigen-induced increases in the percentages of $CFSE^{dim}$ cells of 0.1% or greater and SI values of 2 or greater were considered to represent positive proliferative responses and are highlighted in red in data tables.

The original population of cells labelled with CFSE have a $CFSE^{bright}$ phenotype (population '0' in Figure 2.1). When $CFSE^{bright}$ cells undergo division, the CFSE contained within them is split evenly between progeny cells therefore division generates a population with a CFSE fluorescence intensity 2-fold less than the original population. Successive rounds of proliferation produce populations with ever-decreasing CFSE fluorescence intensities (populations 1-4 in Figure 2.1). Thus the appearance of $CFSE^{dim}$ cells signifies proliferation and comparing the percentage/number of $CFSE^{dim}$ cells in an antigen-stimulated culture with that in an unstimulated culture with, for example, a stimulation index calculation, as described above, allows quantification of antigen-

specific proliferation. However, the CFSE dilution assay can also provide qualitative information about the proliferative response, specifically whether the $CFSE^{dim}$ cells are a result of many $CFSE^{bright}$ cells undergoing few rounds of division or of fewer $CFSE^{bright}$ cells undergoing a greater number of divisions. Therefore, in comparison with 3HTdR incorporation data, expressing the CFSE data as stimulation index values is less appropriate. For example, if 50 $CFSE^{dim}$ cells were detected in an unstimulated sample of CFSE-labelled cells and 640 $CFSE^{dim}$ cells were detected in the stimulated sample, then the SI value of the stimulated cells would be 12.8. However, this SI value gives no indication as to the quality of the stimulus-specific proliferative response; 640 $CFSE^{dim}$ cells could results from 160 $CFSE^{bright}$ cells undergoing 2 rounds of division (160 > 320 > 640) or from 10 $CFSE^{bright}$ cells undergoing 6 rounds of division (10 > 20 > 40 > 80 > 160 > 320 > 640). Indeed, it should technically be possible to determine the precursor frequency of antigen-specific cells within a $CFSE^{bright}$ population that produced a total population of $CFSE^{dim}$ cells. However, while preliminary work with proliferation modelling software showed that this could be achieved to some extent when analysing mitogen-induced proliferative responses in which distinct populations were observed, it was not possible when measuring antigen-specific responses because the number of cells that had undergone cell division were too few for reliable modelling using the software. Therefore SI values were used as a simple quantification of proliferation. To account for small variations in the numbers of cells analysed from different cultures in my CFSE experiments, SI values were calculated based on the percentage of $CFSE^{dim}$ cells and not the raw numbers.

2.4. Flow cytometric analysis

All flow cytometric analyses were performed using a flow cytometer (LSR II, Becton Dickinson, New Jersey, USA) equipped with FACSDiva software (Becton Dickinson). Acquisition thresholds based on forward scatter (FSC) and side scatter (SSC) characteristics were set using samples of unlabelled, unstimulated PBMC. Compensation was set prior to acquisition using samples of single-fluorochrome-labelled PBMC. Further adjustments in compensation were made if necessary using FACSDiva software after acquisition of data. For the estimation of the precursor frequencies of proliferating cells and other parameters of proliferation kinetics, the data were analysed using the Proliferation Platform of FlowJo version 8.7.1 software (Treestar Software, San Carlos, California, USA).

2.5. Quantification of cytokine production in culture supernatant

Supernatant samples were collected from cultures of unlabelled cells and stored in round-bottom 96-well plates at -20ºC until analysis for cytokine content. When using the triplicate culture or T-cell:mo-DC co-culture approaches, 50µl supernatant samples were collected from each of the wells in triplicate cultures prior to addition of 3HTdR and stored in pooled 150µl volumes. When using the bulk culture approach, 150µl supernatant samples were collected from individual bulk cultures prior to resuspension of the cells. Frozen supernatant samples were thawed for 1 hour at room temperature prior to analysis, and samples were discarded after undergoing 2 freeze/thaw cycles.

My choice of cytokines to quantify production of was based on both previous literature and preliminary work. As discussed in chapter 1, the importance of IL-17, IL-6 and TNF-α in the pathogenesis of RA is well documented, therefore it was essential that I assayed for the production of these cytokines. While IL-2 and IFN-γ are considered classical T-cell cytokines, the paucity of these cytokines in the RA synovium - discussed in chapter 1 - suggested that they may not be the most useful cytokines to assay for. However, a previously mentioned study (van Bilsen *et al*, 2004) described how opposing IFN-γ/IL-10 balances distinguished RA patients from healthy subjects in their responses to HCgp39. Therefore I chose to quantify the production of IFN-γ along with IL-10, anticipating that production of immunosuppressive IL-10 may be characteristic of healthy subjects as opposed to RA patients (Feldman *et al*, 1996 and McInnes & Schett, 2007). Preliminary work showed that IL-4 production was observed in response to certain candidate autoantigens so this was another cytokine of interest.

2.5.1. Electrochemiluminescence (ECL) multiplex analysis

The levels of IL-10, IL-17, IL-4, IL-6 and TNF-α in cell culture supernatants were measured via electrochemiluminescence using customized Human MULTI-SPOT 96-Well–7 Spot kits from Meso Scale Discovery according to the manufacturer's instructions. In brief, all incubations were performed at room temperature with shaking at 500rpm. Prior to use, plates were incubated for 30 minutes with 10% FBS-supplemented RPMI 1640 at 25µl/well. For each plate, six serial dilutions (4-fold) of cytokine standards of known concentrations (highest concentration for each cytokine was 10,000 pg/ml), and a ''blank'' sample, all in duplicate, were included for the construction of cytokine standard curves. 10% FBS-supplemented RPMI 1640 was used as diluent. DPBS supplemented with Tween-20 at 0.05% served as a wash buffer and

was used at a volume of 200µl/well. Detection antibody mixture at 50µg/ml was diluted 1/50 with ‘’antibody diluent’’ prior to use. ‘’Read buffer T 4X’’ was diluted 2-fold with dH_2O before use. For each assay, 25µl/well of test sample were added to the plates. After 4 hours of incubation, the plates were washed 3 times, detection antibody mixture was added at 25µl/well and the plates were incubated for a further 2 hours. The plates were then washed 3 times, ‘’read buffer’’ was added at 150µl/well and plates were read using a Sector Imager 2400 (Meso Scale Discovery). Plates were analysed and cytokine concentrations in test samples were estimated using MSD Workbench software (Meso Scale Discovery). Data were analysed using different approaches; raw pg/ml values, absolute ‘pg/ml minus background’ values defined as pg/ml value of supernatant from test culture well minus pg/ml value of supernatant from negative control culture well, or as SI values defined as pg/ml value of supernatant from test culture well divided by pg/ml value of supernatant from negative control culture well. The lower limits of detection for IL-10, IL-17, IL-4, IL-6 and TNF-α were defined by Meso Scale Discovery as 2.5 standard deviations above ‘’blank’’ well/background value and mean (± SEM) values from seventeen assays were 1.5 (± 0.5), 1.8 (± 0.2), 1.4 (± 0.5), 1.4 (± 0.6) and 1.3 (± 0.4) respectively. Therefore, raw or absolute values of IL-10, IL-17, IL-4, IL-6 or TNF-α production of 2pg/ml or greater were considered to represent positive responses. IL-10, IL-17, IL-4, IL-6 or TNF-α production values highlighted in red in data tables represent positive responses. IL-10, IL-17, IL-4, IL-6 or TNF-α production values below the threshold level of detection are represented as ‘0’ in data tables. SI values of 2 or greater were considered to represent positive responses.

2.5.2. Enzyme-linked immunosorbent assay (ELISA) analysis

The levels of IFN-γ and IL-6 in cell culture supernatants were measured using ELISA. All assays were performed using high-bind 96-well plates. Coating buffer consisted of 4.35g Na_2HPO_4 and 5.37g $NaH_2PO_4H_2O$ in 500ml dH_2O. 10X PBS consisted of 163.5g NaCl, 24.2g Na_2HPO_4 and 4.4g $NaH_2PO_4H_2O$ in 2L dH_2O and was diluted 1/9 in dH_2O prior to use. Citrate phosphate buffer consisted of 2.55g Citric acid, 3.66g Na_2HPO_4 and 4.58g $Na_2HPO_42H_2O$ in 500ml dH_2O. PBS containing Tween-20 at 0.1% was used as a wash buffer in which plates were washed by submersion. Wash buffer supplemented with BSA at 1% was used as both a blocking solution and as a diluent for all dilutions unless otherwise stated. For each plate, 9 serial 2-fold dilutions in duplicates of cytokine standards of known concentrations (highest concentration for each cytokine was 2,000pg/ml), and a ‘’blank’’ sample also in duplicate containing diluent only were

included for construction of cytokine standard curves. OPD tablets were each dissolved in 13ml citrate phosphate buffer to produce substrate solution and 6µl of H_2O_2 were added to each 13ml volume immediately prior to use.

To all ELISA plates, purified IFN-γ or IL-6 capture antibody (Table 2.13) diluted to 1µg/ml in coating buffer was added at 50µl/well and incubated overnight at 4ºC. Plates were washed once, followed by incubation for 2 hours at room temperature with blocking solution at 100µl/well. Plates were washed 4 times prior to addition of standards, blanks and test samples, all in duplicates at 50µl/well, and incubated overnight at 4ºC. Plates were washed 4 times, corresponding biotinylated detection antibody (Table 2.13) diluted to 1µg/ml was then added at 50µl/well and plates were incubated for 1 hour at room temperature. Plates were washed 4 times, Streptavidin-horse radish peroxidase (HRP) solution diluted to 1µg/ml was added at 50µl/well and plates were incubated for 30 minutes at room temperature. Plates were washed 5 times and substrate solution was added at 50µl/well. Reactions were terminated by addition of 3M H_2SO_4 at 50µl/well and the optical density of the solution in each well was read spectrophotometrically at 490nm using a Sunrise microplate absorbance reader (Tecan, Switzerland). Levels of IFN-γ or IL-6 in test samples were estimated using the linear regression function of GraphPad Prism version 5.01 (GraphPad Software, San Diego, California, USA) which used the cytokine standard curve to interpolate the concentrations of unknown samples. Data were analysed as described in 2.5.1. The lower limit of detection for IFN-γ was 5pg/ml. IFN-γ production values highlighted in red in data tables represent positive responses. IFN-γ production values below the threshold level of detection are represented as '0' in data tables. SI values of 2 or greater were considered to represent positive responses.

2.6. Limulus amoebocyte lysate (LAL) endotoxin detection assay

The levels of bacterial endotoxin in protein or peptide preparations were assessed quantitatively using chromogenic LAL assays. All assays were carried out in sterile flat-bottom 96-well plates pre-warmed to 37ºC and used QCL-1000 LAL kits (Lonza, Walkersville, Maryland, USA). All incubations were carried out at 37ºC in a humidified environment containing 95% air and 5% CO_2. Briefly, vials of lyophilised *E. coli* endotoxin, chromogenic substrate and LAL were reconstituted according to the manufacturer's protocol using LAL reagent water (all reagents were supplied with the kit). Solutions containing 1, 0.5, 0.25 and 0.1EU of endotoxin per ml were prepared for the construction of the endotoxin standard curve. Test samples and endotoxin standards

were added to plates in duplicates at 25µl/well. LAL reagent water was used as a negative control. Equal volumes of LAL were added to the wells and the plates were incubated for 16 minutes. 50µl chromogenic substrate pre-warmed to 37ºC, were added to each of the wells and plates were incubated for a further 6 minutes. Reactions were terminated by addition of 50µl/well of a solution of 25% glacial acetic acid (BDH Prolabo, VWR, Lutterworth, UK) in dH_2O, and the optical density of the solution in each well was read at 405nm using a Sunrise microplate absorbance reader (Tecan). Levels of endotoxin in test samples were estimated using the linear regression function of GraphPad Prism version 5.01 which used the endotoxin standard curve to interpolate the endotoxin content of unknown samples. 10EU/ml equates to approximately 1ng/ml of endotoxin.

2.7. Removal of endotoxin from contaminated protein/peptide samples

Bacterial endotoxin was removed from stock preparations of LPS-contaminated DeglyAgg/CEP-1 using a Proteospin Endotoxin Removal Micro kit (Norgen, Geneflow, Staffordshire, UK) in accordance with the manufacturer's protocol. The kit included spin columns, collection tubes, column activation & wash buffer, binding buffer, endotoxin removal solution, neutraliser, elution tubes and elution buffer. Briefly, a sample of LPS-contaminated protein or peptide preparation of up to 150µg was made up to a volume of 450µl with dH_2O and mixed with 20µl of binding buffer. The resulting solution was loaded onto one of the provided spin columns (pre-rinsed and centrifuged for 1 minute at 14,000g with two 500µl volumes of column activation & wash buffer), mixed with a 1% volume of endotoxin removal solution and incubated for 5 minutes at room temperature. A 10% volume of isopropanol (Fisher Scientific) was then added to the column which was then centrifuged for 1 minute at 14,000g and the flow-through was discarded. Column-bound protein/peptide was washed with two 500µl volumes of column activation & wash buffer. The column was then inserted into an elution tube containing 9.3µl of neutraliser and clean protein/peptide was eluted from the column into the elution tube by addition of two 50µl volumes of elution buffer. According to the data provided by the manufacturer, 95% of a protein/peptide sample can be recovered after the endotoxin removal process. The eluted protein/peptide samples were made up to 1mg/ml by further dilution with elution buffer based on a 95% recovery rate. Actual concentrations of endotoxin-free protein/peptide samples were determined by the Bradford protein assay (see 2.8).

2.8. Bradford protein assay

Bradford reagent (Sigma-Aldrich) was diluted 1:1 (volume:volume) in sterile nanopure water prior to use. For creation of standard protein/peptide solutions of 2, 4, 6, 8 and 10µg/ml, 2, 4, 6, 8 and 10µl volumes of a 1mg/ml BSA solution in sterile Nanopure water were added to eppendorf tubes each containing 1ml Bradford reagent and solutions were mixed by vortexing. Bradford reagent alone served as a blank. As a positive control, 5µl of a 1mg/ml solution of CEP-1 (pre-endotoxin removal hence 5µg) were added to a 1ml volume of Bradford reagent and the solution was mixed by vortexing. For the test sample, 5µl of a solution of CEP-1 *clean* (post-endotoxin removal but concentration adjusted to 1mg/ml) peptide were added to a 1ml volume of Bradford reagent and solution was mixed by vortexing. After 10 minutes of incubation at room temperature, the standard solutions, the blank and the positive control and test sample solutions were added to a flat-bottom 96-well plate in duplicates at 100µl/well and absorbance was read at 595nm using a Sunrise microplate absorbance reader (Tecan). Levels of protein/peptide in test samples were estimated using the linear regression function of GraphPad Prism version 5.01 which used the protein standard curve to interpolate the concentrations of unknown samples.

2.9. Statistical analysis

Statistical analysis, which included descriptive statistics, linear regression, Chi-square tests, Mann-Whitney tests and paired t tests, were performed using GraphPad Prism version 5.01. P values of less than 0.05 were considered statistically significant.

ID	Sex	Age	RF/CCP	CRP/ESR	Year of diagnosis	Medication
RA 4.1	F	56	Pos/ND	9/34	2000	SSZ
RA 4.2	M	65	Pos/ND	64/54	>15 years ago	MTX/ETA
RA 4.3	F	36	ND/ND	ND/8	2001	SSZ
RA 4.4	F	64	ND/ND	5/40	>15 years ago	MTX
RA 4.5	M	60	Neg/ND	6/10	2007	MTX
RA 4.6	F	45	Pos/Neg	11/38	2008	MTX
RA 4.7	F	57	Pos/ND	ND/20	2001	ADA
RA 4.8	M	75	ND/ND	ND/4	>15 years ago	Gold
RA 4.9	F	50	Pos/Pos	ND/12	2002	SSZ
RA 4.10	F	42	Pos/Pos	ND/31	2007	MTX
RA 4.11	F	43	Pos/Pos	8/ND	2008	None
RA 4.12	F	55	Pos/ND	ND/36	>15 years ago	MTX/ETA
RA 4.13	M	56	Pos/ND	ND/2	2002	MTX
RA 4.14	M	76	Neg/Neg	5/48	2002	SSZ
RA 4.15	F	27	Neg/Neg	ND/10	2008	MTX

Table 2.1. Clinical details of RA patients tested in the HCgp39 and CFSE experiments described in 4.2.1. Abbreviations; F = female, M = male, RF = rheumatoid factor, CCP = cyclic citrullinated peptide antibody, Pos = positive, ND = no data, Neg = negative, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, Many years ago = pre 1995, ADA = Adalimumab, ETA = Etanercept, MTX = Methotrexate, SSZ = Sulfasalazine.

ID	Sex	Age	RF/CCP	CRP/ESR	Year of diagnosis	Medication
RA 4.16	F	68	Pos/Pos	<5/10	>15 years ago	LEF
RA 4.17	F	73	Pos/ND	14/66	>15 years ago	MTX (previous RTX)
RA 4.18	F	59	ND/ND	11/36	>15 years ago	MTX+ETA
RA 4.19	F	49	ND/ND	<5/30	2007	MTX
RA 4.20[1]	F	81	Pos/ND	20/46	2000	LEF
RA 4.21[2]	F	65	Pos/Pos	ND/12	>10 years ago	SSZ
RA 4.22[3]	M	64	ND/ND	9/66	>12 years ago	ETA
RA 4.23[4]	F	82	Pos/Pos	ND/28	2007	MTX
RA 4.24[5]	F	35	Pos/Pos	ND/12	2009	None, to start MTX

Table 2.2. Clinical details of RA patients tested in the bulk culture 3HTdR incorporation experiments described in 4.2.2. Abbreviations; F = female, M = male, RF = rheumatoid factor, CCP = cyclic citrullinated peptide antibody, Pos = positive, ND = no data, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, Many years ago = pre 1995, ETA = Etanercept, LEF = Leflunomide, MTX = Methotrexate, RTX = Rituximab, SSZ = Sulfasalazine. Patients with a superscripted number following their ID ([1, 2, 3, 4 & 5]) were those also tested in the experiments described in 4.2.3.

ID	Sex	Age	RF/CCP	CRP/ESR	Year of diagnosis	Medication
RA 4.25	F	77	Pos/Pos	20/50	2008	MTX
RA 4.26	M	47	Pos/Pos	7/34	2007	SSZ+HCQ
RA 4.27	F	67	ND/ND	11/40	2007	LEF
RA 4.28	F	46	Pos/Pos	ND/22	2008	None, to start MTX
RA 4.29	M	77	Pos/Pos	ND/28	2007	MTX
RA 4.30	F	60	ND/ND	5/22	>12 years ago	anti-TNF
RA 4.31	F	57	Neg/ND	17/34	2006	MTX+SSZ
RA 4.32	F	47	Pos/Pos	13/30	2006	SSZ
RA 4.33	F	52	Pos/Pos	10/56	2007	None
RA 4.34	F	50	Neg/Neg	34/32	2006	SSZ
RA 4.35	M	32	Neg/ND	9/6	2003	MTX
RA 4.36	F	55	ND/ND	5/18	>12 years ago	MTX
RA 4.37	F	65	Pos/ND	ND/14	2001	MTX
RA 4.38	F	36	Neg/Pos	ND/24	2008	MTX
RA 4.39	F	64	Pos/Pos	ND/52	2008	MTX
RA 4.40	F	49	Pos/Pos	ND/12	2007	MTX
RA 4.41	F	52	Pos/ND	ND/10	2006	ADA+MTX
RA 4.42[1]	F	81	Pos/ND	20/46	2000	LEF
RA 4.43[2]	F	65	Pos/Pos	ND/12	>10 years ago	SSZ
RA 4.44[3]	M	64	ND/ND	9/66	>12 years ago	ETA
RA 4.45[4]	F	82	Pos/Pos	ND/28	2007	MTX
RA 4.46[5]	F	35	Pos/Pos	ND/12	2009	None, to start MTX
RA 4.47	F	54	Pos/ND	7/13	2000	MTX
RA 4.48	M	61	Pos/ND	<5/18	>15 years ago	ETA+MTX

Table 2.3. Clinical details of RA patients tested in the triplicate culture 3HTdR incorporation experiments described in 4.2.3. Abbreviations; F = female, M = male, RF = rheumatoid factor, CCP = cyclic citrullinated peptide antibody, Pos = positive, ND = no data, Neg = negative, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, ADA = Adalimumab, ETA = Etanercept, HCQ = Hydroxychloroquine, LEF = Leflunomide, MTX = Methotrexate, SSZ = Sulfasalazine. Patients with a superscripted number following their ID ([1, 2, 3, 4 & 5]) were those also tested in the experiments described in 4.2.2.

ID	Sex	Age	RF/CCP	CRP/ESR	Year of diagnosis	Medication
RA 5.1	F	50	Pos/ND	<5/14	2005	MTX+ETA
RA 5.2	F	63	Pos/ND	10/36	1998	LEF
RA 5.3	F	56	Pos/ND	5/60	1998	MTX+ETA
RA 5.4	F	77	Neg/Neg	<5/36	1998	MTX
RA 5.5	F	51	Pos/ND	<5/12	2003	SSZ+LEF
RA 5.6	F	80	Neg/Neg	<5/40	2008	SSZ
RA 5.7	F	38	ND/ND	<5/6	2001	MTX
RA 5.8[6]	M	62	Pos/ND	25/56	2004	MTX
RA 5.9[7]	F	48	Pos/Pos	<5/6	2009	MTX
RA 5.10	F	71	Pos/ND	7/28	1998	MTX

Table 2.4. Clinical details of RA patients tested in the peptide panel #1 experiments described in 5.2.1. Abbreviations; F = female, M = male, RF = rheumatoid factor, CCP = cyclic citrullinated peptide antibody, Pos = positive, ND = no data, Neg = negative, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, ETA = Etanercept, LEF = Leflunomide, MTX = Methotrexate, SSZ = Sulfasalazine. Patients with a superscripted number following their ID ([6 & 7]) were those also tested in the experiments described in 5.2.3/5.2.5.

ID	Sex	Age	RF/CCP	CRP/ESR	Year of diagnosis	Medication
RA 5.11[8]	F	52	Pos/ND	10/60	2005	LEF/MTX
RA 5.12[9]	M	64	ND/ND	15/66	>15 years ago	ETA+HCQ
RA 5.13	F	62	Pos/Neg	<5/14	2009	MTX
RA 5.14	F	64	ND/ND	<5/26	1998	MTX
RA 5.15[10]	F	50	Pos/Pos	11/25	2007	MTX
RA 5.16[11]	F	61	Pos/Neg	6/15	2009	MTX
RA 5.17[12]	F	71	Pos/ND	<5/9	1998	ETA+MTX
RA 5.18[13]	M	45	Pos/Pos	11/12	2009	MTX
RA 5.19[14]	F	73	Pos/ND	<5/12	1999	ETA+MTX

Table 2.5. Clinical details of RA patients tested in the peptide panel #2 experiments described in 5.2.2. Abbreviations; F = female, M = male, RF = rheumatoid factor, CCP = cyclic citrullinated peptide antibody, Pos = positive, ND = no data, Neg = negative, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, ETA = Etanercept, HCQ = Hydroxychloroquine, LEF = Leflunomide, MTX = Methotrexate. Patients with a superscripted number following their ID ([8, 9, 10, 11, 12, 13 & 14]) were those also tested in the experiments described in 5.2.3/5.2.5.

ID	Sex	Age	RF/CCP	CRP/ESR	Year of diagnosis	Medication
RA 5.20	F	67	Pos/ND	ND/40	1998	MTX/HCQ
RA 5.21	F	58	Neg/Pos	ND/32	2009	MTX
RA 5.22	F	38	ND/ND	ND/8	2001	SSZ/MTX
RA 5.23	F	45	Neg/ND	<5/16	2003	ETA+MTX
RA 5.24	F	54	ND/ND	11/6	1999	MTX
RA 5.25	F	64	Pos/Neg	<5/16	1980	MTX
RA 5.26[8]	F	52	Pos/ND	10/60	2005	LEF/MTX
RA 5.27[9]	M	64	ND/ND	15/66	>15 years ago	ETA+HCQ
RA 5.28	F	48	Pos/ND	<5/10	2003	MTX+HCQ
RA 5.29	F	73	Pos/Pos	<5/8	2007	SSZ
RA 5.30	F	68	ND/ND	<5/9	1993	LEF
RA 5.31	F	64	Pos/ND	79/80	2002	LEF
RA 5.32	M	53	Neg/Neg	<5/12	2007	LEF
RA 5.33	F	59	Pos/ND	<5/16	2001	MTX+ADA
RA 5.34	F	79	Pos/Pos	<5/24	2009	MTX
RA 5.35	F	59	Neg/Pos	<5/40	2009	MTX
RA 5.36	F	56	Neg/Neg	<5/11	2009	SSZ
RA 5.37	M	72	Neg/Neg	<5/6	ND	MTX
RA 5.38[6]	M	62	Pos/ND	25/56	2004	MTX
RA 5.39[7]	F	48	Pos/Pos	<5/6	2009	MTX
RA 5.40	F	59	Neg/ND	23/36	2005	MTX+SSZ
RA 5.41	F	44	Pos/Pos	<5/12	2009	MTX
RA 5.42	F	56	ND/ND	<5/18	1997	MTX
RA 5.43[10]	F	50	Pos/Pos	11/25	2007	MTX
RA 5.44[11]	F	61	Pos/Neg	6/15	2009	MTX
RA 5.45[12]	F	71	Pos/ND	<5/9	1998	ETA+MTX
RA 5.46[13]	M	45	Pos/Pos	11/12	2009	MTX
RA 5.47[14]	F	73	Pos/ND	<5/12	1999	ETA+MTX

Table 2.6. Clinical details of RA patients tested in the REP-1 and CEP-1 experiments described in 5.2.3/5.2.5. Abbreviations; F = female, M = male, RF = rheumatoid factor, CCP = cyclic citrullinated peptide antibody, Pos = positive, ND = no data, Neg = negative, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, ADA = Adalimumab, ETA = Etanercept, HCQ = Hydroxychloroquine, LEF = Leflunomide, MTX = Methotrexate, SSZ = Sulfasalazine. Patients with superscripted numbers following their ID were those also tested in the experiments described in 5.2.1 ([6 & 7]) or 5.2.2 ([8, 9, 10, 11, 12, 13 & 14]).

ID	Sex	Diagnosis	Age	RF/CCP	CRP/ESR	Year of diagnosis	Medication
DisC 5.1	F	MCTD	78	Pos/ND	<5/30	2005	MTX
DisC 5.2	M	PsA	75	Neg/ND	8/43	2009	MTX
DisC 5.3	F	SLE	48	Neg/ND	10/80	2008	HCQ+MTX
DisC 5.4	F	Sweet syndrome	37	Neg/ND	12/12	2009	None
DisC 5.5	F	PsA	38	Neg/Neg	6/26	2009	SSZ
DisC 5.6	F	PsA	55	Neg/ND	<5/18	2002	MTX+SSZ
DisC 5.7	F	PsA	45	ND/ND	<5/40	2009	SSZ
DisC 5.8	F	SAPHO syndrome	60	ND/ND	<5/40	ND	MTX
DisC 5.9	M	PsA	48	ND/ND	6/48	1997	MTX
DisC 5.10	M	PsA	45	Neg/ND	<5/18	2005	MTX
DisC 5.11	F	PsA	64	Neg/Neg	<5/10	2006	SSZ

Table 2.7. Clinical details of disease controls tested in the REP-1 and CEP-1 experiments described in 5.2.3. Abbreviations; DisC = disease control, F = female, M = male, MCTD = mixed connective tissue disease, PsA = Psoriatic arthritis, SLE = systemic lupus erythematosus, SAPHO = synovitis-acne-pustulosis-hyperostosis-osteitis, RF = rheumatoid factor, CCP = cyclic citrullinated peptide antibody, Pos = positive, ND = no data, Neg = negative, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, HCQ = Hydroxychloroquine, MTX = Methotrexate, SSZ = Sulfasalazine.

Reagent	Source	Stock concentration	Working dilution	Final concentration
L-Glutamine	Sigma-Aldrich, Gillingham, UK	200mM	Stock	2mM
Penicillin-Streptomycin		10,000 units/ml & 10mg/ml	Stock	100 units/ml & 100µg/ml
HEPES buffer		1M	Stock	10mM
2-Mercaptoethanol		14M	1/280	50µM
CFSE		5mM	1/100 then 1/25	1µM
PMB		50mg/ml	1/10	25µg/ml
EDTA	Fisher Scientific, Loughborough, UK	0.5M	Stock	2mM
Recombinant human IL-4	ImmunoTools, Friesoythe, Germany	250µg/ml	1/50	50ng/ml
GM-CSF	Genzyme, Oxford, UK	250µg/ml	1/50	50ng/ml
3HTdR	Perkin Elmer, Turku, Finland	37MBq/ml	0.55MBq/ml	65kBq/ml & 55kBq/ml

Table 2.8. Supplementary reagents used in cell culture. Abbreviations; CFSE = carboxyfluorescein succinimidyl ester, PMB = polymyxin B, EDTA = ethylenedinitrilo tetraacetic acid, IL-4 = interleukin 4, GM-CSF = granulocyte-macrophage colony-stimulating factor, 3HTdR = ^{3}H-thymidine.

Stimulus	Source	Stock concentration	Working dilution	Final concentration
PHA P-lectin from *phaseolus vulgaris*	Sigma-Aldrich, Gillingham, UK	1mg/ml	Stock	5µg/ml
LPS		1mg/ml	1/5 or 1/10	Various
PPD	Calbiochem, San Diego, California, USA	1mg/ml	1/5	1µg/ml
HCgp39	A kind gift from Organon	2mg/ml	Stock	10µg/ml
CII	A kind gift from Dr Amit Patel (*1)	0.85mg/ml	Stock	10µg/ml
GlyAgg	Kind gifts from Dr Caroline Wilson (*2)	1.45mg/ml	Stock	10µg/ml
DeglyAgg		1.45mg/ml	Stock	10µg/ml
Peptide 1	JPT Peptide Technologies, Berlin, Germany	4mg/ml	1/4	10µg/ml
Peptide 2		4mg/ml	1/4	10µg/ml
Peptide 3		4mg/ml	1/4	10µg/ml
Peptide 4		4mg/ml	1/4	10µg/ml
Peptide 5		4mg/ml	1/4	10µg/ml
Peptide 6		4mg/ml	1/4	10µg/ml
Peptide 7		4mg/ml	1/4	10µg/ml
Peptide 8	ProImmune, Oxford, UK	4mg/ml	1/4	10µg/ml
Peptide 9		4mg/ml	1/4	10µg/ml
Peptide 10		4mg/ml	1/4	10µg/ml
Peptide 11		4mg/ml	1/4	10µg/ml
Peptide 12		4mg/ml	1/4	10µg/ml
Peptide 13		4mg/ml	1/4	10µg/ml
Peptide 14		4mg/ml	1/4	10µg/ml
Peptide 15		4mg/ml	1/4	10µg/ml
Peptide REP-1	Kind gifts from Professor Patrick Venables (Imperial College)	10mg/ml	1/10	10µg/ml
Peptide CEP-1		10mg/ml	1/10	10µg/ml
Peptide REP-1 (2^{nd} batch)		5mg/ml	1/5	10µg/ml
Peptide CEP-1 (2^{nd} batch)		5mg/ml	1/5	10µg/ml

Table 2.9. Cell culture stimuli. Abbreviations; PHA = phytohaemagglutinin, LPS = lipopolysaccharide, PPD = purified protein derivative (tuberculin), HCgp39 = human cartilage glycoprotein 39, CII = type II collagen, GlyAgg = glycosylated aggrecan, DeglyAgg = deglycosylated aggrecan. *1 = (manufactured in-house from human articular cartilage), *2 = (manufactured in-house from bovine nasal cartilage).

Peptide ID	Sequence	Antigen
1	SAVRA(Cit)SSVPGVR	Cit-Vimentin$_{65-77}$
2	QDFTN(Cit)INKLKNS	Cit-Fibrinogen α–chain$_{80-92}$
3	KIHA(Cit)EIFDS(Cit)GNPTVE	Cit-Enolase$_{5-21}$
4	HSASQDGQDTI(Cit)GHPGSS	Cit-Filaggrin$_{2776-2793}$
5	DSGH(Cit)GYSGSQASDNEGH	Cit-Filaggrin$_{3005-3022}$
6	CVLLVATEGRVRVNSAYQDC	Aggrecan$_{84-103}$ with terminal cysteines
7	CVLLVATEG(Cit)VRVNSAYQDC	Cit-Aggrecan$_{84-103}$ with terminal cysteines
8	AGWLADQTVRYPI	Aggrecan$_{201-213}$
9	AGWLADQTV(Cit)YPI	Cit-Aggrecan$_{201-213}$
10	AGWLADRSVRYPI	Aggrecan$_{299-311}$
11	AGWLAD(Cit)SVRYPI	Cit-Aggrecan$_{299-311}$
12	GRSFTLASSETGVGA	HCgp39$_{262-276}$
13	G(Cit)SFTLASSETGVGA	Cit-HCgp39$_{262-276}$
14	VVLLVATEG(Cit)VRVNSAYQDK	N Cit-Aggrecan$_{84-103}$
15	VVLLVATEGRV(Cit)VNSAYQDK	C Cit-Aggrecan$_{84-103}$
REP-1	CKIHAREIFDSRGNPTVEC	Enolase$_{5-21}$ with terminal cysteines
CEP-1	CKIHA(Cit)EIFDS(Cit)GNPTVEC	Cit-Enolase$_{5-21}$ with terminal cysteines

Table 2.10. Peptide details. Abbreviations; (Cit) = citrulline, Cit- = citrullinated. The purities of peptides 1-15 were >80%. The purities of the REP-1 and CEP-1 peptides were >95%.

Antibody	Source	Clone	Isotype	Final dilution
Pacific Blue anti-human CD3	eBioscience, Hatfield, UK	OKT3	Mouse IgG2a	1/9
APC anti-human CD4	ImmunoTools, Friesoythe, Germany	MEM-241	Mouse IgG1	1/9
PE anti-human CD19		LT19	Mouse IgG1	1/9
APC anti-human CD80		MEM-233	Mouse IgG1	1/9
FITC anti-human CD86		BU63	Mouse IgG1	1/9
PE anti-human HLA-DR		MEM-12	Mouse IgG1κ	1/9

Table 2.11. Flow cytometry antibodies. Abbreviations; CD = cluster of differentiation, APC = allophycocyanin, PE = phycoerythrin, FITC = fluorescein isothiocyanate, HLA = human leukocyte antigen.

Reagent	Source	Stock concentration	Working dilution
IL-10	Meso Scale Discovery, Gaithersberg, Maryland, USA	50µg/ml	1/5
IL-17		50µg/ml	1/5
IL-4		50µg/ml	1/5
IL-6		50µg/ml	1/5
TNF-α		50µg/ml	1/5
Recombinant human IFN-γ	BD Pharmingen, San Diego, California, USA	2µg/ml	1/1000
Recombinant human IL-6		2µg/ml	1/1000
Streptavidin-HRP	Dako, Stockport, UK	1mg/ml	1/1000

Table 2.12. Additional reagents for cytokine detection. Abbreviations; IL = interleukin, TNF-α = tumour necrosis factor alpha, IFN-γ = interferon gamma, HRP = horse radish peroxidase.

Antibody	Source	Clone	Isotype	Stock concentration	Working dilution
SULFO-TAG human IL-10, IL-17, IL-4, IL-6 & TNF-α detection antibody mix	Meso Scale Discovery, Gaithersberg, Maryland, USA	Data not available from manufacturer		50µg/ml	1/50
Purified anti-human IFN-γ	BD Pharmingen, San Diego, California, USA	NIB42	Mouse IgG1κ	1mg/ml	1/1000
Purified anti-human IL-6		MQ2-13A5	Rat IgG1	0.5mg/ml	1/500
Biotinylated anti-human IFN-γ		4S.B3	Mouse IgG1κ	0.5mg/ml	1/500
Biotinylated anti-human IL-6		MQ2-39C3	Rat IgG2a	0.5mg/ml	1/500

Table 2.13. Cytokine detection antibodies. Abbreviations; IL = interleukin, TNF-α = tumour necrosis factor alpha, IFN-γ = interferon gamma.

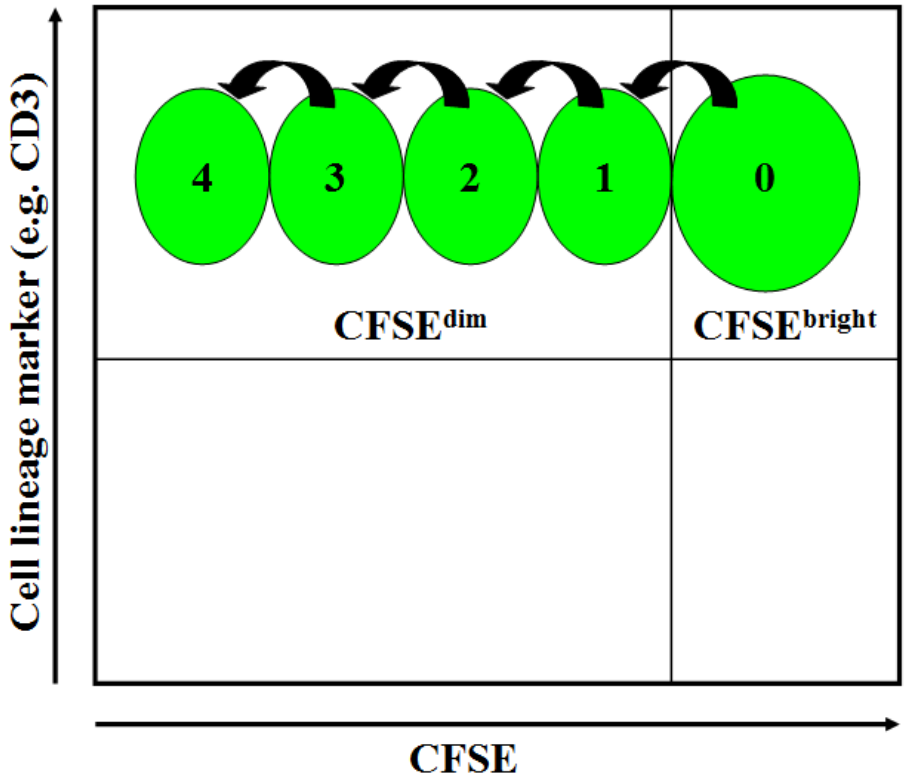

Figure 2.1. Diagram of a CFSE versus cell-lineage-marker flow cytometry dot plot showing dilution of CFSE by cell division. '0' represents an initially labelled population of 'parent' cells with $CFSE^{bright}$ phenotypes. '1', '2', '3' & '4' represent populations of 'progeny' cells which have ever-decreasing CFSE fluorescence intensities and arise from subsequent rounds of cell division.

Chapter 3. Optimisation of a system for detecting autoantigen-specific T-cell immune responses

3.1. Introduction

Although the pathogenesis of RA is thought to involve autoimmune T-cell responses, the autoantigens against which these T-cell responses are directed have not been fully defined. Furthermore, the mechanisms that regulate immune responses to candidate autoantigens are poorly understood. Thus the role of autoantigen-specific T-cell responses in RA remains under debate. A major argument against the role of autoimmune T-cell responses in RA is that such responses have proved difficult to be consistently identified within RA patients. There are several explanations as to why this might be the case, the simplest being that RA is in fact not driven by autoimmune T-cells. However, there are a number of other explanations that do not rule out a role for autoantigen-specific T-cells in RA pathogenesis.

Firstly, the paucity of detectable T-cell responses to putative RA autoantigens in previous studies could be related to the study design. For example, it may be the case that previous studies have focussed on inappropriate autoantigens. Furthermore, studies investigating more than one candidate autoantigen (Kotzin *et al*, 2000; Fritsch *et al*, 2002 and Zou *et al*, 2003) are small in number, with the vast majority of previous studies having focussed on single candidate autoantigens, and it is possible that different autoantigens are involved in different individuals. It is also possible that T-cells specific for many different autoantigens exist in RA patients but that their responses to single autoantigens are too weak to be detected in peripheral blood. Another possibility is that the protocols used in previous studies may have been sub-optimal. For example, subjects tested in previous studies have usually had long-standing RA and/or a history of immunomodulatory therapy. If the T-cell responses responsible for initiating the disease are short-lived, rare or subtle, then the use of such patients may bias against the detection of responses. Perhaps more importantly, it is possible that the methods used to detect autoantigen-specific T-cell responses in previous studies were not sufficiently sensitive. A study of autoantigen-specific immune responses is only as robust as the assays used to detect the responses. Thus, in order to achieve our overall aims, we first needed to optimise an experimental system for the detection of such autoantigen-specific T-cell responses.

3.1.1. Detecting proliferation and cytokine production: Assays and culture media

In the majority of previous studies measuring proliferation of whole PBMC in response to candidate RA autoantigens, proliferation has been assessed using the 3HTdR incorporation assay (Goodstone *et al*, 1996; Verheijden *et al*, 1997; Snowden *et al*, 1997; Boots *et al*, 1997; Guerassimov *et al*, 1998; Cope *et al*, 1999; Kim *et al*, 1999; Vos *et al* #2, 2000; Park *et al*, 2001; Ria *et al*, 2008 and de Jong *et al*, 2010). Furthermore, this approach has been considered a method of detecting T-cell proliferation, yet when measuring proliferation in a mixed PBMC population using this method it is not possible to determine the cellular subsets that have undergone cell division. Nevertheless, it is a well-validated technique, is easy to perform, and has been utilised in many previous studies in this area of work. Therefore, I considered it important to include this assay in this part of my project. In addition, I evaluated the use of the CFSE dilution assay as a means of measuring proliferation. This is because in combination with specific cell surface molecule immunofluorescence staining, this assay is not only able to detect proliferation, but also allows the determination of the cellular subset(s) within the whole PBMC population that have proliferated. In addition, a study by Mannering *et al* (2003) demonstrated the superior sensitivity of the CFSE dilution assay to that of the 3HTdR incorporation assay in measuring autoantigen-induced proliferation. The group tested PBMC from eight healthy subjects for proliferation in response to the type 1 diabetes autoantigen glutamic acid decarboxylase (GAD) using both the CFSE dilution and 3HTdR incorporation assays and found that while the former assay detected GAD-induced proliferation in all subjects, the latter did so in only 50% of the subjects. Furthermore, the responses detected with the CFSE dilution assay were significantly stronger than those detected with the 3HTdR incorporation assay. The group also demonstrated the CFSE dilution assay's ability to detect autoantigen-induced T-cell proliferation at 10-fold lower antigen concentrations than the 3HTdR incorporation assay. Since I am interested in T-cell responses, I used the CFSE dilution approach to look at proliferation within the $CD3^+$ PBMC subset so that I could compare data from this assay with that from the 3HTdR incorporation assay to determine whether what is seen with whole PBMC is representative of what is seen within the $CD3^+$ subset.

However, cell proliferation is only one of several possible manifestations of T-cell responses to antigen-specific stimulation. Therefore, if T-cells from RA patients and healthy controls show similar levels of proliferation in response to a candidate RA

autoantigen, it does not necessarily mean that their responses to the stimulus are identical. For example, cytokine production is another important functional manifestation of a T-cell response which can occur in tandem with or independent of proliferation, and it is possible that the cytokine profiles of candidate RA autoantigen-induced T-cell responses differ between RA patients and healthy controls, despite similar levels of proliferative responses. Indeed, it has been suggested that the cytokine responses of RA patients are biased towards a pro-inflammatory phenotype while those of healthy controls are more immunosuppressive in nature (Feldman *et al*, 1996 and McInnes & Schett, 2007). Thus, given the importance of cytokines in RA pathogenesis, measuring cytokine profiles may be of crucial importance when investigating T-cell responses to candidate RA autoantigens. However, many of the previous studies of candidate RA autoantigen-specific T-cell responses have measured only cellular proliferation (Goodstone *et al*, 1996; Verheijden *et al*, 1997; Snowden *et al*, 1997; Boots *et al*, 1997; Guerassimov *et al*, 1998; Cope *et al*, 1999; Kim *et al*, 1999; Vos *et al* #2, 2000 and Ria *et al*, 2008), while fewer studies have measured both proliferation and cytokine production (Li, N.L. *et al*, 2000; Park *et al*, 2001 and de Jong *et al*, 2010). Furthermore, when cytokine production has been measured, often only a few cytokines have been assayed for (Li, N.L. *et al*, 2000; Park *et al*, 2001 and van Bilsen *et al*, 2004), although a more recent study by de Jong *et al* (2010) investigated the production of a larger panel of cytokines. Therefore, in addition to measuring the proliferation of RA patient and healthy control T-cells in response to candidate RA autoantigen stimulation, I also quantified the production of a broad range of cytokines.

In addition to comparing different methods of detecting T-cell responses, I also compared the use of different types of sera as supplement to the culture media in our experimental system. This is because in previous studies examining candidate autoantigen-specific T-cell responses in PBMC, different sera were used to supplement culture media. For example, some studies utilised FBS-supplemented medium (Kim *et al*, 1999 and Park *et al*, 2001), while others cultured PBMC in HABS-supplemented medium (Boots *et al*, 1997; Snowden *et al* 1997; Guerassimov *et al*, 1998 and Ria *et al*, 2008). AS-supplemented culture medium was used by other groups (Goodstone *et al*, 1996; Cope *et al*, 1999 and Li, N.L. *et al*, 2000). Autologous plasma has also been used as a culture medium supplement in some of the previous studies (Verheijden *et al*, 1997 and Vos *et al* #2, 2000). Thus I compared the use of FBS-supplemented, HABS-supplemented and AS-supplemented culture media. My aim was to determine to what

extent the type of serum used would influence the responses of the PBMC to mitogens and protein antigens.

3.1.2. Aims

- To compare the 3HTdR incorporation assay measuring proliferation of whole PBMC culture with the CFSE dilution assay measuring proliferation within the $CD3^+$ subset of PBMC culture.
- To compare 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media in my experimental system in order to determine which is the most suitable.

3.2. Results

10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media were compared in an experimental system that comprised the 3HTdR incorporation assay measuring proliferation within triplicate cultures of whole PBMC, the CFSE dilution assay measuring proliferation within the $CD3^+$ subset of CFSE labelled PBMC cultures and ECL and ELISA assays measuring cytokine production by whole PBMC (Figure 3.1).

3.2.1. PBMC/$CD3^+$ PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media

With both proliferation assays I measured background, PHA-induced and PPD-induced proliferation. I generated both 3HTdR incorporation and CFSE dilution data from each of 6 subjects allowing a direct comparison of PBMC proliferation measured via 3HTdR incorporation and $CD3^+$ PBMC proliferation measured via CFSE dilution. Representative data of PBMC proliferation assessed using the 3HTdR incorporation assay and $CD3^+$ PBMC proliferation assessed using the CFSE dilution assay are shown in Figure 3.2 and Figure 3.3 respectively.

Figure 3.2 A and B show that when PBMC are cultured in 10% FBS-supplemented medium alone, they show increasing levels of proliferation as the culture period progresses which reach highly detectable peak levels by day 6, yet when they are cultured in 5% AS-supplemented medium alone, they show very little proliferation throughout the culture period. Regardless of which media the PBMC are cultured in, PHA-stimulated PBMC show high levels of proliferation which peak at day 2 (Figure

3.2A), while PPD-stimulated cells show more moderate but still highly detectable levels of proliferation which peak at day 6 (Figure 3.2B). However, the level of proliferation shown at day 6 by the unstimulated PBMC cultured in 10% FBS-supplemented medium is very similar to the peak levels of proliferation shown by the PPD-stimulated PBMC cultured in either of the two media. Due to the increasingly high levels of background PBMC proliferation associated with the use of 10% FBS-supplemented medium, the SI values of PHA-induced and PPD-induced proliferation are low when PBMC are cultured in this medium. In contrast, highly robust SI values are obtained when PBMC are cultured in 5% AS-supplemented medium as it induces very little background proliferation.

Figure 3.3 A and B show that regardless of whether the PBMC are cultured in 10% FBS-supplemented medium or 5% AS-supplemented medium, very little $CD3^+$ PBMC proliferation is detected at days 2 or 4 when the cells are cultured with medium alone, yet over the same two time-points, increasingly high levels of $CD3^+$ PBMC proliferation are detected in populations stimulated with PHA. By day 6, substantial levels of $CD3^+$ PBMC proliferation are detectable in PPD-stimulated cell populations cultured in both 10% FBS-supplemented or 5% AS-supplemented media. However, at this same time-point, highly detectable levels of $CD3^+$ PBMC proliferation are also observed in the unstimulated population of PBMC cultured in 10% FBS-supplemented medium (Figure 3.3A) although not in the corresponding population of PBMC cultured in 5% AS-supplemented medium alone (Figure 3.3B).

3.2.2. Background PBMC/$CD3^+$ PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media

The above data suggests that cells undergo a certain amount of background proliferation when cultured in 10% FBS-supplemented medium. Figure 3.4 confirms this finding, representing PBMC cultured for 7 days without a specific stimulus but with various serum supplementations. When PBMC are cultured in 10% FBS-supplemented medium alone, they initially show very low levels of background proliferation (mean cpm value ± SEM of 2061 ± 354 at day 2). However, as the culture period progresses, the levels of background proliferation increase, and reach high levels by day 6 (mean cpm value ± SEM of 42661 ± 7915). Conversely, when PBMC are cultured in 5% HABS-supplemented or 5% AS-supplemented medium alone, they show very little background proliferation throughout the entire culture period. Figure 3.5A shows that when CFSE

labelled PBMC are cultured in 10% FBS-supplemented medium alone, there is initially very little background proliferation seen within the $CD3^+$ PBMC subset, but as the culture period increases, so does the level of background proliferation occurring within the $CD3^+$ PBMC subset. In contrast, when CFSE labelled PBMC are cultured in 5% AS-supplemented medium alone, the levels of background proliferation within the $CD3^+$ PBMC subset remain low throughout the entire culture period. This correlates very well with what is seen with these two media with the corresponding 3HTdR incorporation assay data shown in Figure 3.5B.

3.2.3. PHA-induced and PPD-induced PBMC/$CD3^+$ PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media

Figure 3.6 shows 3HTdR incorporation data showing the levels of PHA-induced and PPD-induced PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. As shown in Figure 3.6A, PHA-stimulated PBMC show very high levels of proliferation, regardless of which media they are cultured in. PBMC cultured in 10% FBS-supplemented medium show remarkably similar levels of PHA-induced proliferation to those shown by PBMC cultured in 5% AS-supplemented medium at all time-points. With each of these two media, the highest levels of PHA-induced proliferation are seen at day 2, and as the culture period progresses, the amount of PHA-induced proliferation gradually decreases, consistent with the kinetics of responses to mitogenic stimuli. However, PBMC cultured in 5% HABS-supplemented medium show somewhat different levels of PHA-induced proliferation compared to those of PBMC cultured in the other two media at all time-points. PBMC cultured 5% HABS-supplemented medium show the highest levels of PHA-induced proliferation at days 3 and 4, and reach higher peak cpm values compared to the PBMC cultured in 10% FBS-supplemented and 5% AS-supplemented media.

With regard to PPD-induced responses (Figure 3.6B), PBMC that are cultured in 10% FBS-supplemented or 5% AS-supplemented medium show high levels of proliferation by day 6 (mean cpm values ± SEM of 58852 ± 8100 and 53995 ± 12715 respectively). PPD-stimulated PBMC cultured in 10% FBS-supplemented medium show slightly higher levels of proliferation then those cultured in 5% AS-supplemented medium at all time-points. However, this could be due to the high levels of background proliferation

observed in cultures using 10% FBS-supplemented medium. With both of these media, the magnitude and kinetics of the PPD-induced response is consistent with recalled (or memory) antigen-specific T-cell proliferation. However, PBMC cultured in 5% HABS-supplemented medium fail to show any substantial amount of proliferation in response to PPD at any of the four time-points.

Figure 3.7 shows 3HTdR incorporation data and corresponding CFSE dilution data showing PHA-induced and PPD-induced PBMC and $CD3^+$ PBMC proliferation. The levels of $CD3^+$ PBMC proliferation in PHA-stimulated cultures are very high regardless of the type of serum used as culture medium supplement, with peak levels of $CD3^+$ PBMC proliferation (80% $CFSE^{dim}$) at day 3 (Figure 3.7A). As mentioned above, the magnitude and kinetics of the response are typical of PHA-induced T-cell proliferation. The levels of PHA-induced $CD3^+$ PBMC proliferation observed in cultures using 10% FBS-supplemented and 5% AS-supplemented media are similar. These data mirror those observed in the 3HTdR incorporation assay (Figure 3.7B).
The levels of $CD3^+$ PBMC proliferation in PPD-stimulated cultures of CFSE-labelled PBMC are high with both 10% FBS-supplemented and 5% AS-supplemented culture media (Figure 3.7C). Of the three time-points that I have taken measurements, the highest levels of PPD-induced $CD3^+$ PBMC proliferation are observed on day 6, regardless of the media used. At all time-points, the levels of PPD-induced $CD3^+$ PBMC proliferation seen when the PBMC are cultured in 10% FBS-supplemented medium are very similar to those seen when PBMC are cultured in 5% AS-supplemented medium. Similarly, the percentages of $CD3^+$ PBMC that have undergone proliferation are slightly higher when PBMC are cultured in 10% FBS-supplemented medium as opposed to 5% AS-supplemented medium. This could again be due to the high levels of background proliferation in cultures using 10% FBS-supplemented medium. As in the case of PHA-induced proliferation, the patterns of PPD-induced $CD3^+$ PBMC proliferation observed with the CFSE dilution assay are very similar to those observed with the 3HTdR incorporation assay (Figure 3.7D).

3.2.4. SI values of PHA-induced and PPD-induced PBMC/$CD3^+$ PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media

When the levels of PHA-induced and PPD-induced proliferation seen in the two assays are expressed as stimulation indices, the differences between the three different media

become clear and the merits of culture media that induce little background proliferation become apparent.

Figure 3.8 shows SI values of PHA-induced and PPD-induced PBMC proliferation using the 3HTdR incorporation assay. The highest peak SI values of PHA-induced PBMC proliferation (mean ± SEM = 361.1 ± 100.7 [mean ± SEM cpm value of 128813 ± 16237 divided by mean ± SEM cpm value of 654 ± 161]) are seen when PBMC are cultured in 5% AS-supplemented medium. This is because 5% AS-supplemented culture medium induces very little background PBMC proliferation yet supports very high levels of PHA-induced PBMC proliferation (Figure 3.8A). Comparable although more variable peak SI values of PHA-induced PBMC proliferation (mean ± SEM = 314.0 ± 224.2 [mean ± SEM cpm value of 166648 ± 15183 divided by mean ± SEM cpm value of 1236 ± 502]) are also observed when PBMC are cultured in 5% HABS-supplemented medium as this too induces very little background PBMC proliferation yet is capable of supporting the high levels of PBMC proliferation in response to PHA. With both of these media, peak SI values of PHA-induced PBMC proliferation are seen at day 3. While SI values in cultures using 5% AS-supplemented medium decrease rapidly after the peak, the decrease in SI values observed with 5% HABS-supplemented medium is more gradual. Compared to the peak SI values of PHA-induced PBMC proliferation seen with the other two culture media, the peak SI values of PHA-induced PBMC proliferation seen with 10% FBS-supplemented culture medium are much lower (mean ± SEM = 81.8 ± 16.5 [mean ± SEM cpm value of 132274 ± 7241 divided by mean ± SEM cpm value of 2061 ± 354]). Even though the cpm values of PHA-stimulated PBMC cultured in 10% FBS-supplemented medium were very similar to those of PHA-stimulated PBMC cultured in 5% AS-supplemented medium, the high levels of background proliferation in cultures using the former culture medium mean that the proliferative response, when expressed as SI values, is significantly reduced. Peak SI values of PHA-induced PBMC proliferation seen with 10% FBS-supplemented culture medium are reached at day 2.

The SI values of PPD-induced PBMC proliferation are very low at all time-points when PBMC are cultured in 10% FBS supplemented medium (mean ± SEM of 2.7 ± 0.5, 2.2 ± 0.4, 1.6 ± 0.3 and 1.0 ± 0.2 at days 4, 5, 6 and 7 respectively [mean ± SEM cpm values of 30328 ± 4913, 48884 ± 8033, 58852 ± 8100 and 46184 ± 8313 divided by mean ± SEM cpm values of 12112 ± 1758, 23909 ± 4060, 42661 ± 7915 and 50425 ±

8626 at days 4, 5, 6 and 7 respectively]) because the levels of PPD-induced PBMC proliferation seen with this medium at days 5, 6 & 7, although very substantial, are only slightly greater than the levels of background PBMC proliferation seen with this medium at the same time-points. This suggests that the PPD-specific PBMC proliferation could have been masked by the high background proliferative response. The SI values of PPD-induced PBMC proliferation seen with 5% HABS-supplemented medium are also low at all time-points (mean ± SEM of 2.1 ± 1.1, 2.4 ± 1.1, 2.3 ± 0.3 and 1.1 ± 0.3 at days 4, 5, 6 and 7 respectively [mean ± SEM cpm values of 1835 ± 1084, 2087 ± 1177, 1653 ± 450 and 1337 ± 540 divided by mean ± SEM cpm values of 814 ± 216, 786 ± 209, 694 ± 101 and 1130 ± 264 at days 4, 5, 6 and 7 respectively]) because although PBMC cultured in this medium had low background proliferation, the proliferative response of PBMC to PPD was poor. In contrast, when PBMC are cultured in 5% AS-supplemented medium, the SI values of PPD-induced PBMC proliferation are highly significant at all time-points, with the peak value observed at day 5 (mean ± SEM = 36.2 ± 12.7 [mean ± SEM cpm value of 36395 ± 7506 divided by mean ± SEM cpm value of 2179 ± 592]). This is a result of the high levels of PPD-induced proliferation and the very low levels of background proliferation that are seen when PBMC are cultured in this type of medium (Figure 3.8B).

Figure 3.9 shows 3HTdR incorporation data (Figures 3.9A and 3.9B) and corresponding CFSE dilution data (Figures 3.9C and 3.9D) showing the SI values of PHA-induced (Figures 3.9A and 3.9C) and PPD-induced (Figures 3.9B and 3.9D) proliferation. Because of the higher levels of background proliferation in cells cultured in 10% FBS-supplemented medium, the SI values of PHA-induced $CD3^+$ PBMC proliferation observed when PBMC are cultured in this medium (mean ± SEM of 150.6 ± 47.4 at peak [mean ± SEM % $CFSE^{dim}$ $CD3^+$ PBMC value of 81.8 ± 3.1 divided by mean ± SEM % $CFSE^{dim}$ $CD3^+$ PBMC value of 1.4 ± 0.8]) are substantially lower than those seen when PBMC are cultured in 5% AS-supplemented medium (mean ± SEM of 250.7 ± 53.0 at peak [mean ± SEM % $CFSE^{dim}$ $CD3^+$ PBMC value of 83.7 ± 2.9 divided by mean ± SEM % $CFSE^{dim}$ $CD3^+$ PBMC value of 0.5 ± 0.1], Figure 3.9C). This is a similar pattern to that observed in the 3HTdR incorporation data that shows the SI values of PHA-induced PBMC proliferation (Figure 3.9A).

SI values of the PPD-induced $CD3^+$ PBMC proliferation seen when PBMC are cultured in 10% FBS-supplemented medium (mean ± SEM of 9.9 ± 4.3 at peak [mean ± SEM %

$CFSE^{dim}$ $CD3^+$ PBMC value of 20.5 ± 4.8 divided by mean ± SEM % $CFSE^{dim}$ $CD3^+$ PBMC value of 2.7 ± 0.7]) are substantially less than those seen when PBMC are cultured in 5% AS-supplemented medium (mean ± SEM of 57.4 ± 31.6 at peak [mean ± SEM % $CFSE^{dim}$ $CD3^+$ PBMC value of 15.6 ± 6.1 divided by mean ± SEM % $CFSE^{dim}$ $CD3^+$ PBMC value of 0.3 ± 0.1], Figure 3.9D) because of the higher levels of background $CD3^+$ PBMC proliferation that are seen when cells are cultured in FBS-supplemented medium. This is a similar pattern to that observed in the 3HTdR incorporation data that shows the SI values of PPD-induced PBMC proliferation (Figure 3.9B). Peak SI values for PPD-induced $CD3^+$ PBMC proliferation were observed at day 6 with both media. The peak SI values of PPD-induced $CD3^+$ PBMC proliferation detected with the CFSE dilution assay when using 10% FBS-supplemented culture medium are substantial (mean ± SEM peak SI value of 9.9 ± 4.3). On the other hand, the peak SI values of PPD-induced PBMC proliferation detected with the 3HTdR incorporation assay when using 10% FBS-supplemented culture medium are low and were lower than the predefined criteria of a positive responses (mean ± SEM peak SI value of 1.7 ± 0.4).

3.2.5. Background cytokine production with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media

There is currently no standardised method to report cytokine production data. Some investigators report raw values of cytokine production while others apply some form of adjustment to take into account the levels of background cytokine production. The commonest approaches are the calculation of 'absolute values', usually defined as raw values minus background values, or SI values, defined as raw values divided by background values. In this thesis, I first report the raw values and then the absolute values of the data obtained.

Figure 3.10 shows the levels of background cytokine production detected in cell culture supernatant of PBMC cultured in the different culture media. Levels of background production of both IL-10 and IL-4 were very low at all three time-points, regardless of the type of culture media used (note scales on y axis). The levels of background production of IL-17, IL-6, TNF-α and IFN-γ were more variable and can be very substantial when PBMC are cultured in different media. For example, substantial background production of IL-17, TNF-α and IFN-γ was observed at day 6 when PBMC were cultured in 10% FBS-supplemented medium (mean ± SEM = 31 ± 17, 512 ± 99

and 144 ± 81 respectively), while equally detectable levels of background production of IL-6 were seen at day 4 when PBMC were cultured in 5% HABS-supplemented medium (mean ± SEM = 1025 ± 918). PBMC cultured in 5% AS-supplemented medium show low levels of background production of all cytokines, while by day 6, PBMC cultured in 10% FBS-supplemented medium show the highest levels of background production of all cytokines except IL-6. Indeed, when PBMC are cultured in 10% FBS-supplemented medium, background production of IL-10, IL-17, IL-4, TNF-α and IFN-γ increases as the culture period progresses, although it is only by day 6 that the levels of background production of these five cytokines with this medium become substantially higher than those seen with the other two media.

3.2.6. Raw values of PHA-induced and PPD-induced cytokine production with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media

Figure 3.11 shows PHA-induced cytokine production with each of the three different culture media. PHA-stimulated PBMC show much higher levels of production of all cytokines than unstimulated PBMC regardless of the media that the PBMC are cultured in. The levels of PHA-induced IL-10, IL-17, IL-4, IL-6, TNF-α and IFN-γ production shown by PBMC cultured in 10% FBS-supplemented medium were similar to those shown by PBMC cultured in 5% AS-supplemented medium, although for PBMC cultured in 5% HABS-supplemented medium, PHA-induced production of IL-10, IL-17 and IL-6 is lower compared to that seen with the other two media, while the production of IL-4 and IFN-γ are comparable and the production of TNF-α is higher.

Figure 3.12 shows PPD-induced cytokine production with each of the different types of culture media. While PBMC cultured in 10% FBS-supplemented medium showed higher levels of PPD-induced production of both IL-10 and IL-17 than PBMC cultured in 5% HABS-supplemented or 5% AS-supplemented media, these differences failed to reach statistical significance and indeed the levels of PPD-induced IL-4 production were comparable between all three culture media. However, PBMC cultured in 5% HABS-supplemented medium showed higher levels of PPD-induced IL-6 production than PBMC cultured in either of the other two media (mean ± SEM pg/ml values of PPD-induced IL-6 production in 5% HABS-supplemented versus 10% FBS-supplemented versus 5% AS-supplemented medium = 6915 ± 2008 versus 3418 ± 1074 versus 1675 ± 467 respectively), although only the difference between 5% HABS-supplemented

medium and 5% AS-supplemented medium reached statistical significance (P value = 0.02, Mann Whitney, Figure 3.12D). PBMC cultured in 10% FBS-supplemented medium also showed higher levels of PPD-induced TNF-α production than PBMC cultured in either of the other two media (mean ± SEM pg/ml values of PPD-induced TNF-α production in 10% FBS-supplemented versus 5% AS-supplemented versus 5% HABS-supplemented medium = 1634 ± 430 versus 504 ± 100 versus 190 ± 77 respectively) although only the difference between 10% FBS-supplemented medium and 5% HABS-supplemented medium reached statistical significance (P value = 0.04, Mann Whitney, Figure 3.12E). While PBMC cultured in 5% HABS-supplemented medium failed to show any PPD-induced IFN-γ production, PBMC cultured in 10% FBS-supplemented and 5% AS-supplemented media showed comparable levels of PPD-induced IFN-γ production (mean ± SEM pg/ml values of 1027 ± 270 and 725 ± 163 respectively). Thus, cytokine production with 5% AS-supplemented medium is the most representative of the expected TH1 response provoked by PPD stimulation.

3.2.7. Absolute values (raw values minus background) of PHA-induced and PPD-induced cytokine production with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media

Essentially, when the PHA-induced cytokine production data are expressed as absolute values, they show an almost identical pattern to that observed when they are expressed as raw values (absolute value data not shown due to extreme similarity with data shown in Figure 3.11).

Figure 3.13 shows the absolute levels of PPD-induced cytokine production with the different types of culture media. While the absolute levels of PPD-induced production of IL-10, IL-17 and IL-4 were comparable between all three culture media, PBMC cultured in 5% HABS-supplemented medium showed higher absolute levels of PPD-induced IL-6 production than PBMC cultured in either of the other two media (mean ± SEM absolute values of PPD-induced IL-6 production in 5% HABS-supplemented versus 10% FBS-supplemented versus 5% AS-supplemented culture medium = 6866 ± 1999 versus 2949 ± 1105 versus 1632 ± 460 respectively), although only the difference between 5% HABS-supplemented medium and 5% AS-supplemented medium reached statistical significance (P value = 0.02, Mann Whitney, Figure 3.13D). PBMC cultured in 10% FBS-supplemented medium showed the highest absolute levels of PPD-induced TNF-α production while those cultured in 5% HABS-supplemented medium showed the

lowest, although the differences between the different types of culture media failed to reach statistical significance. While PBMC cultured in 5% HABS-supplemented medium failed to show any PPD-induced IFN-γ production, the absolute levels of PPD-induced IFN-γ production shown by PBMC cultured in 10% FBS-supplemented and 5% AS-supplemented media were comparable and very robust (mean ± SEM absolute levels of 882 ± 243 and 715 ± 165 respectively). Thus, when the PPD-induced cytokine production data are expressed as absolute values, the patterns of IL-4, IL-6 and IFN-γ production are similar to those observed when the data are expressed as raw values, while the patterns of IL-10, IL-17 and TNF-α production are slightly different.

3.3. Discussion

These data show that the type of sera used to supplement the culture medium influences the responses of T-cells to specific stimuli and the sensitivities of different assays for detecting antigen-specific responses. The serum affects the assays in two main ways;

(i) the level of background (i.e. unstimulated/negative control) responses.
(ii) the ability of the cell culture to support antigen-specific responses.

PBMC cultured in 10% FBS-supplemented culture medium show progressively higher levels of background proliferation (Figures 3.4 and 3.5) and cytokine production (Figure 3.10A, B, C, E and F) over time, suggesting that FBS contains factor(s) that stimulate the PBMC. This background stimulation could perhaps be attributed to the presence of xenogeneic antigens within the FBS which are immunogenic to the human PBMC. It is also possible that FBS contains soluble molecules that non-specifically stimulate PBMC responses by acting directly on T-cells or indirectly via effects on antigen-presenting cells. Regardless of the underlying biological mechanisms of the background responses, such responses interfere with the determination of proliferative and cytokine production responses to the mitogenic (PHA) as well as antigenic (PPD) stimuli. It is, however, not possible to determine whether the effects are simply additive or synergistic or both. Suffice to say, from my data, both the raw data as well as SI values were affected to different degrees. Firstly, for raw data (i.e. 3HTdR cpm readings, CFSE dilution % $CFSE^{dim}$ $CD3^{+}$ PBMC values or cytokine production), high levels of background proliferation and cytokine production may heighten the proliferative and cytokine production responses to specific stimuli, thus making them appear to be of a greater magnitude than they really are. PHA-induced proliferative or cytokine production

responses are only moderately affected by the high levels of background response, as PHA-induced responses seen with 10% FBS-supplemented culture medium are no greater than those seen with either of the other two culture media (Figures 3.6A, 3.7A, 3.7B and 3.11). There are two possible explanations as to why PHA-induced responses are less susceptible to the effects of the high levels of background proliferation and cytokine production seen with 10% FBS-supplemented culture medium. Firstly, PHA-induced responses are manifested and measured quite early on in the culture period. At these time-points, the levels of background proliferation seen with 10% FBS-supplemented culture medium, while higher than those seen with the other two media, are relatively modest (Figures 3.2A and 3.3A), and background cytokine production is similarly low with all three media (Figure 3.10). Secondly, the ratio of the PHA-induced responses to background responses are very high, and therefore only very high levels of background proliferation or cytokine production will interfere with the measurement of such responses.

In contrast, the PPD-induced responses are affected to a greater extent by the high levels of background proliferation and cytokine production seen with 10% FBS-supplemented culture medium. Indeed, the highest PPD-induced responses are measured when PBMC are cultured in this medium (Figures 3.6B, 3.7C, 3.7D and 3.12A, B, E and F). Again, there are two factors contributing to this. Firstly, PPD-induced responses are manifested and measured later on in the culture period, when the background levels of proliferation and cytokine production seen with 10% FBS-supplemented culture medium have reached high levels (Figures 3.2B, 3.3A and 3.10A, B, C, E and F). Secondly, PPD-induced responses are more modest in their magnitude than PHA-induced responses, and are therefore more susceptible to interference by the background responses.

Unsurprisingly, the impact of high levels of background proliferation seen with 10% FBS-supplemented culture medium on the detection of stimuli specific responses are most apparent when the data are expressed as SI values. Because SI calculate the fold changes in the levels of proliferation in response to a specific stimulus relative to background responses, the high levels of background proliferation seen with 10% FBS-supplemented medium will reduce the SI values of responses to specific stimuli. Indeed, even strong T-cell responses such as PHA-induced responses are affected. Thus, proliferation data show that the SI values of PHA-induced proliferation seen with 10% FBS-supplemented culture medium are substantially lower than those seen with either

of the other two culture media (Figures 3.8A, 3.9A and 3.9C). However, while SI values of PHA-induced proliferation are greatly reduced with 10% FBS-supplemented culture medium, they remain high. This is again due to a combination of the facts that at the early time-points at which PHA-induced responses are measured, background proliferation with 10% FBS-supplemented medium has not reached high levels, and that the PHA-induced responses are very great in magnitude. In contrast, the high levels of background proliferation seen with 10% FBS-supplemented culture medium reduce the SI values of the PPD-induced response much more dramatically (Figures 3.8B, 3.9B and 3.9D). Indeed, the SI values of PPD-induced PBMC proliferation detected with the 3HTdR incorporation assay when using 10% FBS-supplemented culture medium are below the threshold of a positive response as defined in the protocol (Figure 3.9B).

Taken together, the high levels of background proliferation associated with the use of 10% FBS-supplemented medium make this culture medium unsuitable for use in an experimental system for detecting autoantigen-specific immune responses.

5% HABS-supplemented culture medium induces very little background proliferation (Figure 3.4) and cytokine production (Figure 3.10A, B, C, E and F) but does not support antigen-specific responses as well as the other two sera (Figures 3.6B and 3.8B), so is therefore also unsuitable. It should be noted that the HABS used in the experiments described in this chapter was all derived from the same batch, and it is possible that HABS from another batch may have proved to be a suitable culture medium supplement. However, the favourable results obtained when using AS lead me to discard the idea of testing HABS from a different batch and instead use AS as the culture medium supplement.

5% AS-supplemented culture medium is associated with very little background proliferation (Figures 3.4 and 3.5) and cytokine production (Figure 3.10), yet supports both mitogen and antigen-specific responses very well (Figures 3.6, 3.7, 3.8, 3.9), and thus allows the most sensitive and specific detection of antigen-specific responses among the three types of sera tested. While it is possible that sera from different subjects may differ in their ability to support T-cell responses, this was not apparent in any of the experiments utilising AS-supplemented culture medium described in this chapter.

As mentioned previously, when measuring proliferation within an un-separated population of PBMC, the 3HTdR incorporation assay can not allow the identification of the subset(s) of the PBMC that are responsible for the detected 'proliferation', whereas the CFSE dilution assay, when used in combination with immunofluorescence staining for specific cell surface molecules, allows the detection of cellular subset-specific proliferation within an un-separated PBMC population. The availability of contemporaneous 3HTdR incorporation data from whole PBMC as well as CFSE dilution data showing proliferation of $CD3^+$ PBMC from 6 healthy subjects allowed me to determine whether the proliferation seen within the whole PBMC population was representative of that seen within the $CD3^+$ subset of the PBMC. When looking at the levels of background proliferation with 10% FBS-supplemented and 5% AS-supplemented culture medium, both sets of data show a similar pattern in that with 5% AS-supplemented culture medium, the levels of background proliferation remain very low for the duration of the 6 day culture period, yet the levels of background proliferation seen with 10% FBS-supplemented culture medium start to differ from those seen with 5% AS-supplemented culture medium after 4 days of culture and reach very substantial levels by day 6 (Figure 3.5). Further similarities are seen between the two assays when looking at the levels of PHA and PPD-induced proliferation. The kinetic of the PHA-induced proliferative response of whole PBMC (Figure 3.7B) mirrors that of the $CD3^+$ PBMC (Figure 3.7A). The kinetics of the PPD-induced proliferative responses of whole PBMC and $CD3^+$ PBMC are also quite similar (Figures 3.7C and 3.7D). The fact that the levels of PPD-induced PBMC/$CD3^+$ PBMC proliferation seen with 10% FBS-supplemented culture medium are slightly higher than those seen with 5% AS-supplemented culture medium suggests that both assays are susceptible to the high levels of background proliferation shown by 10% FBS-supplemented culture medium (Figures 3.7C and 3.7D). The data showing the SI values of PHA and PPD-induced PBMC/$CD3^+$ PBMC proliferation is further evidence of this susceptibility to high levels of background proliferation that both of the assays have in common (Figure 3.9). All this suggests that when the 3HTdR incorporation assay is used to measure proliferation within un-separated PBMC population, it does reflect T-cell proliferation. Because the 3HTdR incorporation assay is easier to perform, requires less cells and is less expensive compared to the CFSE dilution assay, using the former as a means of measuring 'T-cell' proliferation may be more appropriate. However, the fact that SI values of PPD-induced proliferation suggestive of positive responses were detected with the CFSE dilution assay but not the 3HTdR incorporation assay when

using 10% FBS-supplemented culture medium (Figure 3.9D versus Figure 3.9B) suggests that the former is more sensitive. Therefore, I have decided to compare these two approaches further in their ability to detect autoantigen-specific T-cell responses.

Finally, although the type of serum has a significant impact on the measurements of proliferative responses, the impact on cytokine production appears to be more modest. The reasons for this discrepancy are not clear but it further highlights the differences between proliferative and cytokine responses. It should also be mentioned that the cellular source of the cytokine production in these assays has not been specifically determined.

3.4. Summary

Proliferation seen in un-separated PBMC with the 3HTdR incorporation assay is representative of that seen in the $CD3^+$ subset with the CFSE dilution assay. 10% FBS-supplemented culture medium is associated with substantial levels of background stimulation which cause background proliferation and cytokine production that increase with the length of the culture period and reach highly substantial levels after 6 days of culture. The high levels of background proliferation and cytokine production seen with 10% FBS-supplemented culture medium interfere with the interpretation of proliferative and cytokine production responses to specific stimuli. When proliferative responses are expressed as SI values, the SI values of antigen-specific responses are masked by the high levels of background proliferation. 5% HABS-supplemented culture medium induces very little background proliferation and cytokine production but does not allow antigen-induced responses to be detected, which may be a consequence of its relative inability to support longer term cell culture. 5% AS-supplemented culture medium induces very little background proliferation and cytokine production and supports mitogen and antigen-induced responses. Therefore, I have chosen to use exclusively 5% AS-supplemented culture medium for my subsequent experiments.

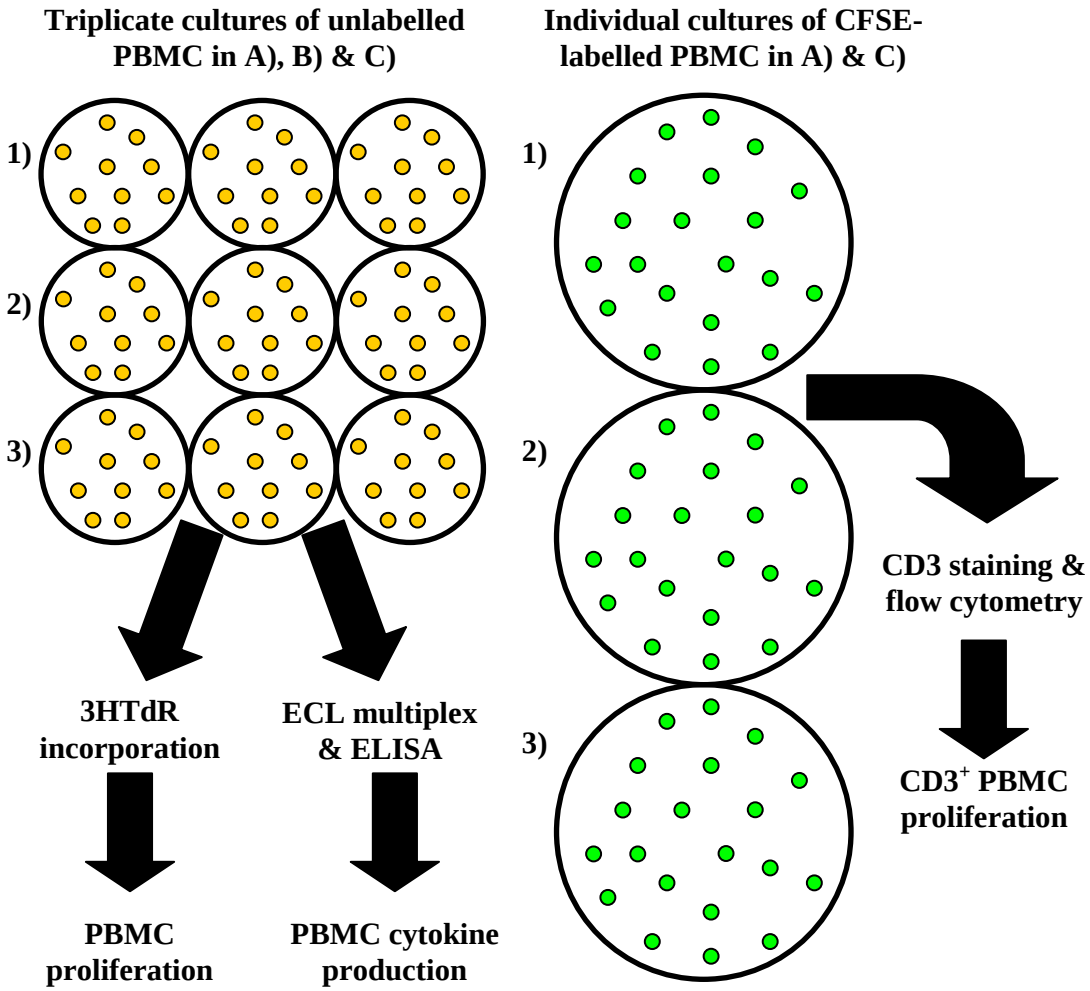

Figure 3.1. The experimental systems used to assess the suitability of 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. Unlabelled PBMC from each of 3 healthy subjects were cultured in A) 10% FBS-supplemented, B) 5% HABS-supplemented and C) 5% AS-supplemented media with 1) medium alone, 2) 5µg/ml PHA or 3) 1µg/ml PPD (left half of figure). CFSE-labelled PBMC from an additional 6 healthy subjects were cultured in A) 10% FBS-supplemented and C) 5% AS-supplemented media with 1) medium alone, 2) 5µg/ml PHA or 3) 1µg/ml PPD (right half of figure). After 2, 3, 4, 5, 6 & 7 days, proliferation of unlabelled PBMC in triplicate cultures on round-bottom 96-well plates was assessed via 3HTdR incorporation. At corresponding time-points, CFSE-labelled PBMC in individual cultures on flat-bottom 48-well plates were stained for CD3 expression and proliferation within the $CD3^+$ subset was assessed via CFSE dilution. Supernatant samples taken from cultures of unlabelled PBMC after 2, 4 & 6 days were analysed for cytokine content via ECL and ELISA.

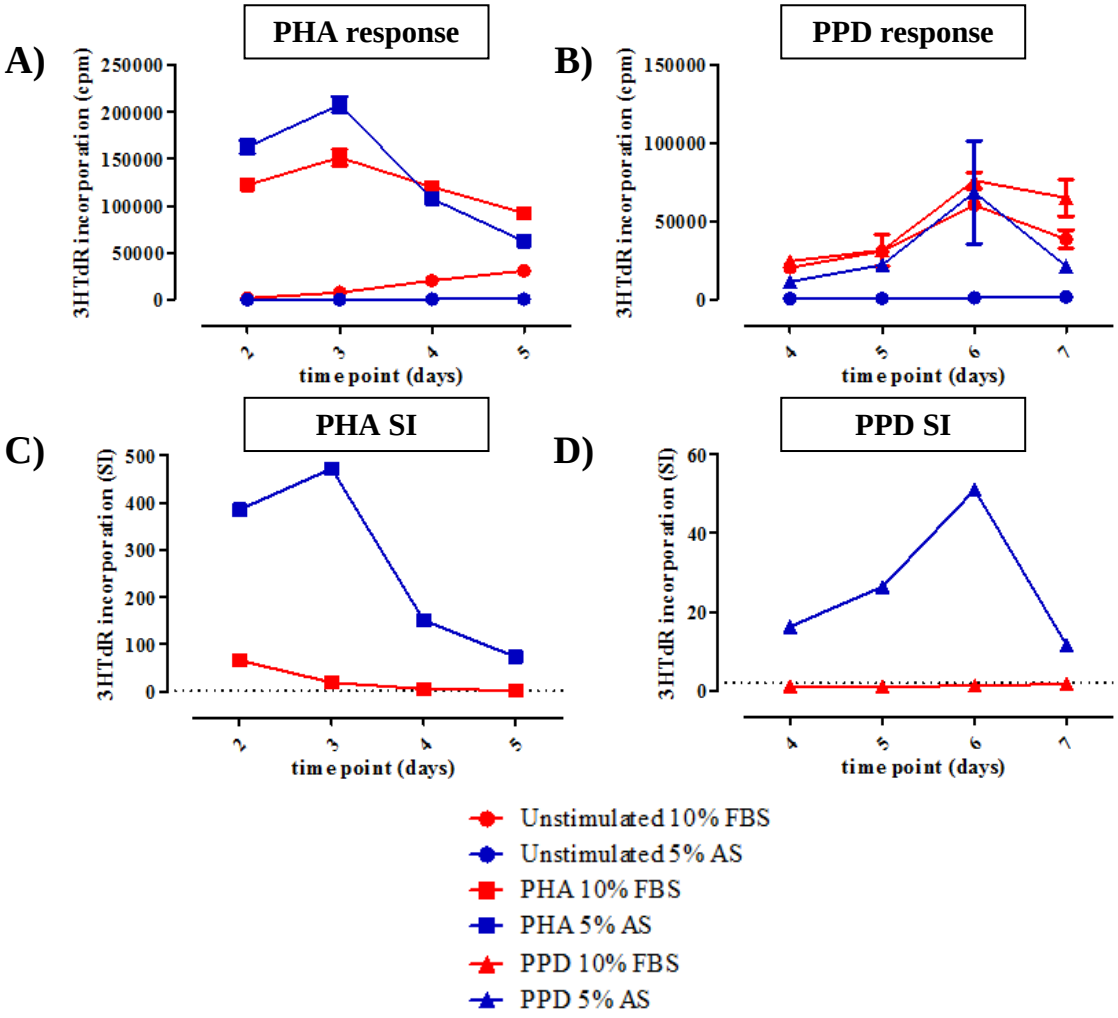

Figure 3.2. An example of 3HTdR incorporation data showing PBMC proliferation in a healthy subject. A, the mean cpm readings of triplicate wells of both unstimulated and PHA-stimulated PBMC after 2, 3, 4 & 5 days of culture in both 10% FBS-supplemented medium and 5% AS-supplemented medium. The vertical bars on each data point represent SEM. **B**, the mean cpm readings of triplicate wells of both unstimulated and PPD-stimulated PBMC after 4, 5, 6 & 7 days of culture in both 10% FBS-supplemented medium and 5% AS-supplemented medium. The vertical bars on each data point represent SEM. **C**, the SI values of PHA-stimulated PBMC after 2, 3, 4 & 5 days of culture in both 10% FBS-supplemented medium and 5% AS-supplemented medium. The dotted horizontal line represents the threshold level for a positive response. **D**, the SI values of PPD-stimulated PBMC after 4, 5, 6 & 7 days of culture in both 10% FBS-supplemented medium and 5% AS-supplemented medium. The dotted horizontal line represents the threshold for a positive response.

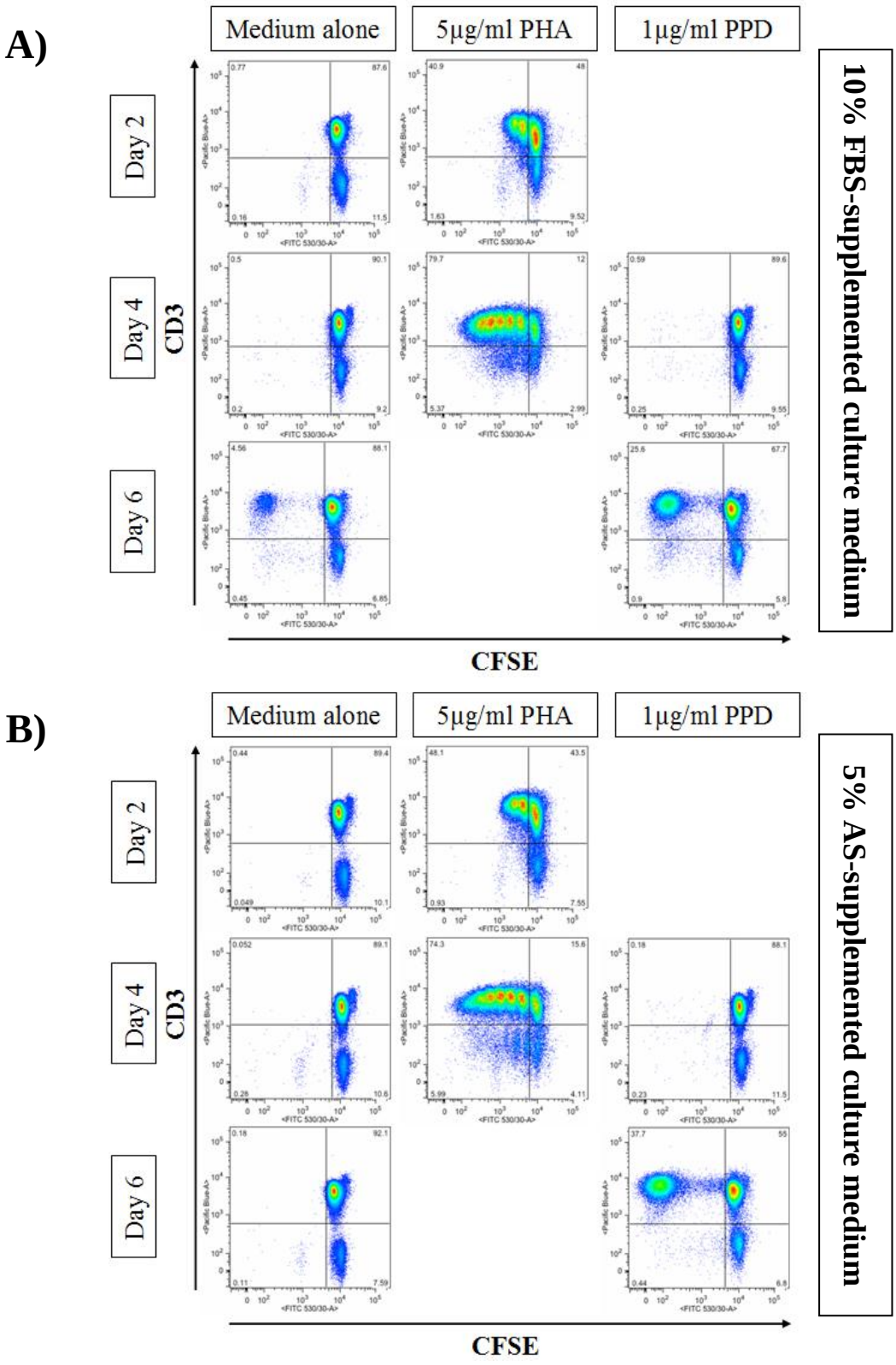

Figure 3.3. An example of CFSE dilution data showing proliferation of CD3$^+$ PBMC from a healthy subject. A, CFSE versus CD3 flow cytometry dot plots of unstimulated, PHA-stimulated and PPD-stimulated CFSE-labelled PBMC after 2, 4 & 6 days of culture in 10% FBS-supplemented culture medium. **B**, CFSE versus CD3 flow cytometry dot plots of unstimulated, PHA-stimulated and PPD-stimulated CFSE-labelled PBMC after 2, 4 & 6 days of culture in 5% AS-supplemented culture medium.

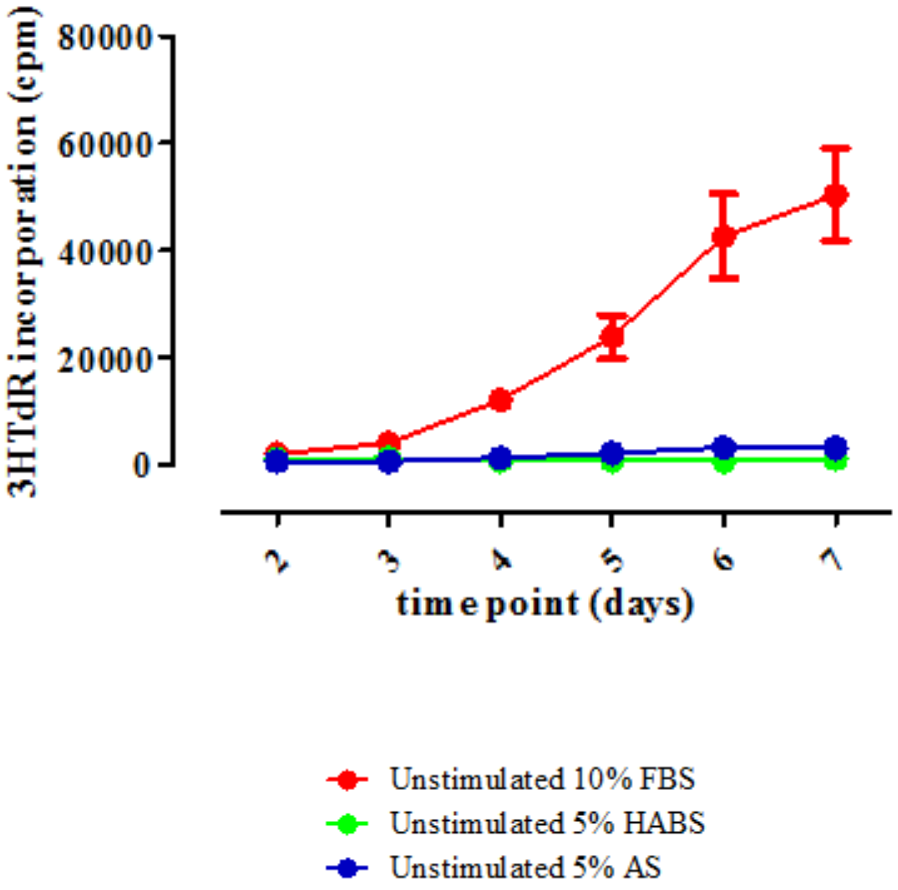

Figure 3.4. Background PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. 3HTdR incorporation data showing the mean cpm readings of unstimulated PBMC from each of the 9 healthy subjects after 2, 3, 4, 5, 6 & 7 days of culture in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). In each data set, each point represents the overall mean cpm reading i.e. the sum of the mean cpm readings from individual subjects divided by *n*. The vertical bars on each data point represent SEM.

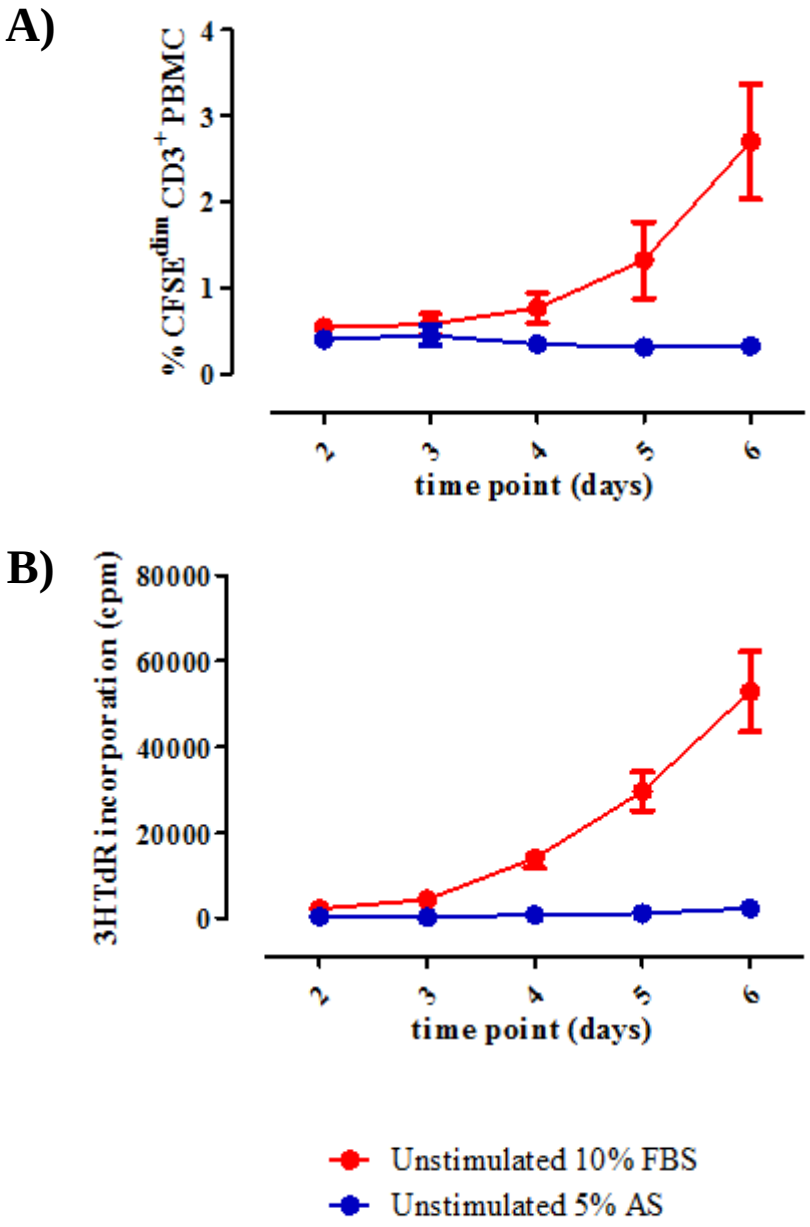

Figure 3.5. Background CD3$^+$ PBMC proliferation versus background PBMC proliferation. A, CFSE dilution data showing the mean % CFSE^{dim} CD3$^+$ PBMC in unstimulated cultures of CFSE-labelled PBMC from each of 6 healthy subjects after 2, 3, 4, 5 & 6 days of culture in 10% FBS-supplemented medium and 5% AS-supplemented medium. In each data set, each point represents the mean value, and the vertical bars on each data point represent SEM. **B**, 3HTdR incorporation data showing the mean cpm readings of unstimulated PBMC from each of the same 6 healthy subjects represented in A after 2, 3, 4, 5 & 6 days of culture in 10% FBS-supplemented medium and 5% AS-supplemented medium. In each data set, each point represents the overall mean cpm reading i.e. the sum of the mean cpm readings from individual subjects divided by 6. The vertical bars represent SEM.

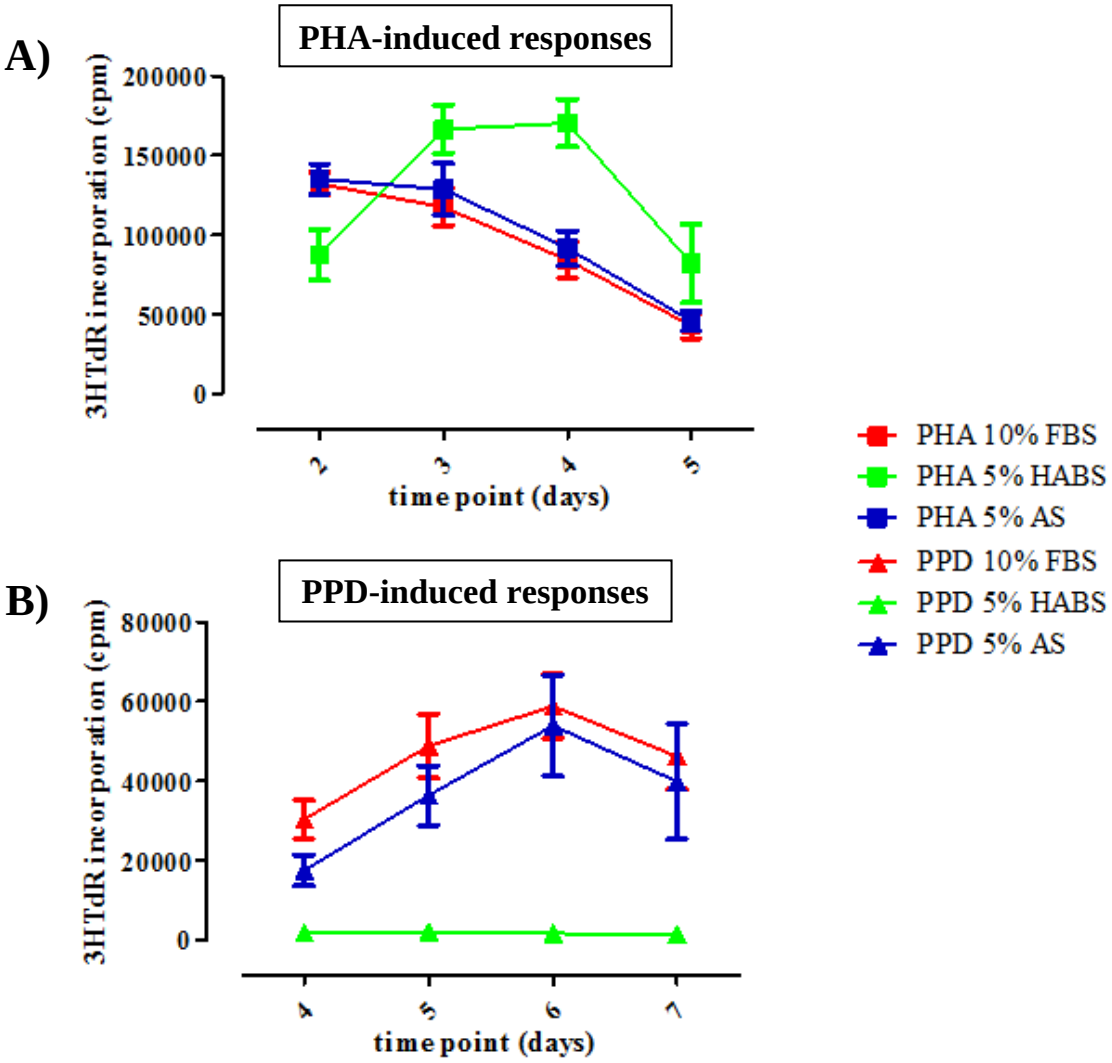

Figure 3.6. PHA-induced and PPD-induced PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. **A**, 3HTdR incorporation data showing the mean cpm readings of PHA-stimulated PBMC from each of 9 healthy subjects after 2, 3, 4 & 5 days of culture in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). Each point in each data set represents the overall mean cpm reading i.e. the sum of the mean cpm readings from individual subjects divided by *n*. The vertical bars on each data point represent SEM. **B**, 3HTdR incorporation data showing the mean cpm readings of PPD-stimulated PBMC from each of 9 healthy subjects after 4, 5, 6 & 7 days of culture in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). Each point in each data set represents the overall mean cpm reading i.e. the sum of the mean cpm readings from individual subjects divided by *n*. The vertical bars on each data point represent SEM.

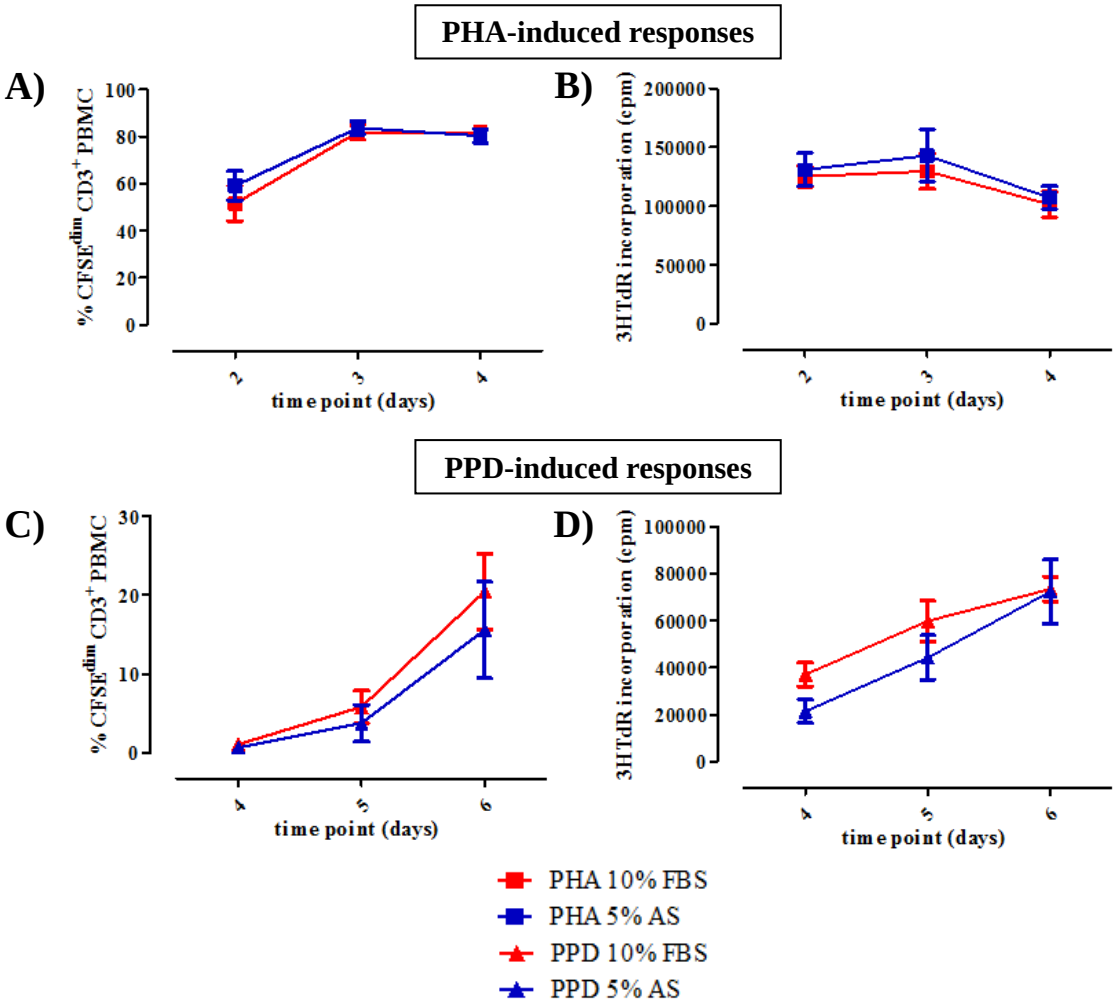

Figure 3.7. PHA-induced and PPD-induced PBMC proliferation versus PHA-induced and PPD-induced CD3$^+$ PBMC proliferation. A & **C**, CFSE dilution data showing the mean % CFSE^{dim} CD3$^+$ PBMC in PHA-stimulated (A) and PPD-stimulated (C) cultures of CFSE-labelled PBMC from each of 6 healthy subjects after 2, 3, 4, 5 & 6 days of culture in 10% FBS-supplemented medium and 5% AS-supplemented medium. For both graphs, each point in each data set represents the mean value, and the vertical bars on each data point represent SEM. **B** & **D**, 3HTdR incorporation data showing the mean cpm readings of PHA-stimulated (B) and PPD-stimulated (D) PBMC from each of the same 6 healthy subjects represented in A & C after 2, 3, 4, 5 & 6 days of culture in 10% FBS-supplemented medium and 5% AS-supplemented medium. For both graphs, each point in each data set represents the overall mean cpm reading i.e. the sum of the mean cpm readings from individual subjects divided by 6. The vertical bars on each data point represent SEM.

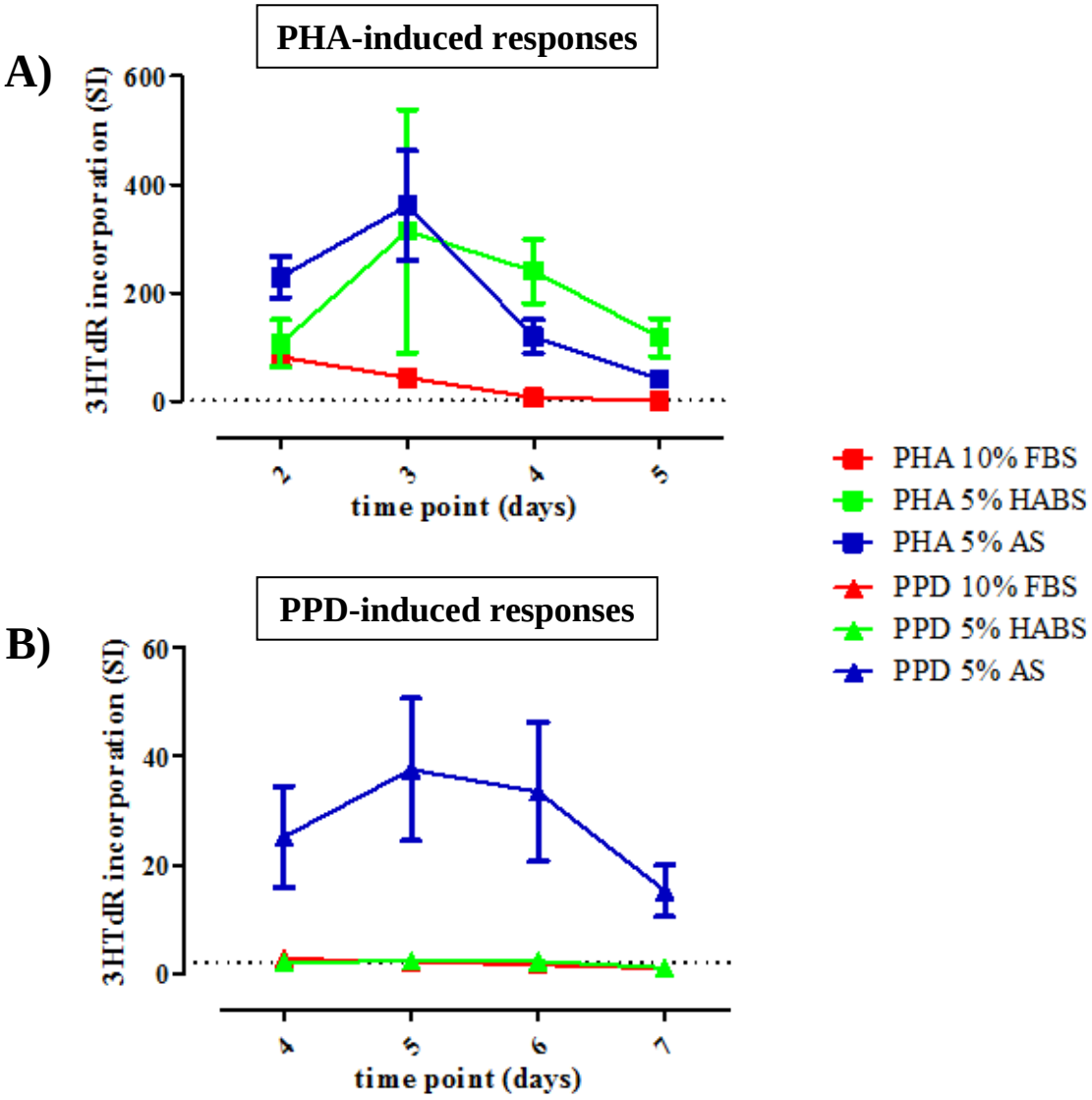

Figure 3.8. SI values of PHA-induced and PPD-induced PBMC proliferation with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. A, 3HTdR incorporation data showing the SI values of PHA-induced PBMC proliferation detected in each of 9 healthy subjects after 2, 3, 4 & 5 days when PBMC are cultured in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). Each point in each data set represents the mean SI value i.e. the sum of SI values from individual subjects divided by *n*. The vertical bars on each data point represent SEM, while the dotted horizontal line represents the threshold level for a positive response. **B**, 3HTdR incorporation data showing the SI values of PPD-induced PBMC proliferation detected in each of 9 healthy subjects after 4, 5, 6 & 7 days when PBMC are cultured in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). Each point in each data set represents the mean SI value i.e. the sum of SI values from individual subjects divided by *n*. The vertical bars on each data point represent SEM, while the dotted horizontal line represents the threshold for a positive response.

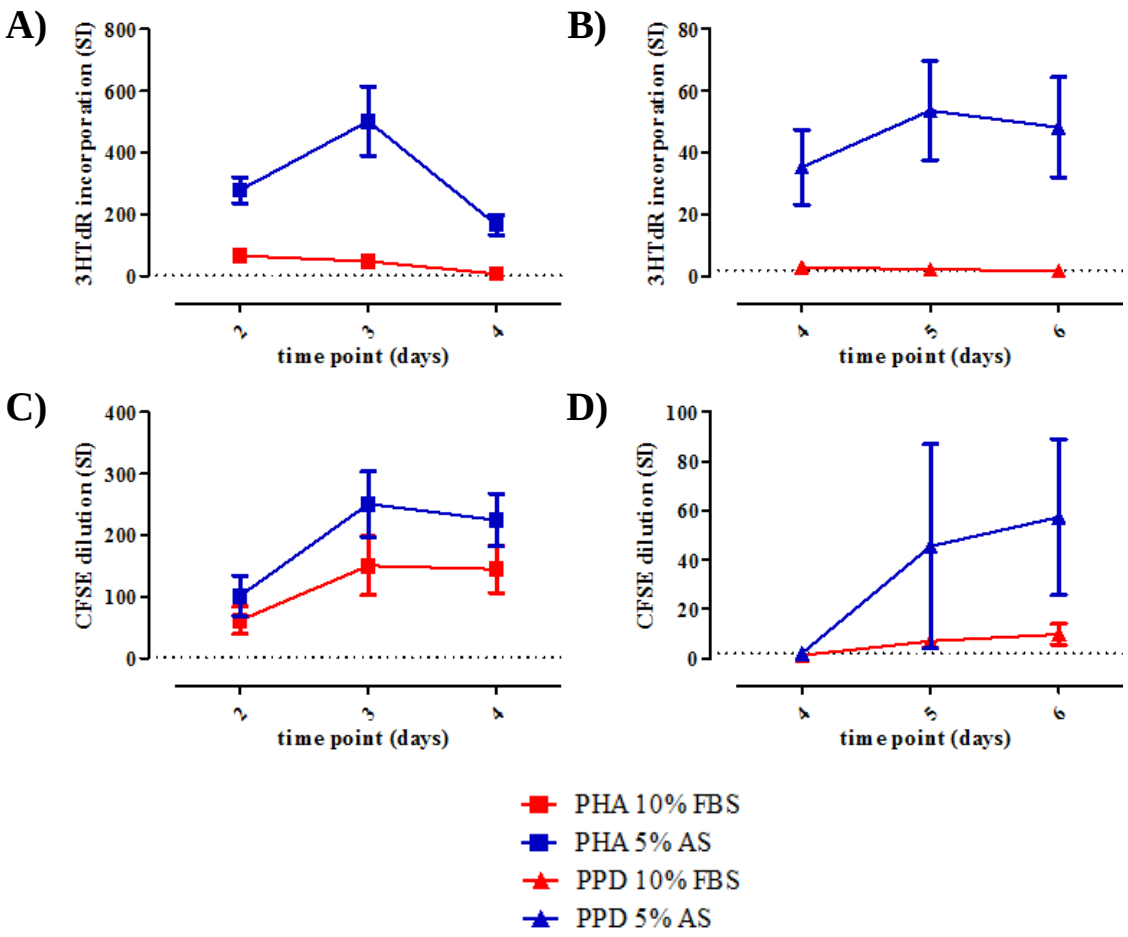

Figure 3.9. SI values of PHA-induced and PPD-induced PBMC proliferation versus SI values of PHA-induced and PPD-induced CD3$^+$ PBMC proliferation. A & **B**, 3HTdR incorporation data showing the SI values of PHA-induced and PPD-induced PBMC proliferation respectively that are detected in each of 6 healthy subjects after 2, 3, 4, 5 & 6 days when PBMC are cultured in 10% FBS-supplemented medium and 5% AS-supplemented medium. For both graphs, each point in each data set represents the mean SI value i.e. the sum of the SI values from individual subjects divided by 6. The vertical bars on each data point represent SEM, while the dotted horizontal line represents the threshold level for a positive response. **C** & **D**, CFSE dilution data showing the SI values of PHA-induced and PPD-induced CD3$^+$ PBMC proliferation respectively that are detected in each of the same 6 healthy subjects represented in A & B after 2, 3, 4, 5 & 6 days when PBMC are cultured in 10% FBS-supplemented medium and 5% AS-supplemented medium. For both graphs, each point in each data set represents the mean SI value i.e. the sum of the SI values from individual subjects divided by 6. The vertical bars on each data point represent SEM, while the dotted horizontal line represents the threshold for a positive response.

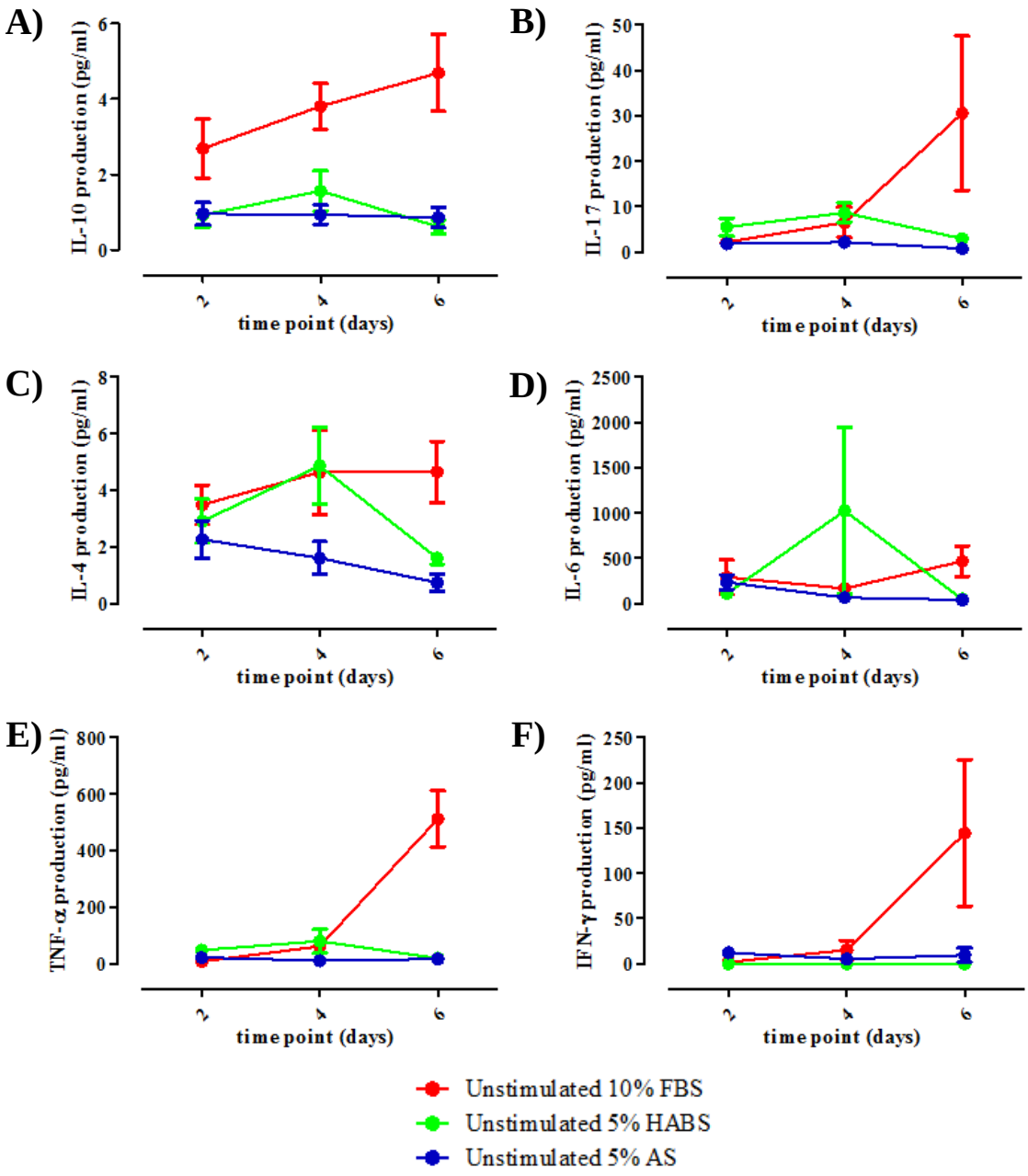

Figure 3.10. Background cytokine production with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. Graphs show in pg/ml the mean levels of production of **A)** IL-10, **B)** IL-17, **C)** IL-4, **D)** IL-6, **E)** TNF-α & **F)** IFN-γ by unstimulated PBMC after 2, 4 and 6 days of culture in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). For all graphs, each point in each data set represents the mean value i.e. the sum of the pg/ml values from individual subjects divided by *n*. The vertical bars on each data point represent SEM.

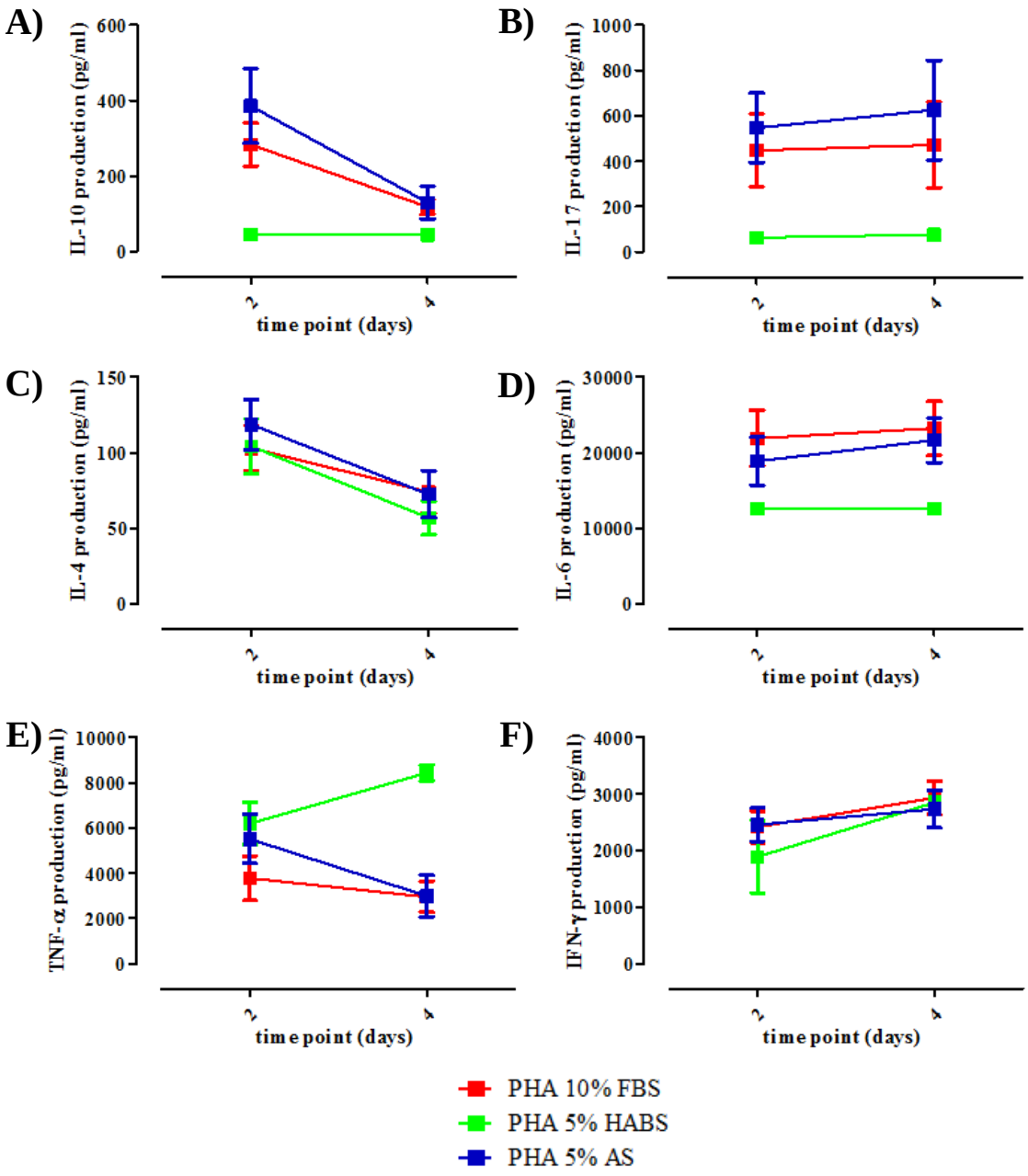

Figure 3.11. PHA-induced cytokine production with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. Graphs show in pg/ml the mean levels of production of **A)** IL-10, **B)** IL-17, **C)** IL-4, **D)** IL-6, **E)** TNF-α & **F)** IFN-γ by PHA-stimulated PBMC after 2 and 4 days of culture in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). For all graphs, each point in each data set represents the mean value i.e. the sum of the pg/ml values from individual subjects divided by *n*. The vertical bars on each data point represent SEM.

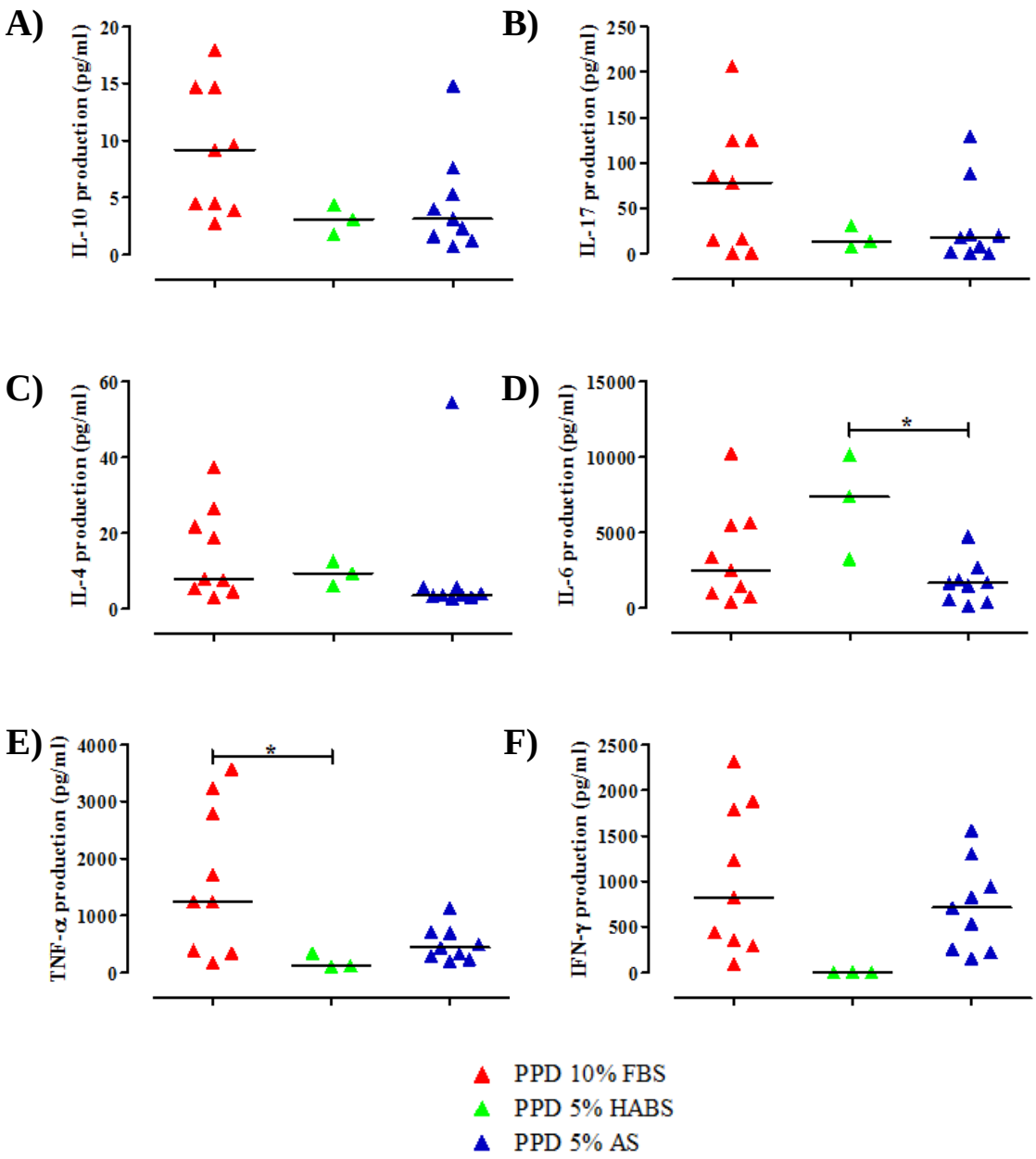

Figure 3.12. PPD-induced cytokine production at day 6 with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. Graphs show in pg/ml the levels of production of **A)** IL-10, **B)** IL-17, **C)** IL-4, **D)** IL-6, **E)** TNF-α & **F)** IFN-γ by PPD-stimulated PBMC after 6 days of culture in 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). For all graphs, each point in each data set represents a pg/ml value from an individual subject, and the bold horizontal line in each data set represents the median value.

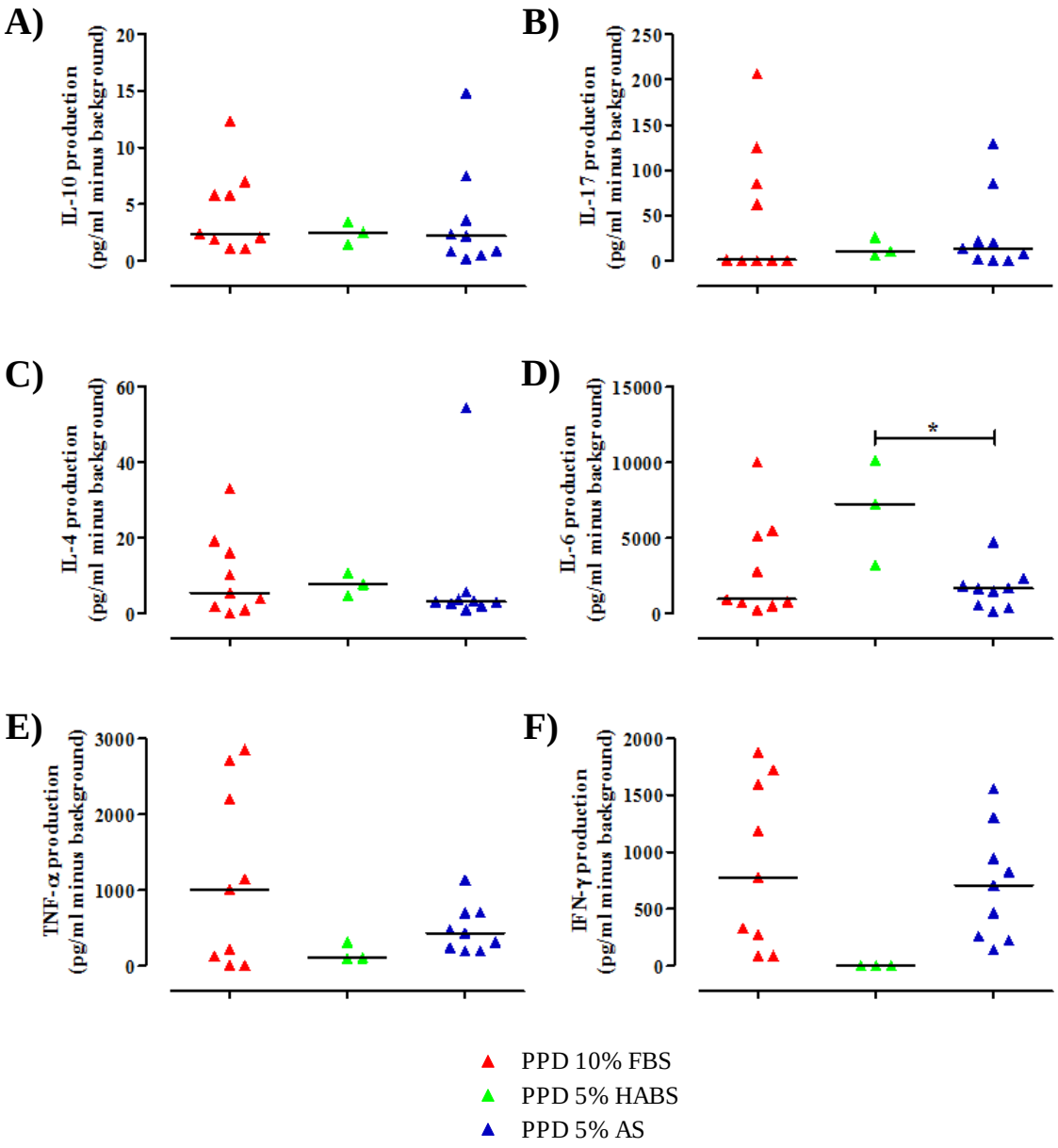

Figure 3.13. Absolute values of PPD-induced cytokine production at day 6 with 10% FBS-supplemented, 5% HABS-supplemented and 5% AS-supplemented culture media. Graphs show the absolute values of PPD-induced production of **A)** IL-10, **B)** IL-17, **C)** IL-4, **D)** IL-6, **E)** TNF-α & **F)** IFN-γ at day 6 when using 10% FBS-supplemented medium (*n*=9), 5% HABS-supplemented medium (*n*=3) and 5% AS-supplemented medium (*n*=9). For all graphs, each point in each data set represents an absolute value from an individual subject, while the bold horizontal line in each data set represents the median absolute value.

Chapter 4. T-cell immune responses to whole-protein candidate RA autoantigens in health and disease

4.1. Introduction

In chapter 3, I examined different protocols for detecting antigen-specific proliferation and cytokine production responses in cultures of PBMC. The results suggested that using 5% AS-supplemented culture medium is the most reliable approach, whether using 3HTdR incorporation or CFSE dilution proliferation assays, and either ECL multiplex or ELISA cytokine production assays. Ultimately, I am interested in autoantigen-specific T-cell responses, and an additional question was whether, when the 3HTdR incorporation assay is used to detect proliferation within whole PBMC cultures, the results could be used as a measure of $CD3^+$ T-cell proliferation. My data suggested that 3HTdR incorporation assays did reflect the data obtained from the CFSE dilution assays, suggesting that the former could be used as an estimate of T-cell proliferation. However, each assay had its own advantages and disadvantages. The 3HTdR incorporation assay requires fewer cells than the CFSE dilution assay and is easier to perform. In contrast, the CFSE dilution assay is more technically demanding, but can provide insights into subset-specific responses (e.g. $CD3^+$ $CD4^+$ T-cells). Consequently, I decided to further explore the relative merits of both assays in the study of T-cell responses to candidate RA autoantigens, the choice of assay reflecting the specific question to be addressed.

4.2. Results

4.2.1. HCgp39-induced T-cell proliferation in RA patients

For my first set of experiments involving a candidate RA autoantigen, I cultured CFSE-labelled PBMC from RA patients with 10µg/ml HCgp39 for 7 days prior to harvesting, staining for expression of CD3 and CD4 and measuring proliferation via CFSE dilution. PHA was used as a positive control in all experiments and proliferation was measured both as an increase in the % $CFSE^{dim}$ cells in response to stimulation (i.e. % $CFSE^{dim}$ cells in PHA/HCgp39-stimulated culture minus % $CFSE^{dim}$ cells in unstimulated culture) and as a CFSE dilution stimulation index (i.e. number of $CFSE^{dim}$ cells in PHA/HCgp39-stimulated culture divided by number of $CFSE^{dim}$ cells in unstimulated culture). Proliferation was measured in both the $CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$ subsets of peripheral blood T-cells. For this set of experiments, a total of 15 RA patients were studied (see Table 2.1 in chapter 2 for RA patient clinical details).

All 15 patients showed good PHA-induced proliferative responses (mean ± SEM SI value of 116.6 ± 48.6, $CD3^+$ $CD4^+$ subset [lowest individual SI value was 60.2]; data not shown). The data in Table 4.1 shows that of the 15 RA patients tested with HCgp39, 5 showed HCgp39-induced increases in the % of $CFSE^{dim}$ $CD3^+$ $CD4^+$ T-cells (mean ± SEM HCgp39-induced increase in the % of $CFSE^{dim}$ $CD3^+$ $CD4^+$ T-cells of 1.17 ± 0.75), and the $CD3^+$ $CD4^+$ subset of T-cells from of one of these 10 patients (RA 4.5) showed a very substantial HCgp39-induced increase in the % $CFSE^{dim}$ cells which measured 4.17%. Four of the RA patients showed HCgp39-induced increases in the % of $CFSE^{dim}$ $CD3^+$ $CD4^-$ T-cells (mean ± SEM HCgp39-induced increase in the % of $CFSE^{dim}$ $CD3^+$ $CD4^-$ T-cells of 0.28 ± 0.08). Two of the patients showed HCgp39-induced proliferation in both subsets, and the HCgp39-induced increases in the % of $CFSE^{dim}$ cells detected in the $CD4^+$ T-cell subsets of these patients (0.41 and 4.17) were greater than those detected in the $CD4^-$ T-cell subsets (0.10 and 0.43). Interestingly, when the HCgp39-induced proliferation was expressed as SI values, only 1 of the 15 patients (RA 4.5) showed a positive proliferative response, yet this was visible in both the $CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$ subsets of T-cells (SI values of 4.0 and 3.3 respectively). However, overall the SI values of HCgp39-induced proliferation detected within the $CD3^+$ $CD4^+$ T-cell subset were significantly greater than those of HCgp39-induced proliferation detected within the $CD3^+$ $CD4^-$ T-cell subset (mean ± SEM SI values of 1.3 ± 0.2 versus 1.1 ± 0.2 respectively, P value = 0.02, paired t test). As the response to HCgp39 is MHC class II-restricted, the proliferation observed in the $CD3^+$ $CD4^-$ subset of the PBMC upon stimulation with HCgp39 could be explained as antigen-independent proliferation induced by IL-2 produced by $CD3^+$ $CD4^+$ cells recognising HCgp39 peptide-MHC class II complexes (Wong & Pamer, 2001).

4.2.2. Bulk culture approach; HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation

In my previous set of experiments that used the CFSE dilution assay to measure the proliferation of RA patient peripheral blood T-cells in response to HCgp39 stimulation, only 1 of the 15 patients tested appeared to show a positive HCgp39-induced proliferative response when the data were expressed as SI values. Several previous studies had reported higher incidences of HCgp39-induced proliferative responses in RA patients (Verheijden *et al*, 1997 and Vos *et al* #2, 2000) therefore I performed some further experiments measuring HCgp39-induced responses in RA patients using a different approach. Since these previous studies had used the 3HTdR incorporation

assay to measure proliferation in cultures of PBMC, I decided to attempt to replicate the findings of these previous HCgp39 studies and to focus on the 3HTdR incorporation assay as a means of measuring HCgp39-induced responses in PBMC as a whole. However, instead of using the triplicate/multiple culture approach with a maximum of $2x10^5$ cells/well which had been employed by these previous studies and additional published studies on candidate RA autoantigens (Goodstone *et al*, 1996; Boots *et al*, 1997; Cope *et al*, 1999; Kim *et al*, 1999 and de Jong *et al*, 2009), I wanted to explore the use of a bulk culture approach similar to that used in my CFSE dilution experiments in which the PBMC were cultured with higher cell numbers per well. My rationale was that increasing the cell number may improve the chance of detecting low precursor frequency T-cell responses. In addition, I was also able to test the responses to CII and GlyAgg at the same time, as samples of these other candidate autoantigens had become available. For these bulk culture experiments, a total of 9 RA patients and 8 healthy subjects were used (see Table 2.2 in chapter 2 for RA patient clinical details). The data in Table 4.2 shows the SI values for PHA-induced, PPD-induced, HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation detected in each of the RA patients and healthy subjects, as determined by 3HTdR incorporation.

Figure 4.1 shows the comparison between the levels of PHA-induced (A) and PPD-induced (B) PBMC proliferation detected in RA patients and healthy subjects when using the bulk culture approach. While the median SI value of PHA-induced PBMC proliferation detected in the RA patients (median = 89.8, IQR: 41.3-170.4) was greater than that of PHA-induced PBMC proliferation detected in the healthy subjects (median = 37.8, IQR: 30.9-59.5), the difference between the two groups was not statistically significant (P value = 0.09, Mann Whitney). As shown in Table 4.2, only 63% of the RA patients showed positive PPD-induced PBMC proliferative responses compared to 100% of the healthy subjects. However, this difference was not statistically significant (P value = 0.45, Chi-square). Furthermore, the peak SI values of PPD-induced PBMC proliferation detected in both the RA patients and healthy subjects (median = 3.1, IQR: 1.8-22.0 and median = 5.4, IQR: 2.8-9.1 respectively) were statistically similar (P value = 0.28, Mann Whitney).

Figure 4.2 shows the kinetics of the HCgp39-induced, CII-induced and GlyAgg-induced proliferative responses detected in both RA patients and healthy subjects when using the bulk culture approach. In the RA patients that showed positive HCgp39-induced PBMC

proliferative responses, peak levels of proliferation were detected at days 9 and 11 (*n*=1 and 3 respectively, Figure 4.2A). In the healthy subjects that showed positive HCgp39-induced PBMC proliferative responses, peak levels of proliferation were also detected at days 9 and 11 (*n*=2 and 1 respectively, Figure 4.2B). In the RA patients that showed positive CII-induced PBMC proliferative responses, peak levels of proliferation were detected at days 7, 9 and 11, Figure 4.2C). In the healthy subjects that showed positive CII-induced PBMC proliferative responses, peak levels of proliferation were detected at days 7 and 9 (*n*=2 and 1 respectively, Figure 4.2D). None of either the RA patients or healthy subjects showed positive GlyAgg-induced PBMC proliferative responses (Figures 4.2E and 4.2F respectively).

As shown in Table 4.2, similar proportions of RA patients and healthy subjects (44% and 38% respectively) showed positive HCgp39-induced PBMC proliferative responses. Furthermore, the peak SI values of HCgp39-induced PBMC proliferation detected in the RA patients (median = 1.7, IQR: 1.2-3.2) and those of HCgp39-induced PBMC proliferation detected in the healthy subjects (median = 1.9, IQR: 1.3-3.3) were similar (P value = 0.70, Mann Whitney, Figure 4.3A). Positive CII-induced PBMC proliferative responses were detected in a similar proportion of the healthy subjects as the RA patients (38% and 33%, healthy subjects and RA patients, Table 4.2). The median peak SI value of CII-induced PBMC proliferation detected in the healthy subjects (median = 1.9, IQR: 1.5-9.9) was slightly greater than that of CII-induced PBMC proliferation detected in the RA patients (median = 1.4, IQR: 1.2-3.7), although the difference between the 2 groups was not statistically significant (P value = 0.18, Mann Whitney, Figure 4.3B). None of the RA patients or healthy subjects showed positive GlyAgg-induced PBMC proliferative responses (Table 4.2), and the peak SI values of GlyAgg-induced PBMC proliferation detected in the RA patients versus the healthy subjects were almost identical (median = 0.8, IQR: 0.7-1.0 versus median = 0.8, IQR: 0.6-1.3 respectively, Figure 4.3C).

Of the four RA patients that showed positive HCgp39-induced PBMC proliferative responses, three (RA 4.16, RA 4.23 and RA 4.24) were both RF and CCP positive, while RF and CCP levels in the fourth (RA 4.19) were not known. All four HCgp39 responders showed CRP levels of less than 5. Interestingly, two of the HCgp39 responders (RA 4.19 and RA 4.24) also showed positive CII-induced PBMC proliferative responses. The third CII responder (RA 4.20) was RF positive but CCP

levels were unknown and CRP levels were more substantial compared to those in other autoantigen responders (20 versus <5). However, while this suggested that positive HCgp39-induced or CII-induced PBMC proliferative responses were associated with RF and CCP positivity and CRP levels of less than 5, such clinical traits were also observed in patients that failed to show positive HCgp39-induced or CII-induced PBMC proliferative responses (RA 4.17 and RA 4.21). Of the six patients showing positive responses to HCgp39 and/or CII, three (RA 4.19, RA 4.23 and RA 4.24) had been only recently diagnosed with RA (i.e. within 2 years of the time of testing) while the other two (RA 4.16 and RA 4.20) had longer standing disease. As was seen with the RA patients, two of the healthy subjects that showed positive HCgp39-induced PBMC proliferative responses also showed positive CII-induced PBMC proliferative responses (HS 4.6 and HS 4.7). In both of these subjects, the CII-induced responses (SI values of 10.6 and 13.1) were more potent than the HCgp39-induced responses (SI values of 2.5 and 3.5).

4.2.3. Triplicate culture approach; HCgp39-induced, CII-induced, GlyAgg-induced and DeglyAgg-induced PBMC proliferation and cytokine production

When using the bulk culture approach to detect proliferation of RA patient and healthy subject PBMC in response to HCgp39, CII and GlyAgg, only HCgp39 and CII appeared to be capable of eliciting positive proliferative responses. However, the numbers of subjects from each of the two cohorts showing positive proliferative responses to these stimuli were low, and the responses were similar between RA patients and healthy subjects in terms of both the prevalence and the magnitudes. I therefore decided to repeat these experiments using the triplicate culture approach so that I could compare similar data from each of the two approaches. I had also obtained some DeglyAgg so this was added to my panel of autoantigens. For these triplicate culture experiments, a total of 24 RA patients and 16 healthy subjects were studied (see Table 2.3 in chapter 2 for RA patient clinical details).

4.2.3.1. PHA-induced and PPD-induced PBMC proliferation and background cytokine production

When using the triplicate culture approach, the median SI value of PHA-induced PBMC proliferation detected in RA patients (median = 232.8, IQR: 162.9-463.1) was slightly greater than that of PHA-induced PBMC proliferation detected in healthy subjects (median = 186.4, IQR: 113.1-321.3), but the difference between the two groups was not

statistically significant (P value = 0.26, Mann Whitney, Figure 4.4A). This is similar to what was seen with the bulk culture approach. However, Figures 4.4B and 4.4C show that for both RA patients and healthy subjects respectively, the SI values of PHA-induced PBMC proliferation detected with the triplicate culture approach were significantly greater than those detected with the bulk culture approach (P values = 0.0039 and 0.0011 respectively, Mann Whitney). Of the 23 RA patients that were tested for PPD-specific PBMC proliferation with the triplicate culture approach, 20 (87%) showed positive responses, while all 16 of the healthy subjects showed positive PPD-induced PBMC proliferative responses. The median peak SI value of PPD-induced PBMC proliferation detected in RA patients (median = 14.9, IQR: 3.6-31.1) was less than that of PPD-induced PBMC proliferation detected in healthy subjects (median = 19.1, IQR: 10.2-31.8) but, as was seen with the bulk culture approach, the difference between the 2 groups was not statistically significant (P value = 0.34, Mann Whitney, Figure 4.5A). Although this is a similar pattern to that seen with the bulk culture approach, it is worth noting that with the RA patients, a higher incidence of 'positive' PPD-specific proliferative responses was detected when using the triplicate culture approach as opposed to the bulk culture approach, although this trend failed to reach statistical significance (87% versus 63% respectively, P value = 0.69, Chi-square). Nevertheless, Figure 4.5B shows that for RA patients, the peak SI values of PPD-induced PBMC proliferation detected with the triplicate culture approach were statistically similar to those detected with the bulk culture approach (P value = 0.16, Mann Whitney). However, for healthy subjects, the peak SI values of PPD-induced PBMC proliferation detected with the triplicate culture approach were significantly greater than those detected with the bulk culture approach (P value = 0.02, Mann Whitney, Figure 4.5C). Therefore, while both the bulk culture and triplicate culture approaches showed similar incidences of positive PPD-specific proliferative responses, the magnitudes of these responses were greatest when using the triplicate culture approach.

Levels of background production of IL-10, IL-17, IL-4, IL-6, TNF-α and IFN-γ by both RA patient and healthy subject PBMC from the triplicate culture experiments were measured after 5 and 11 days of culture. I hypothesised that RA patients would have higher levels of background production of pro-inflammatory cytokines compared to healthy subjects. Figure 4.6A shows that at day 5, the levels of background PBMC cytokine production detected in both RA patients and healthy subjects were very

similar. Background IL-6 production was high in both RA patients and healthy subjects, with mean ± SEM levels measuring 1276 ± 1152 and 893 ± 541 respectively. Day 11 data shows a similar finding but with less pronounced IL-6 production in both groups (Figure 4.6B).

4.2.3.2. HCgp39-induced responses

The 3HTdR incorporation data in Table 4.3 shows that only 40% of RA patients showed positive HCgp39-induced PBMC proliferative responses compared to 75% of healthy subjects (this compares to the 44% of RA patients and 38% of healthy subjects seen with the bulk culture approach), although this difference failed to reach statistical significance (P value = 0.70, Chi-square). The median peak SI value of HCgp39-induced PBMC proliferation detected in RA patients (median = 1.9, IQR: 1.6-2.8) was slightly less than that of HCgp39-induced PBMC proliferation detected in healthy subjects (median = 2.7, IQR: 1.9-3.6) but this difference was not statistically significant (P value = 0.18, Mann Whitney, Figure 4.7A). In the RA patients that showed positive HCgp39-induced PBMC proliferative responses, peak levels of proliferation were detected at days 5, 9 and 11 (*n*=2, 1 and 1 respectively, Figure 4.7B). In the healthy subjects that showed positive HCgp39-induced PBMC proliferative responses, peak levels of proliferation were detected at days 5, 7, 9 and 11 (*n*=2, 1, 1 and 2 respectively, Figure 4.7C).

The cytokine production data in Table 4.3 shows that of the six RA patients and six healthy subjects tested for HCgp39-induced cytokine production, equal proportions of subjects from both groups (67%) showed cytokine production in response to the stimulus. However, this may be an underestimate as some of the RA patients and healthy subjects that were not tested for cytokine production did show HCgp39-induced proliferation. Of the four RA patients that showed HCgp39-induced cytokine production, all showed up-regulation of IL-6 in response to the stimulus (191pg/ml, 86pg/ml, 2649pg/ml and 673pg/ml), while three also showed up-regulation of TNF-α (4pg/ml, 90pg/ml and 64pg/ml). One of the RA patients that showed HCgp39-induced up-regulation of both IL-6 and TNF-α also showed up-regulation of IL-4 in response to the stimulus (10pg/ml). Of the four healthy subjects that showed HCgp39-induced cytokine production, three showed up-regulation of IL-6 in response to the stimulus (4890pg/ml, 58pg/ml and 10pg/ml) and one of these subjects also showed up-regulation of both TNF-α and IL-4 (46pg/ml and 3pg/ml respectively) while another also showed

up-regulation of TNF-α alone (9pg/ml). The fourth healthy subject that showed HCgp39-induced cytokine production up-regulated production of IFN-γ alone in response to the stimulus (14pg/ml). In both the RA patients and healthy subjects, HCgp39-induced cytokine production was detected both in conjunction with and independent of HCgp39-induced proliferative responses. Interestingly, HCgp39 failed to induce production of detectable levels of IL-10 or IL-17 in either RA patients or healthy subjects. Furthermore, only in healthy subjects did HCgp39 induce production of detectable levels of IFN-γ. The levels of HCgp39-induced IL-4, IL-6 and TNF-α production did not differ significantly between the RA patients and the healthy subjects (P values = 1.00, 0.46 and 0.35 for IL-4, IL-6 and TNF-α respectively, Mann Whitney).

4.2.3.3. CII-induced responses

The 3HTdR incorporation data shown in Table 4.4 shows that only 57% of RA patients showed positive CII-induced PBMC proliferative responses compared to 88% of healthy subjects (this compares to the 33% of RA patients and 38% of healthy subjects seen with the bulk culture approach), although this difference failed to reach statistical significance (P value = 0.78, Chi-square). In addition, the median peak SI value of CII-induced PBMC proliferation detected in RA patients (median = 2.1, IQR: 1.2-3.8) was less than that of CII-induced PBMC proliferation detected in healthy subjects (median = 2.7, IQR: 2.3-4.1), yet once again the difference between the 2 groups failed to reach statistical significance (P value = 0.34, Mann Whitney, Figure 4.8A). In the RA patients that showed positive CII-induced PBMC proliferative responses, peak levels of proliferation were detected at days 3, 9 and 11 (*n*=1, 1 and 2 respectively, Figure 4.8B). In the healthy subjects that showed positive CII-induced PBMC proliferative responses, peak levels of proliferation were detected at days 3, 5, 7, 9 and 11 (*n*=1, 1, 2, 1 and 2 respectively, Figure 4.8C).

The cytokine production data in Table 4.4 shows that all four of the RA patients and all six of the healthy subjects that were tested for CII-induced cytokine production showed cytokine production in response to the stimulus. All four of the RA patients showed CII-induced up-regulation of IL-6 (3326pg/ml, 462pg/ml, 79pg/ml and 1817pg/ml) and in three of the patients this was accompanied by an up-regulation of both IL-17 and TNF-α (3pg/ml and 99pg/ml respectively), TNF-α alone (84pg/ml) or IFN-γ alone (8pg/ml). Five of the six healthy subjects showed CII-induced up-regulation of IL-4 (14pg/ml, 3pg/ml, 6pg/ml, 13pg/ml and 18pg/ml), IL-6 (5768pg/ml, 3788pg/ml, 4679pg/ml,

3775pg/ml and 12633pg/ml) and TNF-α (14pg/ml, 68pg/ml, 426pg/ml, 31pg/ml and 61pg/ml). Four of these subjects also showed CII-induced up-regulation of two or three other cytokines, namely IL-17 and IFN-γ (7pg/ml and 12pg/ml respectively), IL-10 and IFN-γ (7pg/ml and 31pg/ml respectively), IL-10 and IL-17 (8pg/ml and 6pg/ml respectively) and IL-10, IL-17 and IFN-γ (3pg/ml, 15pg/ml and 8pg/ml respectively). The sixth healthy subject showing CII-induced cytokine production showed an up-regulation of TNF-α alone in response to the stimulus (10pg/ml). In the RA patients, CII-induced cytokine production was detected both in conjunction with and independent of CII-induced PBMC proliferation, while in the healthy subjects, all subjects that showed CII-induced cytokine production also showed positive CII-induced PBMC proliferative responses. Interestingly, CII-induced production of detectable levels of IL-10 and IL-4 was observed only in the healthy subjects. The levels of CII-induced IL-17, IL-6, TNF-α and IFN-γ production did not differ significantly between the RA patients and the healthy subjects (P values = 0.24, 0.11, 0.75 and 0.57 for IL-17, IL-6, TNF-α and IFN-γ respectively, Mann Whitney).

4.2.3.4. GlyAgg-induced responses

The 3HTdR incorporation data in Table 4.5 shows that the proportions of RA patients and healthy subjects that showed positive GlyAgg-induced PBMC proliferative responses were very similar (44% and 40% respectively). The median peak SI value of GlyAgg-induced PBMC proliferation detected in RA patients was similar to that of GlyAgg-induced PBMC proliferation detected in healthy subjects (median = 1.6, IQR: 1.0-2.8 and median = 1.5, IQR: 1.1-2.7 respectively, P value = 0.90, Mann Whitney, Figure 4.9A). In the RA patients that showed positive GlyAgg-induced PBMC proliferative responses, peak levels of proliferation were detected at days 3 and 5 (*n*=1 and 3 respectively, Figure 4.9B). In the healthy subjects that showed positive GlyAgg-induced PBMC proliferative responses, peak levels of proliferation were detected at days 5, 9 and 11 (*n*=1, 2 and 1 respectively, Figure 4.9C).

The cytokine production data in Table 4.5 shows that of the five RA patients tested for GlyAgg-induced cytokine production, 80% showed cytokine production in response to the stimulus, compared to 63% of the tested healthy subjects. However, this difference was not significant (P value = 0.98, Chi-square). The cytokines produced by RA patients in response to GlyAgg stimulation were IL-10 (2pg/ml), IL-17 (3pg/ml), IL-4 (6pg/ml and 3pg/ml), IL-6 (24pg/ml, 209pg/ml, 1640pg/ml and 3037pg/ml) and TNF-α

(4pg/ml, 3pg/ml and 34pg/ml). Healthy subjects only showed production of IL-6 (25pg/ml, 146pg/ml, 116pg/ml, 393pg/ml and 430pg/ml) and TNF-α (4pg/ml) in response to the stimulus. All four of the RA patients that showed GlyAgg-induced cytokine production also showed GlyAgg-induced proliferation while in the healthy subjects GlyAgg-induced cytokine production was detected both in conjunction with and independent of GlyAgg-induced proliferation. The levels of GlyAgg-induced IL-6 and TNF-α production did not differ significantly between the RA patients and the healthy subjects (P values = 0.37 and 0.13 for IL-6 and TNF-α respectively, Mann Whitney).

4.2.3.5. HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation; triplicate culture 3HTdR incorporation approach versus bulk culture 3HTdR incorporation approach

The proportions of HCgp39-specific proliferative responses detected in the RA patients are comparable with both the triplicate culture approach and the bulk culture approach (40% and 44% respectively). However, in the healthy subjects, a greater proportion of HCgp39-specific proliferative responses were detected when using the triplicate culture approach as opposed to the bulk culture approach (75% versus 38% respectively), although this difference was not statistically significant (P value of 0.68, Chi-square). Greater proportions of CII-specific proliferative responses were detected in both the RA patients and the healthy subjects when using the triplicate culture approach (57% and 88% respectively) as opposed to the bulk culture approach (33% and 38% respectively). However, these differences failed to reach statistical significance (P values of 0.92 and 0.37 for RA patients and healthy subjects respectively, Chi-square). When using the triplicate culture approach, GlyAgg-specific proliferative responses were detected in both the RA patients and the healthy subjects but were not detected in either cohort when using the bulk culture approach.

Figure 4.10 compares the peak SI values of HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation detected with the triplicate culture approach with those of HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation detected with the bulk culture approach. Figure 4.10(A) and (B) shows that in both RA patients and healthy subjects respectively, the peak SI values of HCgp39-induced PBMC proliferation detected with the triplicate culture approach were statistically similar to those detected with the bulk culture approach (P values = 0.68 for RA patients and 0.29

for healthy subjects, Mann Whitney). Figure 4.10(C) and (D) shows that in both RA patients and healthy subjects respectively, the peak SI values of CII-induced PBMC proliferation detected with the triplicate culture approach were statistically similar to those detected with the bulk culture approach (P values = 0.63 for RA patients and 0.80 for healthy subjects, Mann Whitney). Figure 4.10(E) and (F) shows that in both RA patients and healthy subjects respectively, the peak SI values of GlyAgg-induced PBMC proliferation detected with the triplicate culture approach were statistically similar to those detected with the bulk culture approach (P values = 0.06 for RA patients and 0.14 for healthy subjects, Mann Whitney).

4.2.3.6. DeglyAgg-induced responses

The 3HTdR incorporation data in Table 4.6 shows that all RA patients and all healthy subjects showed positive DeglyAgg-induced PBMC proliferative responses. The median peak SI value of DeglyAgg-induced PBMC proliferation detected in RA patients (median = 17.9, IQR: 11.4-30.7) was slightly less than that of DeglyAgg-induced PBMC proliferation detected in healthy subjects (median = 19.1, IQR: 11.9-30.2), although the difference between the 2 groups was not statistically significant (P value = 0.81, Mann Whitney, Figure 4.11A). In the RA patients, peak levels of DeglyAgg-induced PBMC proliferation were detected at days 7, 9 and 11 (n=1, 7 and 4 respectively), and at days 3, 7, 9 and 11 (n=1, 4, 1 and 7 respectively) in the healthy subjects (Figure 4.11(B) and (C) respectively).

The cytokine production data in Table 4.6 shows that all eight of the RA patients and all eight of the healthy subjects tested for DeglyAgg-induced cytokine production showed increased production of IL-10, IL-17, IL-4, IL-6 and TNF-α in response to the stimulus. Four of the RA patients and six of the healthy subjects also showed DeglyAgg-induced up-regulation of IFN-γ production. Both the RA patients and the healthy subjects showed very high levels of DeglyAgg-induced IL-6 production (median absolute values of median = 13245, IQR: 12872-20974 and median = 13589, IQR: 12966-22126 respectively), and highly detectable levels of DeglyAgg-induced TNF-α production (median absolute values of median = 862, IQR: 425-972 and median = 782, IQR: 490-1135 respectively). The levels of DeglyAgg-induced cytokine production did not differ significantly between the RA patients and the healthy subjects (P values = 0.51, 0.24, 0.65, 0.72, 0.96 and 0.06 for IL-10, IL-17, IL-4, IL-6, TNF-α and IFN-γ respectively, Mann Whitney). However, when the cytokine data are expressed as SI values as

opposed to absolute values, the SI values of DeglyAgg-induced IL-6 production of the RA patient group were significantly greater than those of the healthy subject group (median SI values of median = 2366, IQR: 115.4-15944 versus median = 54.8, IQR: 7.9-392.1 respectively, P value = 0.04, Mann Whitney, Figure 4.11D).

Nineteen of the twenty four RA patients and fourteen of the sixteen healthy subjects showed proliferation and/or cytokine production responses to at least one of the four autoantigens. Furthermore, subjects that showed proliferative and/or cytokine production responses to more than one autoantigen were not rare in either of the groups. In the RA patient group, RA 3.30, RA 4.32, RA 4.33 and RA 4.34 all showed positive proliferative and cytokine production responses to both GlyAgg and DeglyAgg and RA 4.34 also showed a positive proliferative response to CII. RA 4.35 and RA 4.38 each showed positive responses to both HCgp39 and CII. Of the 20 RA patients that showed positive responses to at least one of the autoantigens, at least 9 were both RF and CCP positive (a further 3 were not tested for both RF and CCP levels and 4 more were positive for RF but untested for CCP levels). However, 2 of the 4 non-responders were also both RF and CCP positive and indeed one of the responders was negative for both RF and CCP. No trends were visible in either the CRP levels or ESR values of responders or in the duration of the disease. In the healthy subject group, HS 4.13, HS 4.15, HS 4.16 and HS 4.17 all showed positive responses to all four of the autoantigens, while HS 4.12 and HS 4.23 both showed positive response to HCgp39, CII and DeglyAgg. HS 4.10 and HS 4.22 showed positive responses to both GlyAgg and DeglyAgg and HCgp39 and DeglyAgg respectively.

4.2.4. Co-culture approach with mo-DC and T-cells

With the bulk culture and triplicate culture PBMC approaches, proliferative responses to HCgp39, CII and GlyAgg, when detected, were very weak in magnitude. Furthermore, the magnitude of proliferative responses detected to these autoantigens in RA patients was not significantly different to that detected in healthy subjects. I hypothesised that the magnitude of the HCgp39-induced, CII-induced and GlyAgg-induced responses detected with these two whole PBMC approaches were low due to the fact that the presentation of the candidate autoantigens by un-separated PBMC was sub-optimal due to the lack of professional antigen-presenting cells. Therefore, I investigated the use of mo-DC as antigen-presenting cells.

As an initial step I set up a co-culture system that would allow me to detect antigen-specific T-cell proliferation, using PPD as the test antigen. I co-cultured $CD3^+$ PBMC (T-cells) with both immature and mature mo-DC, each either unloaded or PPD-loaded, at three different mo-DC: T-cell ratios; 5:1, 10:1 and 50:1. This would allow me to determine which type of mo-DC and which ratio of mo-DC:T-cells would allow for the best antigen presentation. Data from these experiments is shown in Figure 4.12A. At all three ratios, PPD-loaded, immature mo-DC failed to induce T-cell proliferation. When using PPD-loaded, LPS-matured mo-DC, positive T-cell proliferative responses were only detected consistently with a mo-DC:T-cell ratio of 50:1. However, the mean SI value of PPD-induced T-cell proliferation was only 5, and while this is a clear positive response, it was lower than the mean SI values of PPD-induced PBMC proliferation detected with either the triplicate culture or bulk culture approaches.

Having established that LPS-matured mo-DC were the most efficient at presenting antigen to autologous T-cells, and that a mo-DC: T-cell ratio of 50:1 would elicit the greatest T-cell response, these parameters were used in another set of experiments in which HCgp39-loaded, CII-loaded, GlyAgg-loaded and DeglyAgg-loaded, LPS-matured mo-DC were employed to elicit responses in autologous T-cells. Data from these experiments are shown in Figure 4.12B. PPD-loaded, LPS-matured mo-DC mixed with autologous T-cells served as a positive control. Of the 2 RA patients and 3 healthy subjects tested, all showed SI values of PPD-induced T-cell proliferation of 2 or greater, although surprisingly the SI values of PPD-induced proliferation seen with the whole PBMC triplicate culture approach were significantly greater than those seen with the mo-DC and T-cell co-culture approach (median = 16.0, IQR: 5.5-31.1 versus median = 4.8, IQR: 2.4-6.1 respectively, P value = 0.04, Mann Whitney). Most importantly no T-cell proliferation was shown by any of the 5 subjects in response to HCgp39, CII, GlyAgg or DeglyAgg when using the mo-DC and T-cell co-culture approach.

4.2.5. The role of bacterial lipopolysaccharide (LPS) contamination in DeglyAgg-induced responses

Because of the nature of the DeglyAgg-induced responses (i.e. the universal nature of eliciting T-cell responses in RA patients and healthy subjects as well as the predominant production of IL-6), it was possible that the DeglyAgg preparation was contaminated with LPS and that the PBMC were responding to this and not the DeglyAgg antigen. In order to first determine whether the DeglyAgg preparation contained any LPS, a LAL

assay was performed on samples of the DeglyAgg preparation. At a concentration of 1mg/ml in RPMI 1640, the DeglyAgg preparation was found to contain 1.7 EU/ml of endotoxin. According to the LAL kit manufacturers, 10EU equals 1ng of endotoxin, meaning that 1ml of a 1mg/ml solution of the DeglyAgg contained 170pg of endotoxin. However, previous studies have suggested that such concentrations of LPS are insufficient to induce T-cell proliferation (Tough *et al*, 1997 and Tulic *et al*, 2002). To determine to what extent this level of LPS was influencing the DeglyAgg response, I stimulated PBMC from 5 healthy volunteers with 10μg/ml DeglyAgg with and without the presence of PMB, a compound that neutralises LPS (Cardoso *et al*, 2007). PBMC proliferation and IL-6 production in response to the stimulus were assessed after 5, 7, 9 and 11 days of culture via 3HTdR incorporation and ELISA respectively. Figure 4.13A shows the mean SI values of DeglyAgg-induced PBMC proliferation seen with or without the presence of PMB at each of the time-points. When no PMB was present in the cell cultures, DeglyAgg-induced PBMC proliferation was detectable at all 4 time-points (mean ± SEM SI values of 2.8 ± 0.6, 8.3 ± 2.9, 11.6 ± 5.5 and 10.9 ± 5.1 at days 5, 7, 9 and 11 respectively), and peak levels of DeglyAgg-induced PBMC proliferation were detected at day 9. However, when PMB was present in the cell cultures, no DeglyAgg-induced PBMC proliferation was detected at days 5, 7 and 9 (mean ± SEM SI values of 1.2 ± 0.5, 1.5 ± 0.2 and 1.9 ± 0.8 respectively). Only at day 11 was any PBMC proliferation in response to the DeglyAgg detected (mean ± SEM SI value of 6.4 ± 2.1). Therefore, while the PMB was certainly reducing the magnitude of the DeglyAgg-induced proliferative response, it did not completely abrogate it. Importantly, data from three separate experiments showed that PMB treatment of PBMC cultures did not reduce the magnitude of the PHA-induced proliferative response (mean ± SEM SI value of PHA-induced PBMC proliferation without PMB versus mean ± SEM SI value of PHA-induced PBMC proliferation with PMB = 185.6 ± 62.8 versus 240.9 ± 116.8 respectively, P value of 0.4, paired t test). Figure 4.13B shows the mean absolute levels of DeglyAgg-induced IL-6 production seen with or without PMB at each of the 4 time-points. When PMB was not present in the cultures, high levels of DeglyAgg-induced IL-6 production were detected at all 4 time-points. The peak levels of DeglyAgg-induced IL-6 production were detected at day 5 (mean ± SEM absolute value of 27464 ± 1281), and the levels decreased steadily over the subsequent 2 time-points (mean ± SEM absolute values of 26421 ± 1038 and 22914 ± 4125 at days 7 and 9 respectively). However, by day 11, the levels of IL-6 production detected in response to the DeglyAgg increased again (mean ± SEM absolute value of 25258 ± 974). The kinetic of the

DeglyAgg-induced IL-6 production response detected when PMB was present in the cultures was very similar, although the presence of the PMB clearly reduced the levels of IL-6 production detected in response to the DeglyAgg at all time-points (mean ± SEM absolute values of 20578 ± 1247, 17866 ± 1438, 14516 ± 3697 and 17256 ± 1300 at days 5, 7, 9 and 11 respectively). Indeed, this reduction reached significant levels at days 5, 7 and 11 (P values = 0.02, 0.0079 and 0.0079 respectively, Mann Whitney).

4.3. Discussion

The experiments described in this chapter failed to provide evidence suggesting that HCgp39, CII or aggrecan are RA autoantigens involved in disease pathogenesis. In the experiments described in 4.2.1, I measured HCgp39-induced proliferation of peripheral blood T-cells in each of 15 RA patients using the CFSE dilution assay and assessed proliferation within both the $CD4^-$ and $CD4^+$ subsets as both an increase in the % of $CFSE^{dim}$ cells and as a stimulation index. When proliferation was assessed as an increase in the % of $CFSE^{dim}$ cells, 7 of the 15 RA patients showed HCgp39-induced T-cell proliferation (Table 4.1). Proliferation was more frequently seen in the $CD4^+$ subset than the $CD4^-$ subset (5 subjects versus 4 subjects). In 50% of cases, proliferation within the $CD4^-$ subset occurred in conjunction with proliferation in the $CD4^+$ subset. However, with one exception (RA 4.5), the percentages of these HCgp39-induced T-cell responses were very low and indeed when they were expressed as SI values, none exceeded the threshold for a positive response (Table 4.1). As previously mentioned, many of the studies measuring proliferation of immune cells in response to candidate autoantigens have used the 3HTdR incorporation assay to measure proliferation within un-separated populations of PBMC and the data were often expressed as SI values with an SI value of 2 or more being used as the threshold for a positive response (Verheijden *et al*, 1997; Snowden *et al*, 1997; Guerassimov *et al*, 1998; Cope *et al*, 1999; Kim *et al*, 1999; Vos *et al* #2, 2000 and Ria *et al*, 2008). However, it could be the case that expressing CFSE dilution data measuring proliferation in a specific subset of PBMC as an SI value is not appropriate. Because CFSE dilution assays measure cell division whereas 3HTdR incorporation assays measure indirectly DNA incorporation over a period of time, then the magnitude of 'responses' relative to unstimulated culture may not be comparable. Furthermore, in 3HTdR incorporation assays, other subsets of cells may also contribute to the proliferative response, although in chapter 3 I found that 3HTdR incorporation data mirrors that of CFSE dilution assays for $CD3^+$ T-cells. Although the precursor frequencies of the antigen-specific T-cell responses were not

formally enumerated, the data from these experiments suggest that the precursor frequencies of autoantigen-specific T-cell responses are likely to be low. The fact that good PHA-induced proliferative responses were observed in all 15 of the RA patients suggests that the serum from each patient was capable of supporting T-cell proliferation, therefore it is unlikely that the observed lack of HCgp39-induced T-cell proliferation was due to any problems associated with the use of AS as a culture medium supplement.

In the 3HTdR incorporation experiments described in 4.2.2, I chose the bulk culture approach as opposed to a triplicate culture approach as I anticipated that the former would be more sensitive in the detection of low precursor-frequency autoantigen-specific T-cell responses. My rationale was that the higher numbers of cells per well achieved with the bulk culture approach would increase the likelihood of autoantigen-specific T-cells encountering antigen-presenting cells presenting the autoantigens. While substantial numbers of both HCgp39-induced and CII-induced proliferative responses were detected using the bulk culture approach, no GlyAgg-specific proliferative responses were detected (Table 4.2). However, similar proportions of RA patients and healthy subjects showed positive PBMC proliferative responses to HCgp39 and CII (Table 4.2). Furthermore, the magnitudes of the proliferative responses to HCgp39 and CII were comparable between the RA patients and the healthy subjects (Figure 4.3(A) and (B)).

In the 3HTdR incorporation experiments described in 4.2.3, I revisited the triplicate culture approach with $2x10^5$ cells/well as used in many previous RA autoantigen studies. I also compared the data obtained using the triplicate culture approach with that obtained using the bulk culture approach but because the 2 different approaches were not tested with PBMC from the same subjects (in part due to the number of cells required for such assays) and compared in parallel, observations made from this comparison should be interpreted with caution. Nevertheless, I found that triplicate culture 3HTdR incorporation assays are at least as sensitive as the bulk culture approach.

While there was a general trend suggesting that greater proportions of HCgp39-specific, CII-specific and GlyAgg-specific proliferative responses were detected when using the triplicate culture approach as opposed to the bulk culture approach in both the RA

patients and the healthy subjects, the differences between the 2 approaches were not statistically significant. Indeed, the magnitudes of HCgp39-specific, CII-specific and GlyAgg-specific proliferative responses detected with the 2 approaches were similar (Figure 4.10). Ultimately, as seen with the bulk culture approach, HCgp39-specific, CII-specific and GlyAgg-specific proliferative responses were no more prevalent in RA patients than in healthy subjects (Tables 4.3, 4.4 and 4.5) and the magnitudes of the responses were similar in both subject groups (Figures 4.7A, 4.8A and 4.9A).

Of course, proliferation is only one of several immune responses that are made by T-cells therefore I also examined cytokine production in response to the candidate RA autoantigens. I had collected supernatant samples from all of the bulk culture and triplicate culture experiments but because the triplicate culture approach was associated with greater proportions of candidate RA autoantigen-specific responses, I decided to use the supernatant samples from only the triplicate culture experiments to investigate candidate RA autoantigen-specific cytokine production. However, as previously mentioned, the comparison of the triplicate culture and bulk culture approaches was not an optimal comparison. In addition, and as alluded to in my previous chapter and demonstrated by the data in this chapter, cytokine production may occur independently of proliferation, although it is possible that the optimal conditions for the detection of cytokine production responses may be identical to the conditions that are optimal for the detection of proliferative responses. Nevertheless, the cytokine production data obtained from the triplicate culture experiments showed that the HCgp39-specific, CII-specific and GlyAgg-specific cytokine production responses of the RA patients did not differ significantly from those of the healthy subjects.

The levels of background cytokine production detected in each of the RA patients and healthy subjects tested for candidate autoantigen-induced cytokine production in the experiments described in 4.2.3 are shown in Table 4.7 and Table 4.8 respectively. As can be seen, many of the subjects from both the RA patient and healthy subject groups showed highly detectable levels of background production of both IL-6 and TNF-α. When candidate autoantigen-induced increases in IL-6 and TNF-α production were observed, they were in the majority of cases highly substantial (see the absolute values of HCgp39-induced, CII-induced, GlyAgg-induced and DeglyAgg-induced IL-6 and TNF-α production in Table 4.3, Table 4.4, Table 4.5 and Table 4.6 respectively), and highly likely to be representative of positive responses. However, occasional subjects

showed substantial levels of background production of IL-10, IL-17, IL-4 and IFN-γ, and candidate autoantigen-induced increases in production of these cytokines were more subtle, thus care must be taken when interpreting such data. For example, when PBMC from RA 4.38 were cultured with medium alone, the supernatant was shown to contain IL-17 at a concentration of 56pg/ml (Table 4.7), yet the absolute value of CII-induced IL-17 production for RA 4.38 was 3pg/ml (Table 4.4A), as the supernatant from the CII-stimulated culture of PBMC from this subject was shown to contain IL-17 at a concentration of 59pg/ml. However, because background production of IL-17 by the PBMC from RA 4.38 was so substantial, it is debatable whether an absolute value of CII-induced IL-17 production of 3pg/ml in this patient can really be classed as a positive response, despite the fact that it exceeds the detection threshold of a positive response.

DeglyAgg-induced proliferative responses were observed in all subjects tested and the magnitudes of these responses were similar between RA patients and healthy subjects (Table 4.6 and Figure 4.11A). DeglyAgg-induced cytokine production responses of the RA patients and the healthy subjects were also comparable (Table 4.6). However, the DeglyAgg-specific proliferative responses were more prominent than those induced by HCgp39, CII and GlyAgg. In addition, while all subjects produced detectable levels of IL-10, IL-17, IL-4, IL-6 and TNF-α in response to DeglyAgg, in both cohorts, IL-6 and TNF-α were the most abundantly produced cytokines. This raised the possibility of LPS contamination of the DeglyAgg which was confirmed by LAL assay. In experiments using PMB to determine to what extent the LPS was influencing the DeglyAgg-specific response, I demonstrated that PMB could reduce the DeglyAgg-induced proliferation and IL-6 production responses but could not completely abrogate them (Figure 4.13). One possible explanation of this observation is that the LPS acts by amplifying the response to the DeglyAgg through acting as an adjuvant, perhaps through activating antigen-presenting cells and up-regulating antigen processing and presentation among this cell population.

The kinetics of the candidate RA autoantigen-induced proliferative responses varied between individual subjects, and no clear differences were observed between RA patients and healthy subjects on the basis of response kinetics. In both groups, some subjects showed peak levels of proliferation early on in the culture period suggestive of memory responses, while many showed peak proliferation at the later time-points

associated with primary responses of naïve T-cells (Croft *et al*, 1994). This suggests that T-cell memory to HCgp39, CII and aggrecan is no more common in RA patients than in healthy subjects.

While it is indeed possible that HCgp39-specific, CII-specific and aggrecan-specific T-cell responses do not differ between RA patients and healthy subjects *in vivo*, another explanation as to why I was not seeing differences between the HCgp39-specific, CII-specific and aggrecan-specific T-cell responses of the RA patients and the healthy subjects with the bulk culture and triplicate culture approaches could have been that the responses were sub-optimal when using *in vitro* cultures of whole PBMC. Indeed, the magnitudes of the HCgp39, CII and GlyAgg-induced proliferation and cytokine production responses were in general very moderate, especially when compared to those seen in response to DeglyAgg. One explanation as to why the magnitudes of HCgp39-specific, CII-specific and GlyAgg-specific responses seen in these experiments were very moderate could have been that antigen presentation in the whole PBMC cultures was not optimal. Enhanced antigen presentation may amplify the T-cell responses and enable any differences between the responses of RA patients and healthy subjects to be observed more easily. To investigate whether sub-optimal presentation of the antigens was a reason for the weak responses seen, I repeated the experiments using a co-culture approach using autoantigen-loaded mo-DC and T-cells, anticipating that the presence of specialised antigen-presenting cells would solve the potential problem of sub-optimal antigen presentation. However, no HCgp39, CII, GlyAgg or even DeglyAgg-specific proliferation was seen with this mo-DC and T-cell co-culture approach (Figure 4.12B). Furthermore, the magnitudes of the PPD-specific proliferative responses seen with the mo-DC and T-cell co-culture approach were significantly less than those seen with the triplicate culture approach using un-separated PBMC. However, the comparison of data from the mo-DC:T-cell co-culture approach with that from the triplicate culture approach was not a direct comparison due to the cell numbers required. In any case, if the mo-DC were impairing the PPD-induced proliferative responses, then it would suggest that the mo-DC preparation was toxic in some way.

The experiments described in 4.2.2 and 4.2.3.2 showed that HCgp39-specific PBMC proliferation was detected in both RA patients and healthy subjects, similar to what has been described in previous studies (Verheijden *et al*, 1997; Cope *et al*, 1999 and Vos *et al* #2, 2000). However, Verheijden *et al* (1997) demonstrated that a substantially greater

proportion of RA patients than healthy subjects showed HCgp39-specific PBMC proliferation, while a subsequent study showed a significantly greater proportion of RA patients than healthy subjects showed HCgp39-specific PBMC proliferation (Vos *et al* #2, 2000). However, data from my experiments do not support these observations. In the experiments described in 4.2.2, HCgp39-specific PBMC proliferation was detected in a very similar proportion of RA patients as healthy subjects (44% and 38% respectively, Table 4.2). The difference between my data and those from the Verheijden *et al* and Vos *et al* #2 studies could be due to the fact that my data were obtained using whole protein HCgp39 while the others used HCgp39-derived peptides. Indeed, in these studies one or two of the peptides induced the majority of the responses. Perhaps these peptides were not being generated in abundance in my *in vitro* cultures and were not readily available for presentation. In the experiments described in 4.2.3.2, HCgp39-specific PBMC proliferation was detected in a substantially greater proportion of healthy subjects than RA patients (75% versus 40%, Table 4.3). It has been previously reported that the HCgp39-specific PBMC reactivity of healthy subjects is characterised by production of IL-10 while that of RA patients is associated with significantly greater levels of IFN-γ production (van Bilsen *et al*, 2004). However, the experiments described in 4.2.3.2 showed that of the four healthy subjects that showed HCgp39-induced cytokine production, none showed any up-regulation of IL-10 in response to the stimulus (Table 4.3B). Furthermore, of the four RA patients that showed HCgp39-induced cytokine production, none showed any up-regulation of IFN-γ or IL-10 in response to the stimulus (Table 4.3A). This lack of detectable HCgp39-induced IFN-γ production by RA patients is similar to what was described by Zou *et al* (2003). However, while Zou *et al* also reported a lack of detectable HCgp39-induced TNF-α production in both RA patients and healthy subjects, my experiments showed that HCgp39 induced production of detectable levels of TNF-α production in three of the RA patients and two of the healthy subjects (Table 4.3).

The experiments described in 4.2.2 showed that CII-induced proliferative responses were detected in 33% of RA patients (Table 4.2), and this is similar to what was observed in previous studies that used whole protein CII to stimulate RA patient and healthy subject PBMC (Kim *et al*, 1999 and Park *et al*, 2001). However, these previous studies also demonstrated that CII-specific proliferative responses were significantly more prevalent in RA patients than in healthy subjects. In contrast, the data from my experiments showed that the proportions of subjects showing CII-induced proliferative

responses were comparable between RA patient and healthy subject groups (Table 4.2 and Table 4.4), and this is similar to what was reported by Snowden *et al* (1997) who also used whole protein CII to stimulate RA patient and healthy subject PBMC. The experiments described in 4.2.3.3 detected CII-induced proliferative responses in an even greater proportion of RA patients, similar to that described by Snowden *et al* (1997). It has been shown that RA patients show significantly greater levels of CII-induced IFN-γ than healthy subjects (Park *et al*, 2001), although the data from my experiments did not support this finding. Indeed, while comparable levels of CII-induced IFN-γ production were detected in both RA patients and healthy subjects, a greater proportion of healthy subjects than RA patients showed CII-induced IFN-γ production (Figure 4.8A and Table 4.4). In addition, while Zou *et al* (2003) reported that neither RA patients nor healthy subjects showed any CII-induced TNF-α production, my data showed that of the four RA patients and six healthy subjects that showed CII-induced cytokine production, two of the former and all of the latter up-regulated production of TNF-α in response to the stimulus (Table 4.4).

In the experiments described in 4.2.3.4, GlyAgg-induced proliferative responses were detected in similar proportions of RA patients and healthy subjects and the SI values of GlyAgg-induced proliferation were comparable between both groups (Table 4.5 and Figure 4.9A). This finding is consistent with the study published by Guerassimov *et al* (1998) which showed that the G1 domain of aggrecan in its native, glycosylated form, is no more immunogenic to RA patient PBMC than it is to healthy subject PBMC. However, a further observation of the Guerassimov study was that keratin sulphate-depleted G1 induced proliferative responses in a greater proportion of RA patients than healthy subjects and that the SI values of keratin sulphate-depleted G1 were significantly greater in RA patients than in healthy subjects. In addition, using whole protein DeglyAgg to stimulate RA patient and healthy subject PBMC, Goodstone *et al* (1996) observed that a significantly greater proportion of RA patients than healthy subjects showed positive proliferative responses to the stimulus, and that the SI values of DeglyAgg-induced PBMC proliferation observed in the former were significantly greater than those observed in the latter. However, while the experiments described in 4.2.3.6 showed that deglycosylation did enhance the immunogenicity of the aggrecan, this effect was observed in both the RA patient and healthy subject groups, and the SI values of DeglyAgg-induced proliferation were similar in both cohorts (Table 4.6 and Figure 4.11A). In terms of cytokine production, it has been reported that certain

aggrecan-derived peptides induce significantly higher levels of pro-inflammatory cytokines such as IL-6 and TNF-α from RA patient PBMC than from healthy subject PBMC but induce very little detectable IL-4 and IFN-γ (de Jong *et al*, 2009). As shown in Table 4.5, I observed an absence of detectable IFN-γ in response to GlyAgg in both RA patients and healthy subjects, while detectable GlyAgg-induced IL-4 production was absent in the healthy subject group but observed in two subjects from the RA patient group. GlyAgg-induced IL-6 and TNF-α production was observed in both groups and while IL-6 production by the RA patients tended to be greater than IL-6 production by the healthy subjects and more RA patients than healthy subjects showed TNF-α production, no significant differences were observed between the two groups (Table 4.5). The high levels of DeglyAgg-induced IL-6 and TNF-α production observed in the RA patients was matched by similar levels in the healthy subjects, and detectable levels of IL-4 and IFN-γ production in response to the stimulus were observed in both groups (Table 4.6).

4.4. Summary

Positive proliferative and/or cytokine production responses to the whole protein candidate RA autoantigens HCgp39, CII, GlyAgg and DeglyAgg were readily detectable in both RA patients and healthy subjects. However, the responses were generally weak and both the proportions of subjects showing positive responses to any of the autoantigens as well as the magnitudes of these responses did not differ significantly between the two groups. Furthermore, the candidate RA autoantigen-induced cytokine production responses of RA patients were similar to those of healthy subjects.

ID	Increase in % CFSE^{dim} cells		CFSE dilution SI value	
	CD4$^-$	CD4$^+$	CD4$^-$	CD4$^+$
RA 4.1	1.45-1.60 = 0	1.22-1.40 = 0	1.45/1.60 = 0.9	1.22/1.40 = 0.9
RA 4.2	0.26-0.66 = 0	1.20-1.64 = 0	0.26/0.66 = 0.4	1.20/1.64 = 0.7
RA 4.3	0.27-0.17 = 0.10	1.04-0.63 = 0.41	0.27/0.17 = 1.6	1.04/0.63 = 1.7
RA 4.4	0.17-0.22 = 0	1.51-0.93 = 0.58	0.17/0.22 = 0.8	1.51/0.93 = 1.6
RA 4.5	0.62-0.19 = 0.43	5.58-1.41 = 4.17	0.62/0.19 = 3.3	5.58/1.41 = 4.0
RA 4.6	0.31-0.30 = 0.01	0.43-0.32 = 0.11	0.31/0.30 = 1.0	0.43/0.32 = 1.3
RA 4.7	0.64-0.62 = 0.02	1.70-1.10 = 0.60	0.64/0.62 = 1.0	1.70/1.10 = 1.6
RA 4.8	0.15-0.13 = 0.02	0.77-0.71 = 0.06	0.15/0.13 = 1.2	0.77/0.71 = 1.1
RA 4.9	0.71-0.72 = 0	0.52-0.47 = 0.05	0.71/0.72 = 1.0	0.52/0.47 = 1.1
RA 4.10	0.40-1.13 = 0	0.95-1.09 = 0	0.40/1.13 = 0.4	0.95/1.09 = 0.9
RA 4.11	0.95-0.98 = 0	1.40-1.61 = 0	0.95/0.98 = 1.0	1.40/1.61 = 0.9
RA 4.12	0.62-0.70 = 0	1.54-1.46 = 0.08	0.62/0.70 = 0.9	1.54/1.46 = 1.1
RA 4.13	0.10-0.11 = 0	0.16-0.14 = 0.02	0.10/0.11 = 0.9	0.16/0.14 = 1.1
RA 4.14	1.26-1.06 = 0.20	1.29-1.21 = 0.08	1.26/1.06 = 1.2	1.29/1.21 = 1.1
RA 4.15	2.89-2.51 = 0.38	1.23-1.42 = 0	2.89/2.51 = 1.2	1.23/1.42 = 0.9

Table 4.1. HCgp39-induced proliferation of peripheral blood T-cells in RA patients. CFSE-labelled PBMC from 15 RA patients were cultured with either medium alone, 5µg/ml PHA or 10µg/ml HCgp39. After 7 days, cells were harvested, stained for expression of CD3 and CD4 and analysed for proliferation via CFSE dilution. Proliferation within the CD4$^-$ and CD4$^+$ subsets of CD3$^+$ lymphocytic PBMC was measured as both an increase in the % CFSE^{dim} cells in response to the stimulus (i.e. % CFSE^{dim} cells in PHA/HCgp39-stimulated culture minus % CFSE^{dim} cells in unstimulated culture) and as a CFSE dilution stimulation index (i.e. percentage of CFSE^{dim} cells in PHA/HCgp39-stimulated culture divided by percentage of CFSE^{dim} cells in unstimulated culture).

A) RA patients

ID	3HTdR incorporation SI value (peak)				
	PHA	PPD	HCgp39	CII	GlyAgg
RA 4.16	34.6	2.2	5	1.7	-
RA 4.17	44.8	1.9	1	1.4	-
RA 4.18	288.7	27.7	1.4	1.1	-
RA 4.19	37.8	4.7	3.7	3.3	-
RA 4.20	120.9	4	1.7	4	1.1
RA 4.21	89.8	79	1.6	1.4	0.8
RA 4.22	168.8	1.7	0.9	1.2	0.8
RA 4.23	60.1	0.8	2.1	0.9	0.6
RA 4.24	171.9	-	2.7	8.1	-

B) Healthy subjects

ID	3HTdR incorporation SI value (peak)				
	PHA	PPD	HCgp39	CII	GlyAgg
HS 4.1	29.2	9.8	1.9	1.9	-
HS 4.2	36.1	4.9	1.7	1.4	-
HS 4.3	38	18.7	7.9	1.9	-
HS 4.4	4.8	2.4	1.9	1.2	-
HS 4.5	40.7	7	1.1	7.6	0.9
HS 4.6	37.5	2.4	2.5	10.6	0.7
HS 4.7	65.7	5.8	3.5	13.1	1.4
HS 4.8	175.6	4.1	1.1	1.9	0.6

Table 4.2. PBMC proliferation detected in RA patients and healthy subjects when using the bulk culture 3HTdR incorporation approach. PBMC from 9 RA patients and 8 healthy subjects were cultured with either medium alone, 5µg/ml PHA, 1µg/ml PPD, 10µg/ml HCgp39, 10µg/ml CII or 10µg/ml GlyAgg. Proliferation was assessed after 5, 7, 9 & 11 days via 3HTdR incorporation. **A**, the peak SI values of PHA-induced, PPD-induced, HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation detected in RA patients. **B**, the peak SI values of PHA-induced, PPD-induced, HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation detected in healthy subjects. In both tables, a dash (-) represents an untested value.

A) RA patients

ID	HCgp39-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 4.25	4.4	0	0	0	191	4	0
RA 4.26	1.8	0	0	0	86	0	0
RA 4.35	2	0	0	10	2649	90	0
RA 4.36	1.7	0	0	0	0	0	-
RA 4.37	1.4	0	0	0	0	0	0
RA 4.38	1.6	0	0	0	673	64	0
RA 4.40	1.9	-	-	-	-	-	-
RA 4.41	12	-	-	-	-	-	-
RA 4.42	1.1	-	-	-	-	-	-
RA 4.46	2.3	-	-	-	-	-	-

B) Healthy subjects

ID	HCgp39-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 4.12	2	0	0	0	0	0	0
HS 4.13	3.1	0	0	3	4890	46	0
HS 4.14	1.7	0	0	0	58	0	0
HS 4.15	3.6	0	0	0	0	0	0
HS 4.16	4.4	0	0	0	10	9	0
HS 4.17	1.9	0	0	0	0	0	14
HS 4.22	3.4	-	-	-	-	-	-
HS 4.23	2.3	-	-	-	-	-	-

Table 4.3. HCgp39-induced PBMC responses identified with the triplicate culture approach. PBMC from 10 RA patients and 8 healthy subjects were cultured with either medium alone, 5µg/ml PHA, 1µg/ml PPD or 10µg/ml HCgp39. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of HCgp39-induced PBMC proliferation and the day 5 absolute values of HCgp39-induced cytokine production detected in the RA patients. **B**, the peak SI values of HCgp39-induced PBMC proliferation and the day 5 absolute values of HCgp39-induced cytokine production detected in the healthy subjects. In both tables, a dash (-) represents an untested value.

A) RA patients

ID	CII-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 4.34	4.5	-	-	-	-	-	-
RA 4.35	2.1	0	0	0	3326	84	0
RA 4.36	2.5	0	0	0	462	0	-
RA 4.37	3.8	0	0	0	79	0	8
RA 4.38	1.9	0	3	0	1817	99	0
RA 4.43	1	-	-	-	-	-	-
RA 4.46	1.2	-	-	-	-	-	-

B) Healthy subjects

ID	CII-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 4.12	2.6	0	0	0	0	10	0
HS 4.13	7.4	0	7	14	5768	14	12
HS 4.14	2.8	0	0	3	3788	68	0
HS 4.15	2.2	7	0	6	4679	426	31
HS 4.16	4.4	8	6	13	3775	31	0
HS 4.17	3.1	3	15	18	12633	61	8
HS 4.22	1.1	-	-	-	-	-	-
HS 4.23	2.4	-	-	-	-	-	-

Table 4.4. CII-induced PBMC responses identified with the triplicate culture approach. PBMC from 7 RA patients and 8 healthy subjects were cultured with either medium alone, 5μg/ml, 1μg/ml PPD or 10μg/ml CII. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of CII-induced PBMC proliferation and the day 5 absolute values of CII-induced cytokine production detected in the RA patients. **B**, the peak SI values of CII-induced PBMC proliferation and the day 5 absolute values of CII-induced cytokine production detected in the healthy subjects. In both tables, a dash (-) represents an untested value.

A) RA patients

ID	GlyAgg-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 4.30	3.5	0	0	0	24	0	0
RA 4.31	1.6	0	0	0	0	0	0
RA 4.32	2.1	0	0	0	209	4	0
RA 4.33	2	0	3	6	1640	3	0
RA 4.34	3.7	2	0	3	3037	34	0
RA 4.40	1.1	-	-	-	-	-	-
RA 4.41	0.9	-	-	-	-	-	-
RA 4.44	0.3	-	-	-	-	-	-
RA 4.45	1.2	-	-	-	-	-	-

B) Healthy subjects

ID	GlyAgg-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 4.9	1.2	0	0	0	0	0	0
HS 4.10	4	0	0	0	0	0	0
HS 4.11	1.3	0	0	0	25	0	0
HS 4.12	1.7	0	0	0	0	0	0
HS 4.13	2.3	0	0	0	146	0	0
HS 4.15	2.7	0	0	0	116	0	0
HS 4.16	2.7	0	0	0	393	4	0
HS 4.17	1.2	0	0	0	430	0	0
HS 4.20	0.8	-	-	-	-	-	-
HS 4.21	0.2	-	-	-	-	-	-

Table 4.5. GlyAgg-induced PBMC responses identified with the triplicate culture approach. PBMC from 9 RA patients and 10 healthy subjects were cultured with either medium alone, 5μg/ml PHA, 1μg/ml PPD or 10μg/ml GlyAgg. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of GlyAgg-induced PBMC proliferation and the day 5 absolute values of GlyAgg-induced cytokine production detected in the RA patients. **B**, the peak SI values of GlyAgg-induced PBMC proliferation and the day 5 absolute values of GlyAgg-induced cytokine production detected in the healthy subjects. In both tables, a dash (-) represents an untested value.

A) RA patients

ID	DeglyAgg-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 4.27	17.9	121	36	55	13674	1103	16
RA 4.28	17.8	18	11	67	13040	914	16
RA 4.29	9.3	26	37	70	12835	385	0
RA 4.30	92.4	449	58	819	33570	206	0
RA 4.31	10.5	28	13	35	11754	938	0
RA 4.32	14.1	144	13	94	23408	810	0
RA 4.33	10.5	30	30	46	12982	983	55
RA 4.34	23.9	18	24	68	13449	544	0
RA 4.39	16	-	-	-	-	-	-
RA 4.46	66.2	-	-	-	-	-	-
RA 4.47	26.9	-	-	-	-	-	-
RA 4.48	31.9	-	-	-	-	-	-

B) Healthy subjects

ID	DeglyAgg-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 4.9	20	173	105	118	33485	531	0
HS 4.10	30.4	133	46	239	19284	357	0
HS 4.11	16.7	73	13	92	23073	477	144
HS 4.12	30	26	47	94	12934	927	27
HS 4.13	40.2	84	45	64	13695	739	70
HS 4.15	22.4	25	11	48	9712	1205	505
HS 4.16	35.4	151	25	33	13063	824	34
HS 4.17	13	64	44	92	13483	2276	89
HS 4.18	11.2	-	-	-	-	-	-
HS 4.19	12.5	-	-	-	-	-	-
HS 4.22	4.5	-	-	-	-	-	-
HS 4.23	6.6	-	-	-	-	-	-
HS 4.24	19.1	-	-	-	-	-	-

Table 4.6. DeglyAgg-induced PBMC responses identified with the triplicate culture approach. PBMC from 12 RA patients and 13 healthy subjects were cultured with either medium alone, 5µg/ml PHA, 1µg/ml PPD or 10µg/ml DeglyAgg. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of DeglyAgg-induced PBMC proliferation and the day 5 absolute values of DeglyAgg-induced cytokine production detected in the RA patients. **B**, the peak SI values of DeglyAgg-induced PBMC proliferation and the day 5 absolute values of DeglyAgg-induced cytokine production detected in the healthy subjects. In both tables, a dash (-) represents an untested value.

ID	Background cytokine production in pg/ml (day 5)					
	IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 4.25	0	8	0	181	6	9
RA 4.26	0	0	0	45	3	394
RA 4.27	0	0	0	0	4	12
RA 4.28	0	0	0	55	53	5
RA 4.29	2	0	0	439	55	0
RA 4.30	0	0	0	176	13	0
RA 4.31	0	0	0	0	0	0
RA 4.32	0	0	0	463	95	21
RA 4.33	0	0	0	47	28	0
RA 4.34	0	0	0	50	2	5
RA 4.35	0	0	0	3	26	0
RA 4.36	0	0	0	5	0	0
RA 4.37	0	0	0	148	11	0
RA 4.38	5	56	18	16247	94	15
RA 4.39	-	-	-	-	-	-
RA 4.40	-	-	-	-	-	-
RA 4.41	-	-	-	-	-	-
RA 4.42	-	-	-	-	-	-
RA 4.43	-	-	-	-	-	-
RA 4.44	-	-	-	-	-	-
RA 4.45	-	-	-	-	-	-
RA 4.46	-	-	-	-	-	-
RA 4.47	-	-	-	-	-	-
RA 4.48	-	-	-	-	-	-

Table 4.7. Background cytokine production detected in the RA patients tested for candidate RA autoantigen-induced cytokine production in the experiments described in 4.2.3. A dash (-) represents an untested value.

ID	Background cytokine production in pg/ml (day 5)					
	IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 4.9	0	0	0	331	33	5
HS 4.10	0	0	0	200	5	0
HS 4.11	0	0	0	76	90	0
HS 4.12	0	0	0	23	84	0
HS 4.13	0	0	0	0	0	0
HS 4.14	0	0	0	44	4	0
HS 4.15	7	5	31	4426	58	23
HS 4.16	0	0	10	2909	46	9
HS 4.17	0	0	0	30	6	0
HS 4.18	-	-	-	-	-	-
HS 4.19	-	-	-	-	-	-
HS 4.20	-	-	-	-	-	-
HS 4.21	-	-	-	-	-	-
HS 4.22	-	-	-	-	-	-
HS 4.23	-	-	-	-	-	-
HS 4.24	-	-	-	-	-	-

Table 4.8. Background cytokine production detected in the healthy subjects tested for candidate RA autoantigen-induced cytokine production in the experiments described in 4.2.3. A dash (-) represents an untested value.

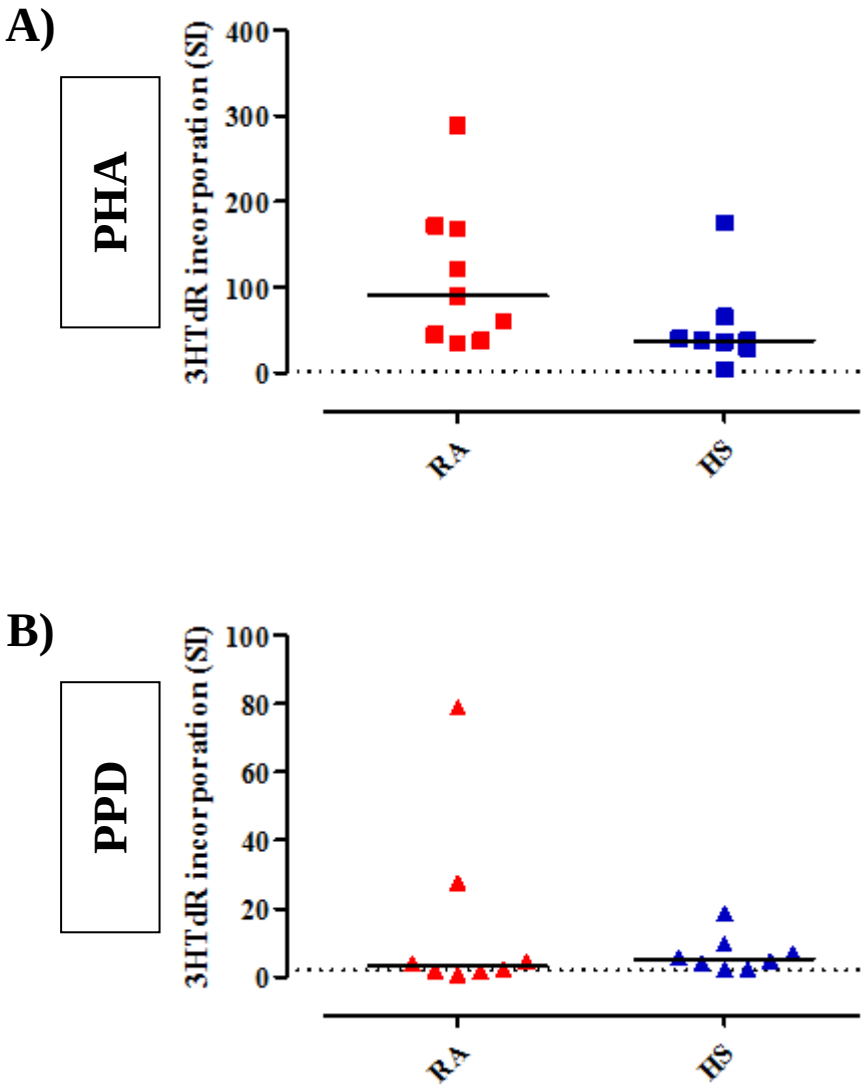

Figure 4.1. PHA-induced and PPD-induced PBMC proliferation in RA patients and healthy subjects; bulk culture 3HTdR incorporation approach. A, the SI values of PHA-induced PBMC proliferation detected in both RA patients (RA, *n*=9) and healthy subjects (HS, *n*=8). **B,** the peak SI values of PPD-induced PBMC proliferation detected in both RA patients (RA, *n*=8) and healthy subjects (HS, *n*=8). In each graph, the dotted horizontal line represents the threshold for a positive response, while the bold horizontal line in each data set represents the median value.

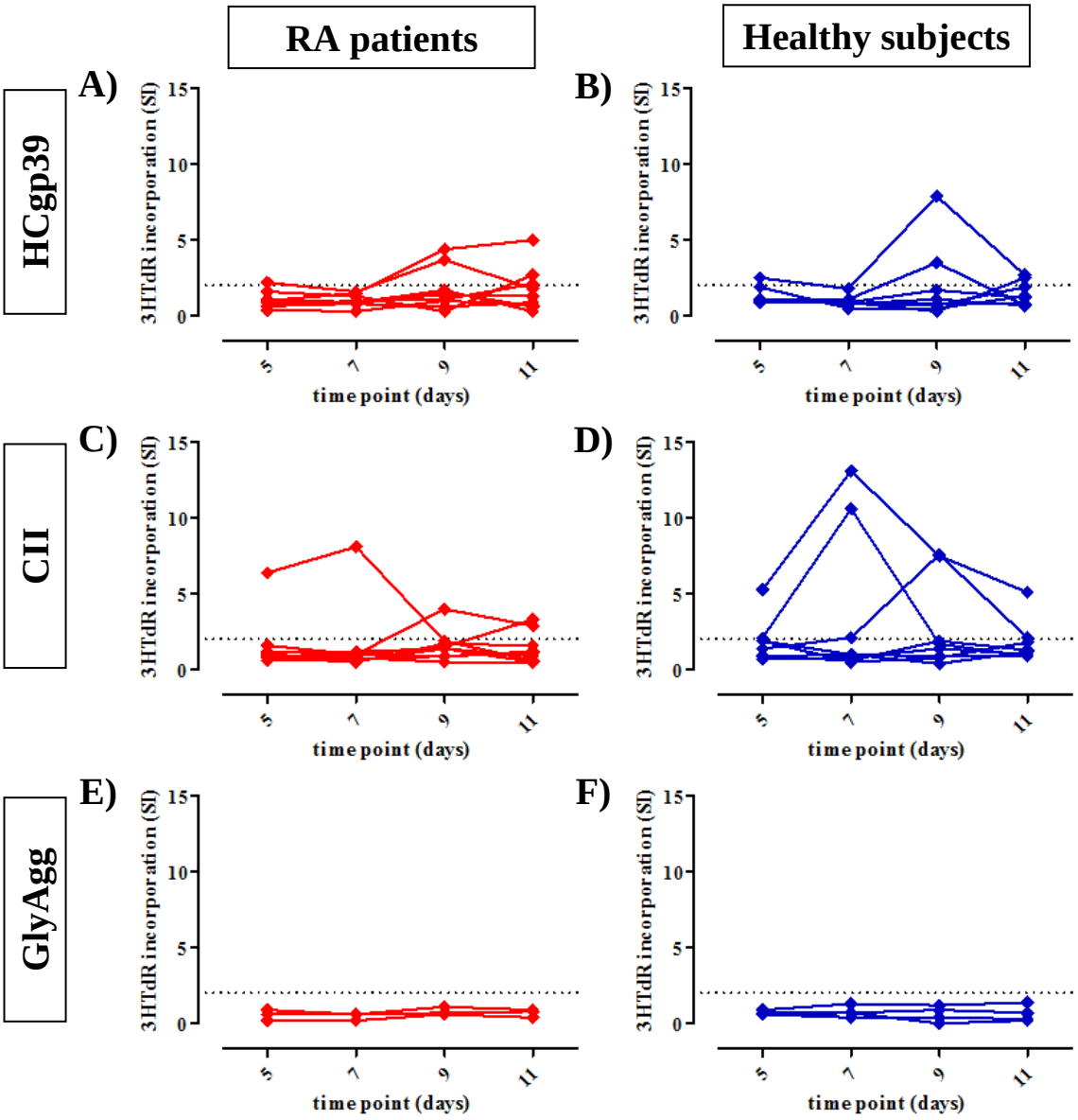

Figure 4.2. Kinetics of the HCgp39-induced, CII-induced and GlyAgg-induced proliferative responses; bulk culture 3HTdR incorporation approach. A & **B**, the kinetics of the HCgp39-induced PBMC proliferation detected in RA patients (*n*=9) and healthy subjects (*n*=8) respectively. **C** & **D**, the kinetics of the CII-induced PBMC proliferation detected in RA patients (*n*=9) and healthy subjects (*n*=8) respectively. **E** & **F**, the kinetics of the GlyAgg-induced PBMC proliferation detected in RA patients (*n*=4) and healthy subjects (*n*=4) respectively. In each graph, the dotted horizontal line represents the threshold for a positive response, and each data point represents an individual SI value.

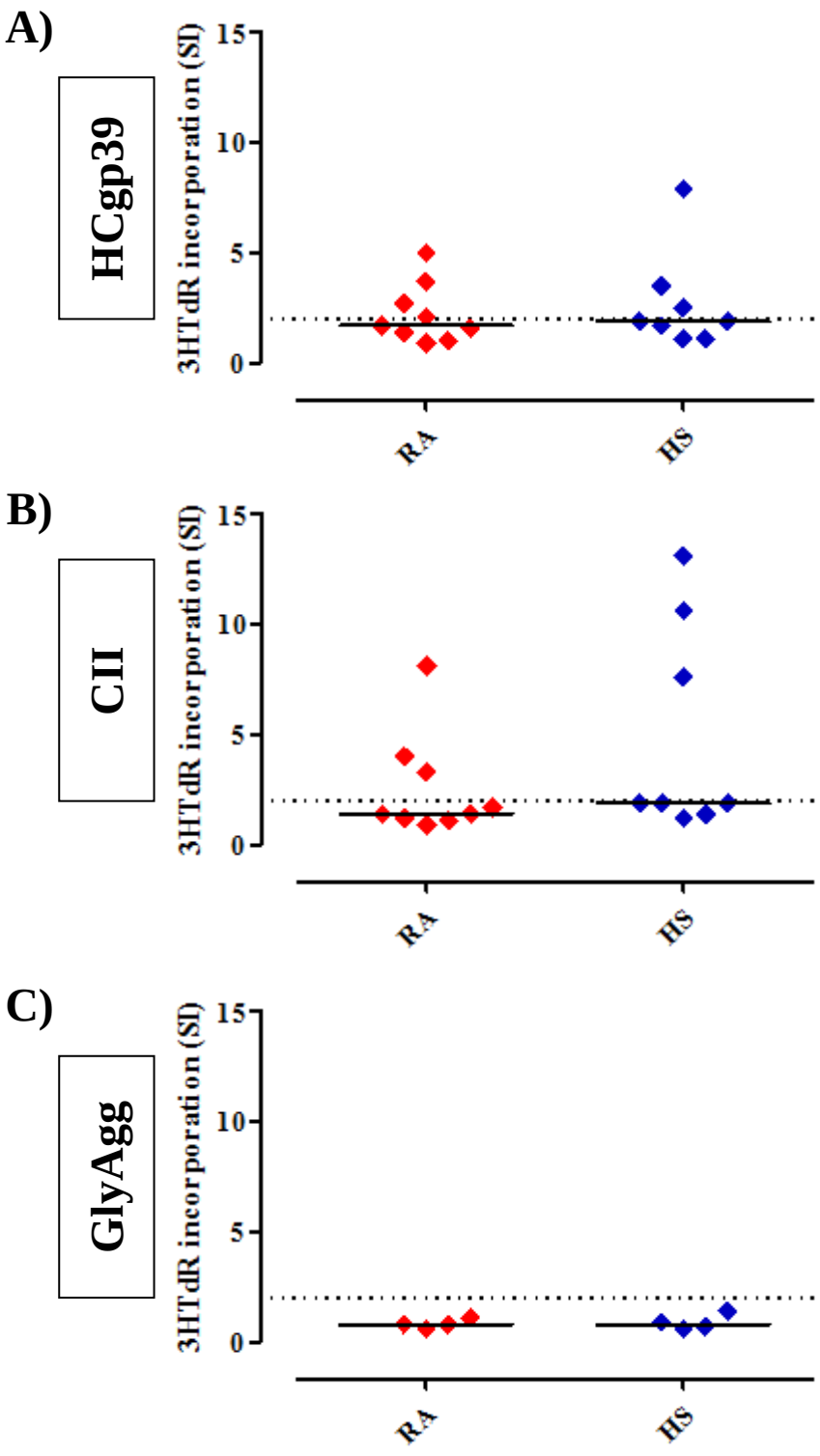

Figure 4.3. HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation in RA patients and healthy subjects; bulk culture 3HTdR incorporation approach. A, the peak SI values of HCgp39-induced PBMC proliferation detected in both RA patients (RA, *n*=9) and healthy subjects (HS, *n*=8). **B**, the peak SI values of CII-induced PBMC proliferation detected in both RA patients (RA, *n*=9) and healthy subjects (HS, *n*=8). **C**, the peak SI values of GlyAgg-induced PBMC proliferation detected in both RA patients (RA, *n*=4) and healthy subjects (HS, *n*=4). In each graph, the dotted horizontal line represents the threshold for a positive response, while the bold horizontal line in each data set represents the median value.

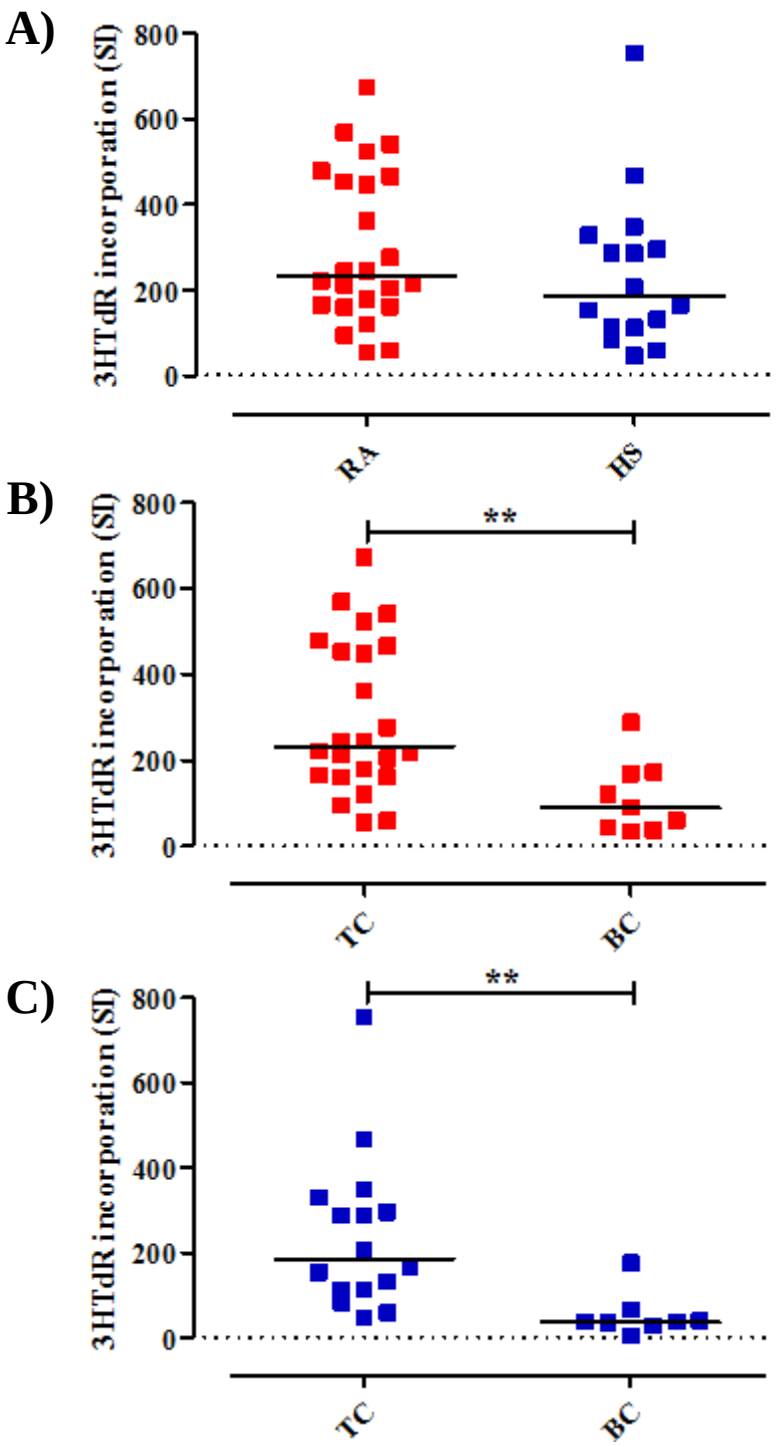

Figure 4.4. PHA-induced PBMC proliferation in RA patients and healthy subjects; triplicate culture 3HTdR incorporation approach versus bulk culture 3HTdR incorporation approach. A, the SI values of PHA-induced PBMC proliferation detected in both RA patients (RA, *n*=24) and healthy subjects (HS, *n*=16) when using the triplicate culture approach. **B**, the SI values of PHA-induced PBMC proliferation detected in RA patients with both the triplicate culture (TC) approach (*n*=24) and the bulk culture (BC) approach (*n*=9). **C**, the SI values of PHA-induced PBMC proliferation detected in healthy subjects with both the triplicate culture (TC) approach (*n*=16) and the bulk culture (BC) approach (*n*=8). In each graph, the dotted horizontal line represents the threshold for a positive response, while the bold horizontal line in each data set represents the median value.

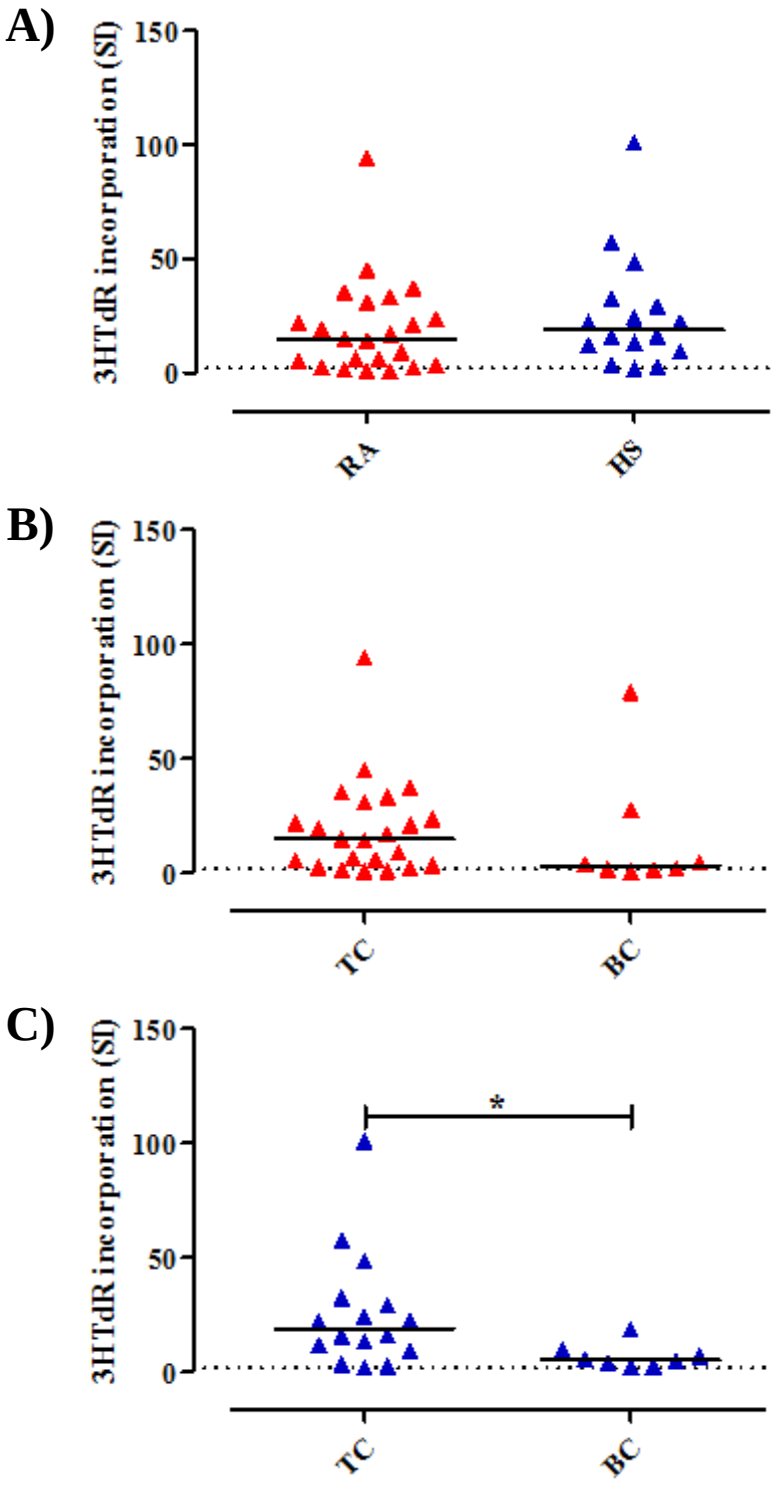

Figure 4.5. PPD-induced PBMC proliferation in RA patients and healthy subjects; triplicate culture 3HTdR incorporation approach versus bulk culture 3HTdR incorporation approach. A, the peak SI values of PPD-induced PBMC proliferation detected in both RA patients (RA, *n*=23) and healthy subjects (HS, *n*=16) when using the triplicate culture approach. **B**, the peak SI values of PPD-induced PBMC proliferation detected in RA patients with both the triplicate culture (TC) approach (*n*=23) and the bulk culture (BC) approach (*n*=8). **C**, the peak SI values of PPD-induced PBMC proliferation detected in healthy subjects with both the triplicate culture (TC) approach (*n*=16) and the bulk culture (BC) approach (*n*=8). In each graph, the dotted horizontal line represents the threshold for a positive response, while the bold horizontal line in each data set represents the median value.

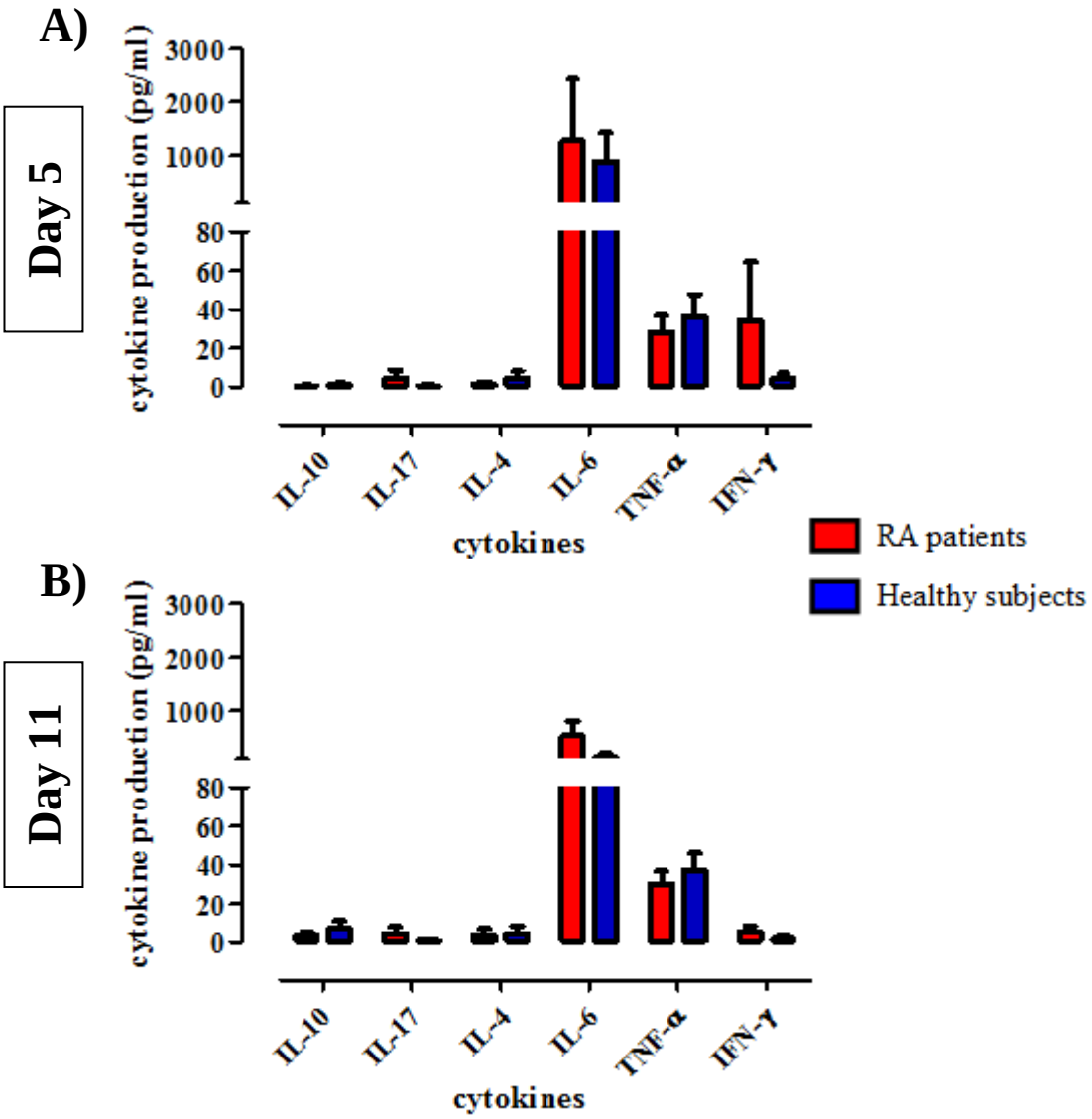

Figure 4.6. Background cytokine production in RA patients and healthy subjects. **A**, the mean levels of cytokine production detected in unstimulated cultures of PBMC from RA patients (*n*=14) and healthy subjects (*n*=9) after 5 days of culture. **B**, the mean levels of cytokine production detected in unstimulated cultures of PBMC from the same RA patients and healthy subjects represented in A after 11 days of culture. For both graphs, vertical bars in each data set represent SEM.

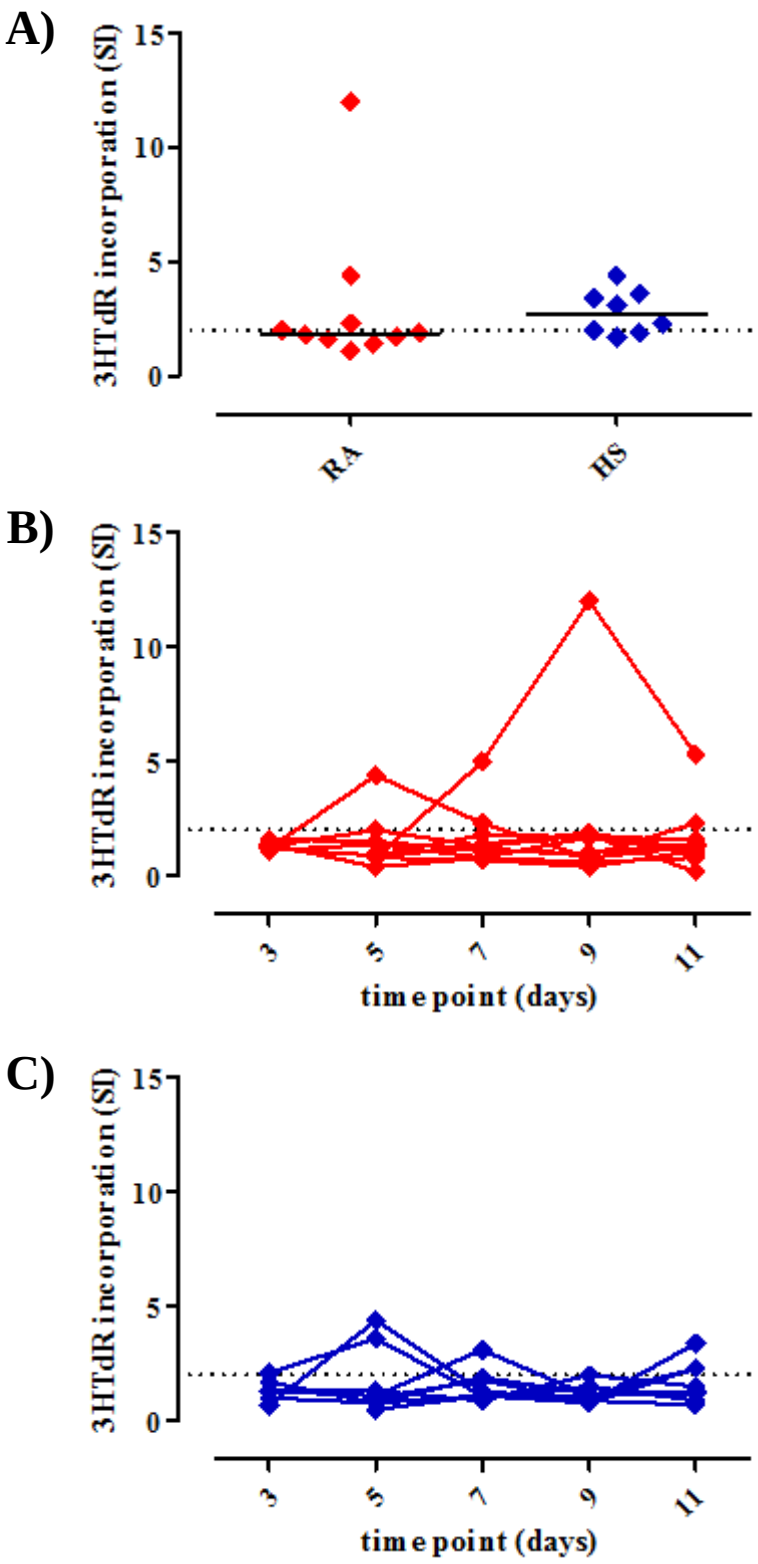

Figure 4.7. HCgp39-induced PBMC proliferation in RA patients and healthy subjects; triplicate culture 3HTdR incorporation approach. A, the peak SI values of HCgp39-induced PBMC proliferation detected in both RA patients (RA, *n*=10) and healthy subjects (HS, *n*=8) when using the triplicate culture approach. The bold horizontal line in each data set represents the median value. **B** & **C**, the kinetics of the HCgp39-induced PBMC proliferation detected in the RA patients and the healthy subjects respectively. In all graphs, the dotted horizontal line represents the threshold for a positive response.

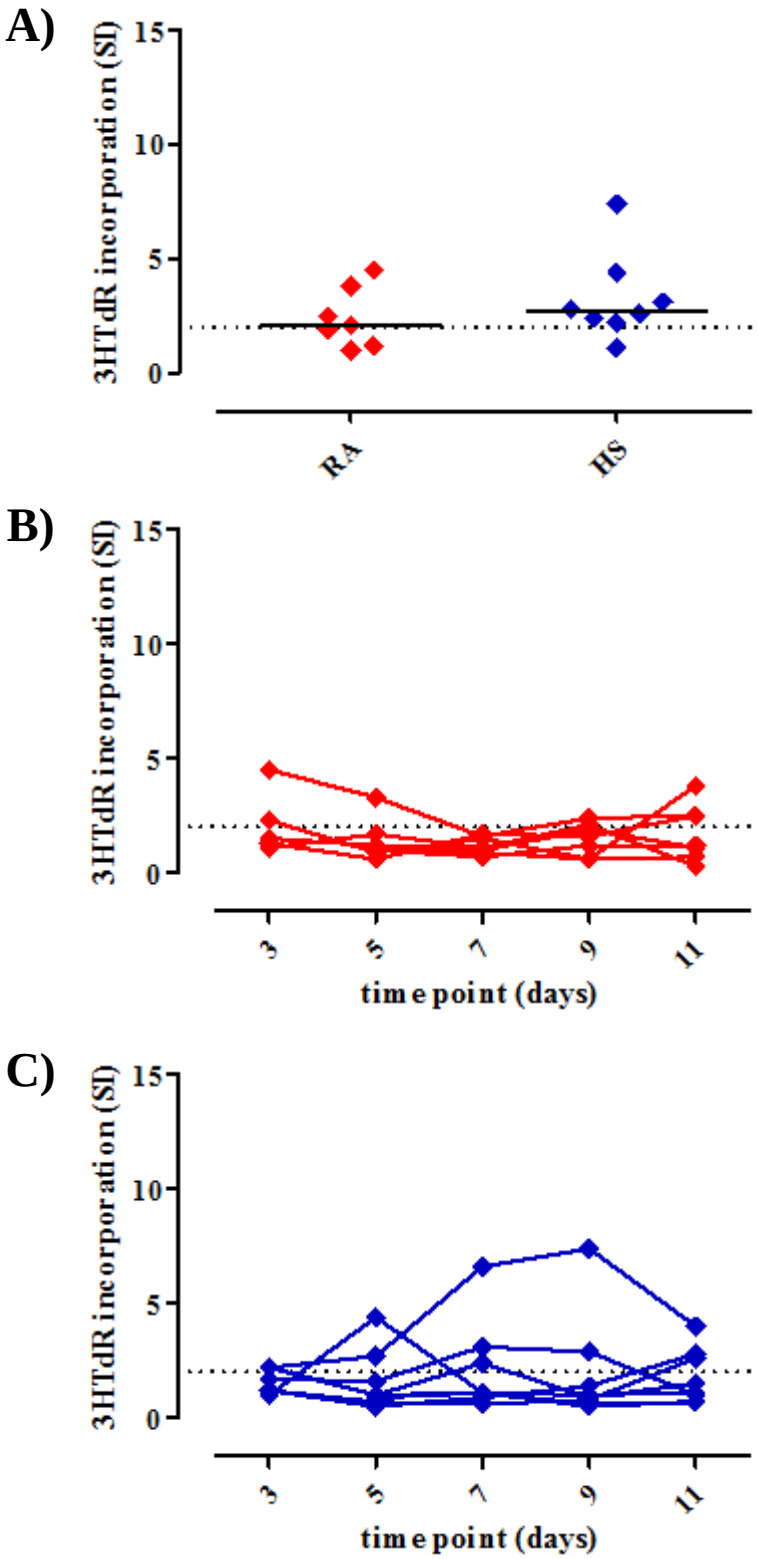

Figure 4.8. CII-induced PBMC proliferation in RA patients and healthy subjects; triplicate culture 3HTdR incorporation approach. A, the peak SI values of CII-induced PBMC proliferation detected in both RA patients (RA, *n*=7) and healthy subjects (HS, *n*=8) when using the triplicate culture approach. The bold horizontal line in each data set represents the median value. **B** & **C**, the kinetics of the CII-induced PBMC proliferation detected in the RA patients and the healthy subjects respectively. In all graphs, the dotted horizontal line represents the threshold for a positive response.

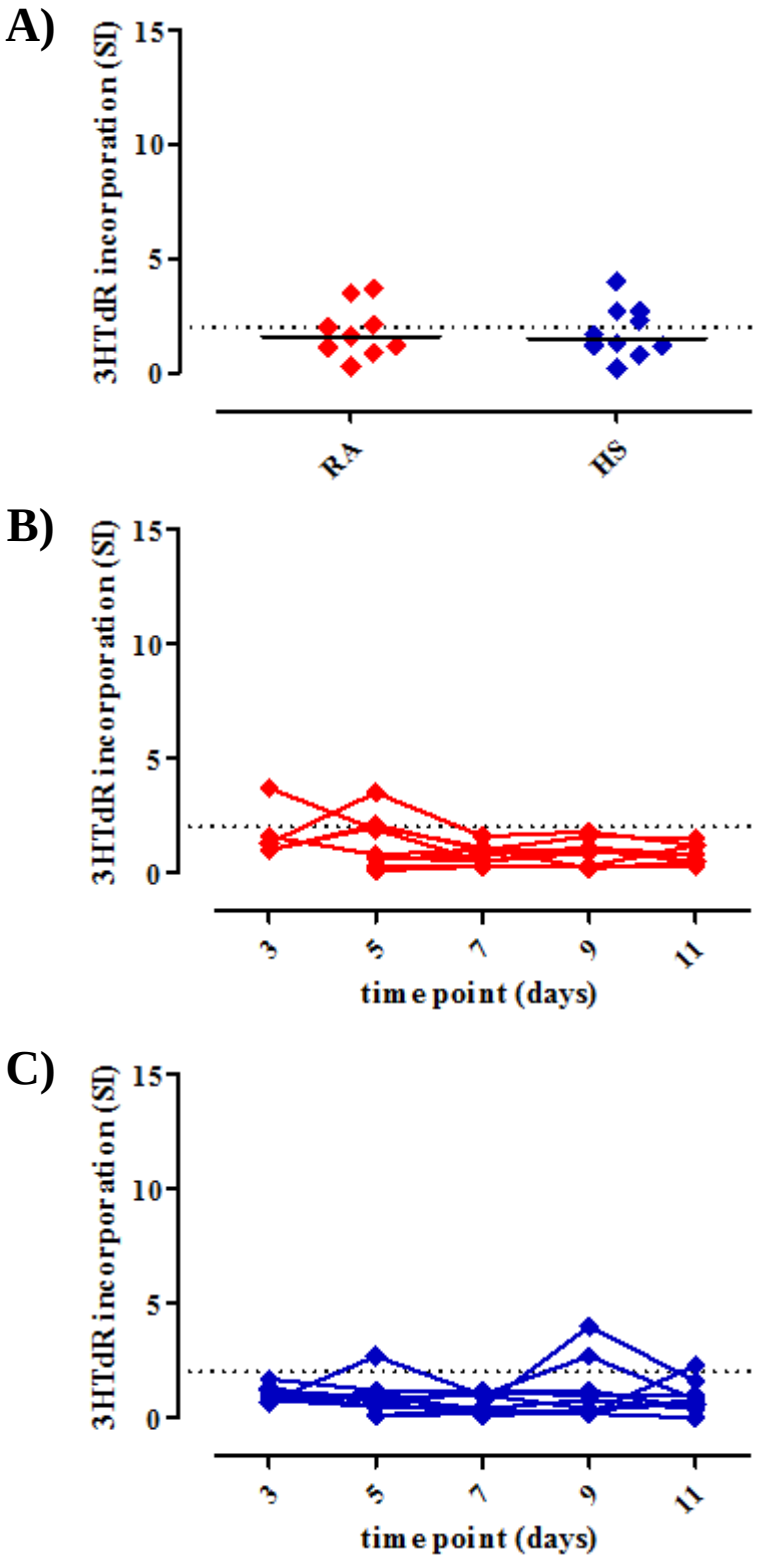

Figure 4.9. GlyAgg-induced PBMC proliferation in RA patients and healthy subjects; triplicate culture 3HTdR incorporation approach. A, the peak SI values of GlyAgg-induced PBMC proliferation detected in both RA patients (RA, *n*=9) and healthy subjects (HS, *n*=10) when using the triplicate culture approach. The bold horizontal line in each data set represents the median value. **B** & **C**, the kinetics of the GlyAgg-induced PBMC proliferation detected in the RA patients and the healthy subjects respectively. In all graphs, the dotted horizontal line represents the threshold for a positive response.

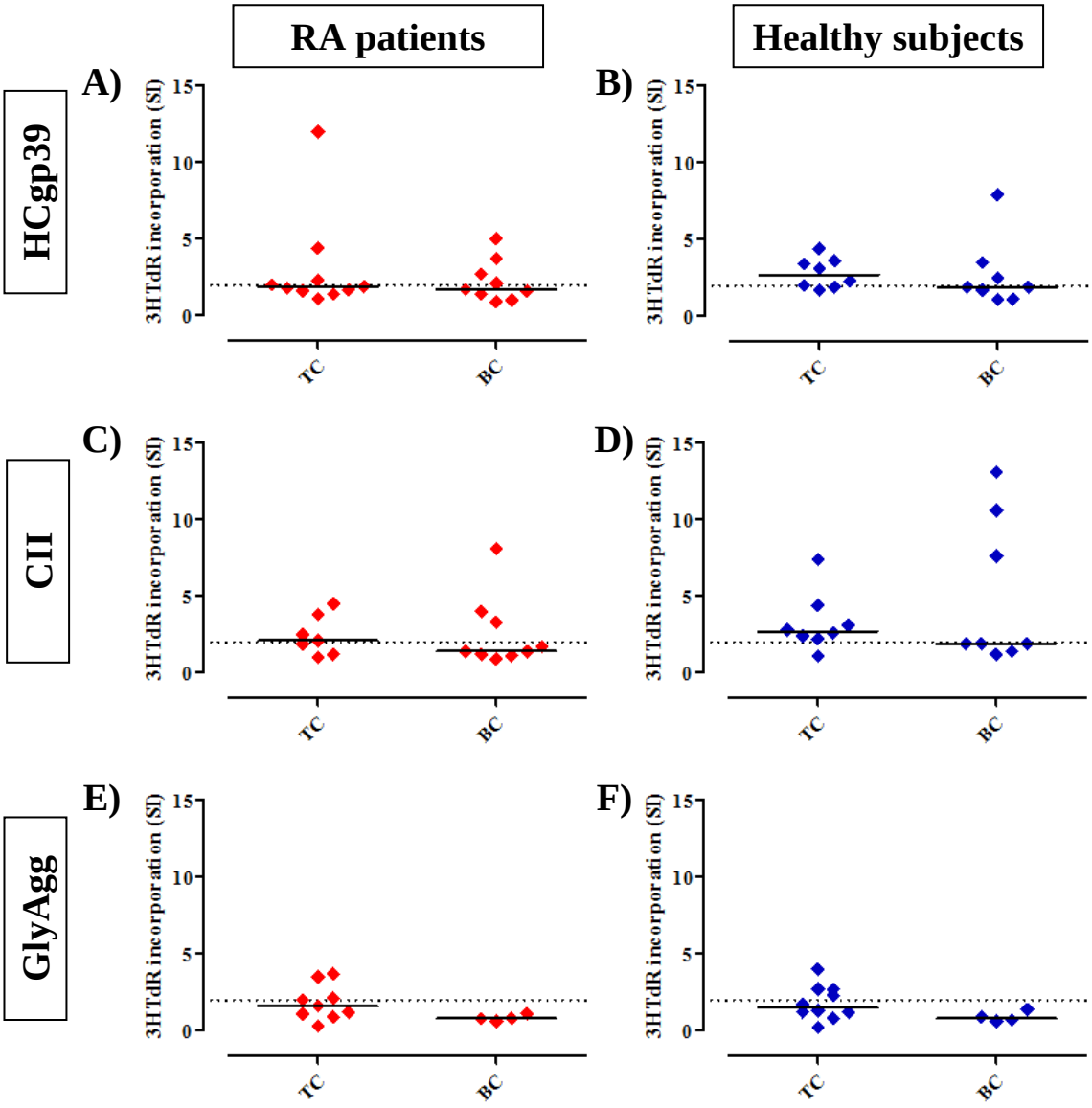

Figure 4.10. HCgp39-induced, CII-induced and GlyAgg-induced PBMC proliferation in RA patients and healthy subjects; triplicate culture 3HTdR incorporation approach versus bulk culture 3HTdR incorporation approach. A & **B**, the peak SI values of HCgp39-induced PBMC proliferation detected in RA patients and healthy subjects respectively with both the triplicate culture (TC) approach and the bulk culture (BC) approach. **C** & **D**, the peak SI values of CII-induced PBMC proliferation detected in RA patients and healthy subjects respectively with both the triplicate culture (TC) approach and the bulk culture (BC) approach. **E** & **F**, the peak SI values of GlyAgg-induced PBMC proliferation detected in RA patients and healthy subjects respectively with both the triplicate culture (TC) approach and bulk culture (BC) approach. In all graphs, the dotted horizontal line represents the threshold for a positive response, while the bold horizontal line in each data set represents the median value.

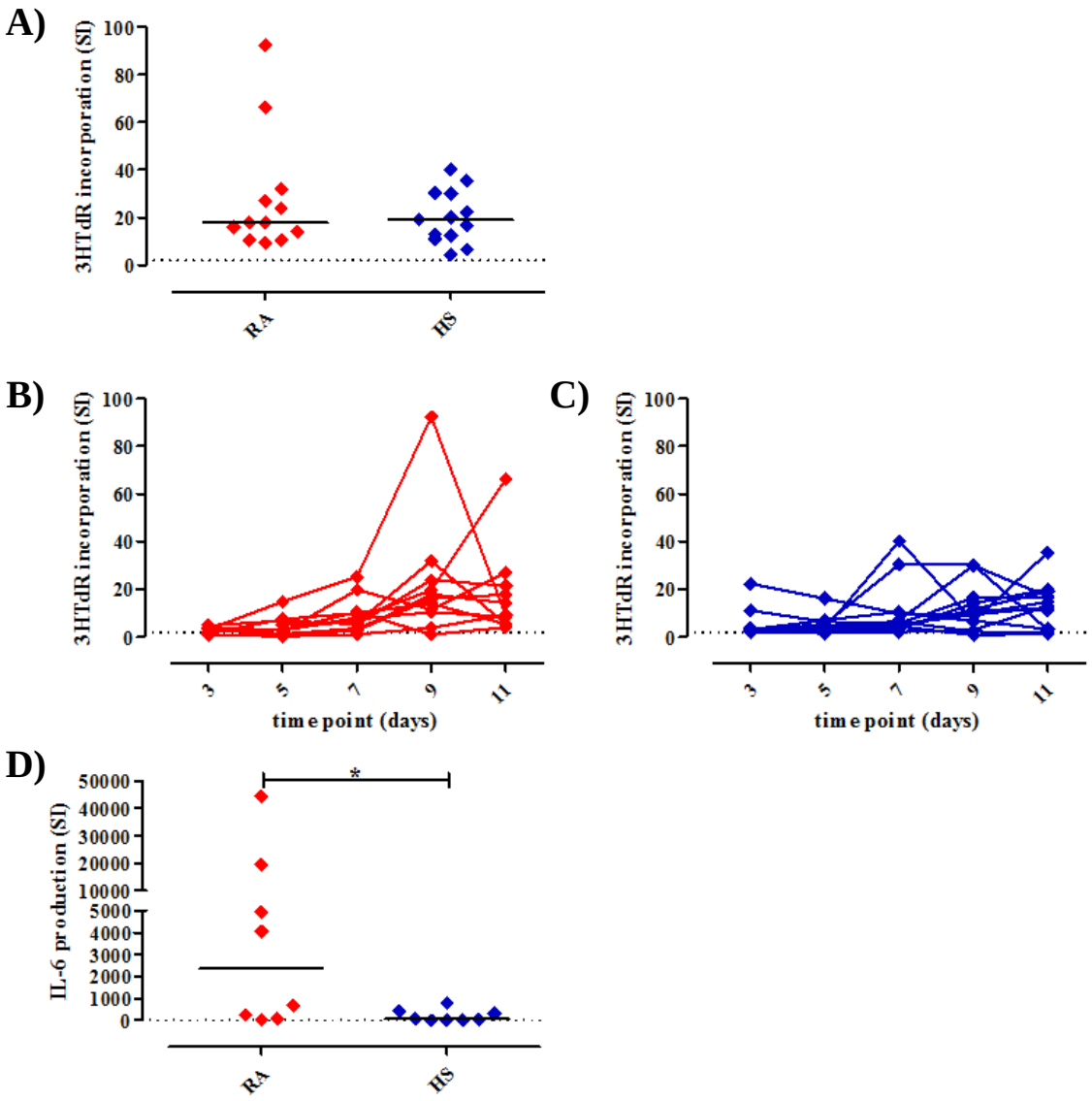

Figure 4.11. DeglyAgg-induced PBMC responses in RA patients and healthy subjects; triplicate culture approach. A, the peak SI values of DeglyAgg-induced PBMC proliferation detected in both RA patients (RA, *n*=12) and healthy subjects (HS, *n*=13). The bold horizontal line in each data set represents the median value. **B** & **C**, the kinetics of the DeglyAgg-induced PBMC proliferation detected in the RA patients and the healthy subjects respectively. **D**, the SI values of DeglyAgg-induced IL-6 production detected in RA patients (RA, *n*=8) and healthy subjects (HS, *n*=8) at day 5. The bold horizontal line in each data set represents the median value. In all graphs, the dotted horizontal line represents the threshold for a positive response.

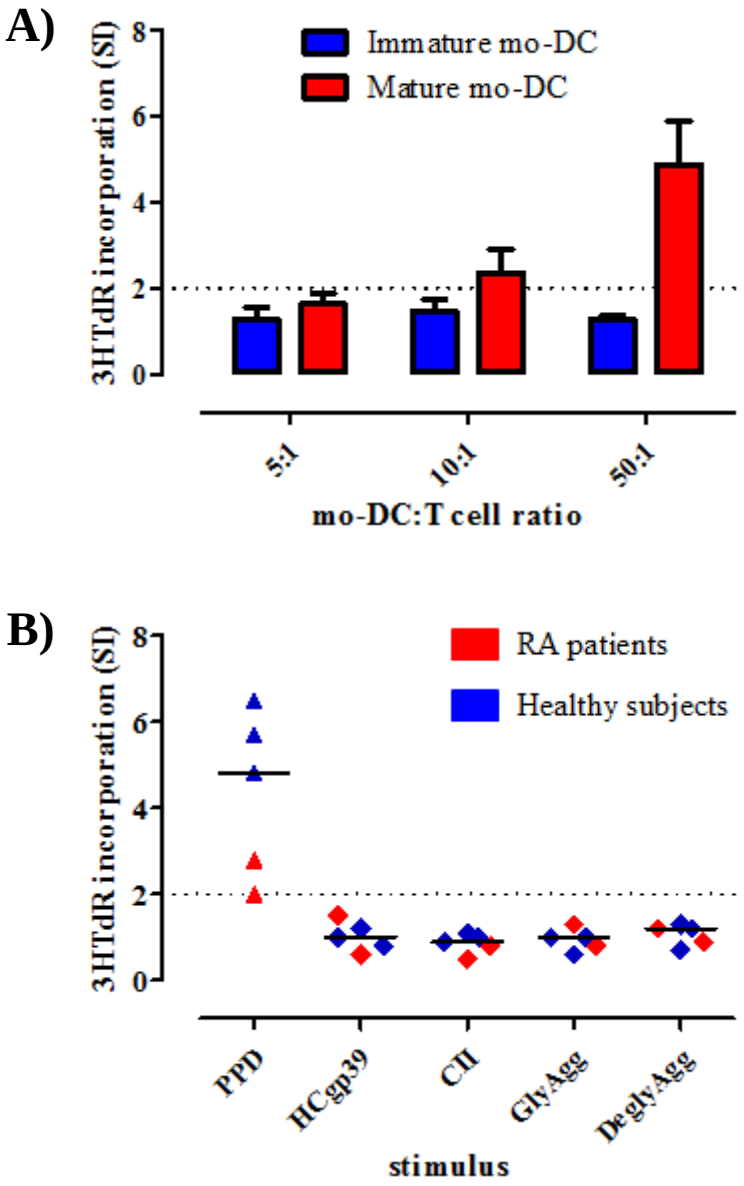

Figure 4.12. PPD-induced and autoantigen-induced T cell proliferation; mo-DC & T cell co-culture approach. $CD3^+$ PBMC from 4 healthy subjects were co-cultured with either unloaded or 1µg/ml PPD-loaded, immature or LPS-matured, autologous mo-DC at mo-DC:T cell ratios of 5:1, 10:1 or 50:1. Proliferation was assessed at day 5 via 3HTdR incorporation. The mean SI values of PPD-induced T cell proliferation detected with the different mo-DC at the different mo-DC:T cell ratios are shown in **A** (the vertical bars in each data set represent SEM, while the dotted horizontal line represents the threshold for a positive response). $CD3^+$ PBMC from 2 RA patients and 3 healthy subjects were co-cultured with either unloaded, 1µg/ml PPD-loaded, 10µg/ml HCgp39-loaded, 10µg/ml CII-loaded, 10µg/ml GlyAgg-loaded or 10µg/ml DeglyAgg-loaded, LPS-matured, autologous mo-DC at a mo-DC:T cell ratio of 50:1. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation. The peak SI values of T cell proliferation detected in the RA patients (red data points) and the healthy subjects (blue data points) with the different mo-DC are shown in **B** (the bold horizontal line in each data set represents the median value, while the dotted horizontal line represents the threshold for a positive response).

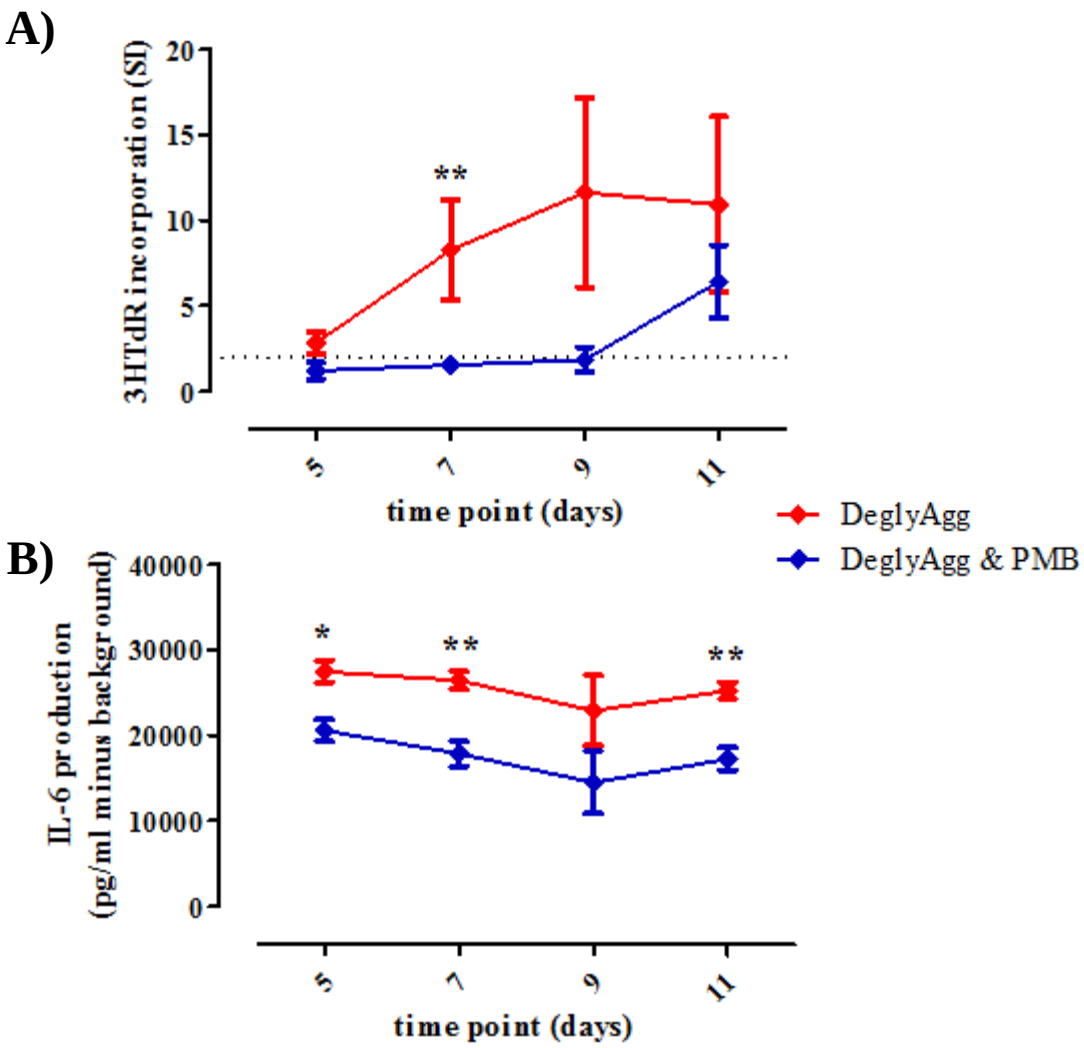

Figure 4.13. The effect of polymyxin B on the DeglyAgg response. PBMC from 5 subjects were cultured with either medium alone or 10µg/ml DeglyAgg, with or without the presence of PMB. After 5, 7, 9 & 11 days, proliferation was assessed via 3HTdR incorporation and IL-6 levels in cell culture supernatant samples were assessed via ELISA. **A**, the mean SI values of DeglyAgg-induced PBMC proliferation detected at the different time-points when PBMC are cultured with and without the presence of PMB. **B**, the mean absolute values of DeglyAgg-induced IL-6 production detected at the different time-points when PBMC are cultured with and without the presence of PMB. In each graph, the vertical bars at each data point represent SEM.

Chapter 5. T-cell Immune responses to candidate RA autoantigen-derived peptides in health and disease.

5.1. Introduction

In the experiments described in chapter 4 I measured T-cell proliferation and cytokine production in response to several whole protein candidate RA autoantigens. Using both the CFSE dilution assay and the 3HTdR incorporation assay to detect proliferation and ECL multiplex assays and ELISAs to detect cytokine production, I measured responses to HCgp39, CII, GlyAgg and DeglyAgg in bulk and triplicate cultures of whole PBMC from RA patients and healthy subjects. Positive responses to these candidate autoantigens were frequently detected in both RA patients and healthy subjects, yet with the exception of the responses to DeglyAgg, they were generally weak. Furthermore, the proportions, natures and magnitudes of the responses detected did not differ significantly between the two groups. Hypothesising that the weak responses could have resulted from sub-optimal antigen presentation in the whole PBMC cultures, I tried an alternative mo-DC and T-cell co-culture approach. However, the magnitude of candidate RA autoantigen-specific T-cell proliferative responses detected with this approach was no better. This led me to postulate that the magnitude of the responses to the whole protein HCgp39, CII and GlyAgg could have been low due to sub-optimal antigen processing in the *in-vitro* cultures. Therefore, I proposed to use candidate RA autoantigen-derived peptides in my subsequent studies.

5.1.1. Peptide stimuli versus whole-protein stimuli

As alluded to in the previous section, my main reason for choosing to use peptide stimuli in my subsequent experiments was in an attempt to elicit more substantial T-cell responses in my assays. Because T-cells can only recognise their specific antigens when they are presented as peptides in the context of MHC molecules, the generation of T-cell responses to any given antigen is only possible after the antigen molecules have been processed into peptide fragments by antigen presenting cells. For example, when an exogenous antigen is processed by a professional antigen presenting cell, the resulting peptides bind to specific MHC class II molecules and form the MHC class II:peptide complexes that antigen-specific $CD4^+$ T-cells recognise. Thus the likelihood of any T-cell responses being generated relies heavily on the efficiency of the antigen processing pathway within the professional antigen presenting cells. The use of peptides as stimuli may overcome the potential problem of sub-optimal antigen processing in our

assay system. The use of peptides also has further benefits. When a whole protein molecule containing potentially autoantigenic epitopes is processed, large numbers of peptides will be generated, and so the peptides in which the potentially autoantigenic epitopes are found will have to compete with many other peptides for binding to MHC molecules. If the potentially autoantigenic peptides are not very abundant and/or have low affinities for MHC molecules, then it is possible that they will be out-competed for binding to the peptide binding grooves of MHC molecules, and may therefore not be presented to antigen-specific T-cells (Adorini & Nagy, 1990). In contrast, if I use peptide epitopes as stimuli in my assays, then much higher molar concentrations of the peptides of interest can be added to the cell cultures. As a result, peptide competition will not be a rate-limiting factor in whether responses to these epitopes are detected.

5.1.2. Peptides of interest

The peptides tested in this part of the project were chosen based on the findings of previous studies (Table 5.1). For example, Hill *et al* (2003 and 2008) have demonstrated that the citrullinated forms but not the un-modified forms of both vimentin$_{65\text{-}77}$ and fibrinogen α-chain$_{79\text{-}91}$ elicit HLA-DR-restricted T-cell recall responses characterised by proliferation and IFN-γ production in DR4(DRB1*0401) transgenic mice. Furthermore, the group's earlier study also showed that while the unmodified form of the vimentin peptide displayed only low affinity for a number of both SE-positive and SE-negative HLA-DR molecules, the citrullinated form displayed high affinity for the RA-associated HLA-DR molecules DR1(DRB1*0101), DR4(DRB1*0401) and DR4(DRB1*0404). These studies suggest that citrullination of autoantigen-derived peptides may enhance their antigenicity in SE-positive RA patients. While the study by Fritsch *et al* (2002) demonstrated that citrullination of filaggrin$_{2776\text{-}2793}$ and filaggrin$_{3005\text{-}3022}$ had varying effects on the immunogenicities of the peptides, studies by Lundberg *et al* (2008) and von Delwig *et al* (2010) provide evidence that citrullination of both α-enolase$_{5\text{-}21}$ and aggrecan$_{84\text{-}103}$ respectively enhances their immunogenicity in RA patients. In addition, aggrecan$_{201\text{-}213}$ and aggrecan$_{299\text{-}311}$ have both been shown to bind with high affinity to the RA-associated DR4(DRB1*0401) molecule (Boots *et al*, 1997), while it was observed that the HCgp39$_{262\text{-}276}$ binds with high affinity to the RA-associated HLA-DR molecules DR1(DRB1*0101), DR4(DRB1*0401) and DR4(DRB1*0404), and elicits peripheral blood T-cell proliferation in RA patients but not healthy subjects (Verheijden *et al*, 1997).

5.2. Results

In the experiments covered in this chapter, PBMC from RA patients, healthy subjects and disease controls were tested for reactivity to various candidate RA autoantigen-derived peptides; peptide panel #1 (peptides 1-7, Table 5.1), peptide panel #2 (peptides 8-15, Table 5.1) and REP-1 and CEP-1 (Table 5.1). Based on my data reported in chapter 4, all experiments utilised the triplicate culture approach and proliferation was measured via 3HTdR incorporation while cytokine levels in cell culture supernatant samples were assessed via ECL multiplex and ELISA. Preliminary work had suggested that assessing cytokine production at day 5 would increase the chances of responses being detected (data not shown).

5.2.1. Peptide panel #1

PBMC from 10 RA patients and 10 healthy subjects were tested for proliferation and cytokine production in response to the 7 peptides comprising peptide panel #1 (see Table 2.4 in chapter 2 for RA patient clinical details). Proliferation was assessed after 5, 7, 9 & 11 days via 3HTdR incorporation, while cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. Figure 5.1 shows the proportions of RA patients and healthy subjects showing positive proliferative and/or cytokine production responses to each of the 7 peptides. The peak SI values of PBMC proliferation and the absolute values of day 5 cytokine production detected in response to peptides 1-7 in both the RA patients and the healthy subjects are displayed in Tables 5.2-5.8. Similar proportions of RA patients and healthy subjects showed positive proliferative and/or cytokine production responses to peptides 1-7 (RA versus HS for peptides 1-7 = 56% versus 40%, 67% versus 60%, 78% versus 60%, 78% versus 80%, 67% versus 80%, 67% versus 80% and 78% versus 90% respectively). Ultimately, the peak SI values of PBMC proliferation and the absolute values of day 5 IL-10, IL-17, IL-4, IL-6, TNF-α and IFN-γ production detected in response to each of the peptides in panel #1 were comparable between the RA patients and the healthy subjects.

While RA 5.8 failed to show a positive PBMC proliferative and/or cytokine production response to any of the 7 peptides, all of the other RA patients showed positive PBMC proliferative and/or cytokine production responses to two or more of the peptides (table 5.9A). For example, RA 5.9 showed positive responses to all 7 peptides while RA 5.2, RA 5.3 and RA 5.7 all showed positive responses to all peptides except peptides 5, 2

and 1 respectively. Based on clinical details, the non-responder RA 5.8 differed from the other nine patients by sex (male as opposed to female) and by having a substantially higher CRP value. The ESR value of RA 5.8 was also substantially higher than that of the majority of the other patients although one of the responders displayed a similar ESR value. Complete data on the RF and/or CCP status of the majority of the patients was un-available, yet based on the clinical details that were available, the patients responding to multiple peptides did not differ from those responding to just one or two of the peptides. Of the healthy subjects, all ten showed positive responses to at least two of the 7 peptides, and only HS 5.8 and HS 5.9 showed positive responses to less than four of the peptides (Table 5.9B). Indeed, HS 5.5 and HS 5.7 showed positive responses to all 7 peptides.

Peptides 6 and 7 respectively were non-citrullinated and citrullinated versions of the same peptide. In both the RA patient group and the healthy subject group, similar proportions of subjects showed positive responses to peptide 7 as to peptide 6 (peptide 7 versus peptide 6 in RA and HS = 78% versus 67% and 90% versus 80% respectively). While two of the seven RA patients that showed positive responses to peptide 7 did not show positive responses to peptide 6, the other five peptide 7-responding RA patients showed positive proliferation and/or cytokine production responses to both peptides 6 and 7. RA 5.5 showed a positive response to peptide 6 but not peptide 7. RA 5.2 responded to peptide 6 with production of IL-17 alone (6pg/ml), yet responded to peptide 7 with production of TNF-α and IFN-γ (2pg/ml and 12pg/ml respectively) as well as IL-17 (5pg/ml). RA 5.3 responded to both peptides 6 and 7 with both proliferation (SI values of 2.2 and 2.4 respectively) and IL-17 production, although peptide 7 induced more IL-17 production than peptide 6 (14pg/ml versus 3pg/ml). RA 5.7 responded to both peptides 6 and 7 with IL-6 production alone, although peptide 7 induced a slightly more potent response (5pg/ml versus 3pg/ml). RA 5.9 responded to peptide 6 with proliferation (SI value of 3.0) and production of IL-17, IL-6 and TNF-α (7pg/ml, 13pg/ml and 2pg/ml), yet responded to peptide 7 with IL-17 production alone (5pg/ml). RA 5.10 responded to both peptides 6 and 7 with similar levels of proliferation and IL-6 and IFN-γ production. No patterns were visible in the CRP level or ESR values or the disease duration of patients showing positive responses to the citrullinated peptide. Moreover, the CRP level and ESR values and disease duration of patients that did show positive responses to the citrullinated peptide did not differ from those of patients that did not show positive responses to the citrullinated peptide. Of the

three peptide 7-responders with known CCP status, one was positive and two negative. Of the nine healthy subjects that showed positive responses to peptide 7, seven also showed positive responses to peptide 6. HS 5.4 showed a positive response to peptide 6 but not peptide 7. HS 5.1 responded to peptide 6 with IFN-γ production alone (37pg/ml), yet responded to peptide 7 with both proliferation (SI value of 2.3) as well as with more robust IFN-γ production (45pg/ml). While HS 5.2 responded to both peptides 6 and 7 with production of IL-17, IL-6 and IFN-γ, peptide 7 induced a more robust response than peptide 6 and also induced production of TNF-α. HS 5.3 responded to peptide 6 with production of IL-6, TNF-α and IFN-γ (11pg/ml, 5pg/ml and 37pg/ml respectively), yet responded to peptide 7 with production of only IL-6 and IFN-γ at reduced levels (2pg/ml and 31pg/ml respectively). HS 5.5 responded to both peptides 6 and 7 with both proliferation and IFN-γ production, although peptide 7 induced a more robust proliferative response than peptide 6 (SI values of 4.4 versus 3.4) while the latter induced a more robust IFN-γ response than the former (60pg/ml versus 31pg/ml). HS 5.6 responded to peptide 6 with production of IL-17, IL-6 and TNF-α (87pg/ml, 21pg/ml and 14pg/ml respectively), yet responded to peptide 7 with production of IL-6 alone (4pg/ml). HS 5.7 responded to peptide 6 with production of IL-6 and TNF-α (34pg/ml and 2pg/ml respectively), yet responded to peptide 7 with IL-10 and IL-17 production (2pg/ml and 12pg/ml respectively) and much more robust IL-6 production (960pg/ml). HS 5.9 responded to peptide 6 with production of IL-17 and IL-6 (12pg/ml and 133pg/ml respectively), yet responded to peptide 7 with IL-10 production alone (3pg/ml).

5.2.2. Peptide panel #2

PBMC from 9 RA patients and 9 healthy subjects were tested for proliferation and cytokine production in response to the 8 peptides comprising peptide panel #2 (see Table 2.5 in chapter 2 for RA patient clinical details). Proliferation was assessed after 5, 7, 9 & 11 days via 3HTdR incorporation, while cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. Figure 5.2 shows the proportions of RA patients and healthy subjects showing positive proliferative and/or cytokine production responses to each of the 8 peptides. The peak SI values of PBMC proliferation and the absolute values of day 5 cytokine production detected in response to peptides 8-15 in both the RA patients and the healthy subjects are displayed in Tables 5.10-5.17. Similar proportions of RA patients and healthy subjects showed positive proliferative and/or cytokine production responses to peptides

8-15 (RA versus HS for peptides 8-15 = 75% versus 50%, 89% versus 56%, 75% versus 50%, 67% versus 56%, 67% versus 83%, 43% versus 78%, 56% versus 78% and 67% versus 67% respectively). Ultimately, the peak SI values of PBMC proliferation and the absolute values of day 5 IL-10, IL-17, IL-4, IL-6, TNF-α and IFN-γ production detected in response to each of the peptides in panel #2 were comparable between the RA patients and the healthy subjects.

RA 5.13 failed to show positive responses to any of the peptides from peptide panel #2 and while RA 5.15 and RA 5.16 both showed positive responses only to peptide 9, the rest of the RA patients all responded to four or more of the peptides that they were tested against (Table 5.18). Indeed, RA 5.12 showed positive responses to all of the peptides in panel #2, while RA 5.11 showed positive responses to all except for peptide 13. Based on the RA patient clinical details that were available, the non-responder did not differ from the responders and those patients showing positive responses to multiple peptides did not differ from those showing positive responses to only one of the peptides. While HS 5.19 showed a positive response to peptide 14 alone and HS 5.15 to peptides 8 and 15, the rest of the healthy subjects showed positive responses to at least three of the peptides that they were tested with (Table 5.19). For example, HS 5.12 and HS 5.16 both showed positive responses to all of the 8 peptides in panel #2 except for peptide 8, while HS 5.14 showed positive responses to all.

In the RA patients group, citrullinated peptide 9 elicited a similar proportion of positive responses as its non-citrullinated counterpart, peptide 8 (89% versus 75%), while citrullinated peptide 11 elicited a similar proportion of positive responses as its non-citrullinated counterpart, peptide 10 (67% versus 75%). Furthermore, citrullinated peptide 13 elicited a similar proportion of positive responses as its non-citrullinated counterpart, peptide 12 (43% versus 67%). In addition, the alternatively citrullinated peptides 14 and 15 elicited responses in comparable proportions of patients (56% and 67% respectively). In the healthy subject group, citrullinated peptides 9, 11 and 13 elicited comparable proportions of positive responses as their non-citrullinated counterparts, peptides 8, 10 and 12 respectively (50% versus 56%, 50% versus 56% and 83% versus 78% for peptides 8 versus 9, 10 versus 11 and 12 versus 13 respectively). The alternatively citrullinated peptides 14 and 15 elicited responses in comparable proportions of subjects (78% and 67% respectively). No clear trends or statistical

differences were visible between the responses to non-citrullinated versus citrullinated peptides within the two groups.

Some of the RA patients and healthy subjects showed positive responses to the non-citrullinated versions of certain peptides but not to the corresponding citrullinated versions (RA 5.11, peptide 12 but not peptide 13; HS 5.13, peptide 8 but not peptide 9; HS 5.15, peptide 8 but not peptide 9). Other healthy subjects, although none of the RA patients, failed to show positive responses to the non-citrullinated versions of certain peptides yet did show positive responses to the corresponding citrullinated versions (HS 5.12, not peptide 8 but peptide 9; HS 5.13, not peptide 10 but peptide 11; HS 5.16, not peptide 8 but peptide 9). When positive responses to both non-citrullinated peptides and the corresponding citrullinated peptides were detected in individual subjects (RA 5.11, peptides 8 and 9 and peptides 10 and 11; RA 5.12, peptides 8 and 9, peptides 10 and 11 and peptides 12 and 13; RA 5.14, peptides 8 and 9 and peptides 10 and 11; HS 5.11, peptides 12 and 13; HS 5.12, peptides 10 and 11 and peptides 12 and 13; HS 5.13, peptides 12 and 13; HS 5.14, peptides 8 and 9, peptides 10 and 11 and peptides 12 and 13; HS 5.16, peptides 10 and 11 and peptides 12 and 13), they were often different. However, responses to citrullinated peptides in individual subjects varied and there were no patterns observed in citrullinated responders versus non-responders.

For five of the nine RA patients, data on the CCP status was unfortunately not available so it was not possible to determine whether there was any correlation between CCP positivity and responses to citrullinated peptides. None of the other clinical characteristics distinguished patients responding to citrullinated versions of the peptides from those patients not responding to the citrullinated versions.

5.2.3. Non-citrullinated and citrullinated forms of an α-enolase peptide; REP-1 and CEP-1

Citrullinated enolase has recently emerged as a target of ACPA in RA and anti-citrullinated enolase antibodies have been found to be highly specific for RA (Kinloch *et al*, 2005 and Lundberg *et al*, 2008). Since such autoantibodies are often of IgG subtypes, this suggests that T-cell help may be important, therefore I postulated that T-cell responses to citrullinated enolase peptides might be heightened in RA patients. Citrullinated enolase$_{5\text{-}21}$ is the immunodominant epitope recognised by antibodies reacting with citrullinated enolase (Lundberg *et al*, 2008) therefore I reasoned that the

same peptide may also contain a candidate T-cell epitope. Thus, I measured proliferation and cytokine production responses of PBMC stimulated with citrullinated α-enolase$_{5-21}$ and its non-citrullinated analogue (peptides CEP-1 and REP-1 respectively, Table 5.1). A total of 20 RA patients, 9 healthy subjects and 11 disease controls were tested (the clinical details of the RA patients - RA 5.20 to RA 5.39 - and disease controls are summarised in Tables 2.6 and 2.7 respectively in chapter 2).

5.2.3.1. Proliferative responses to REP-1 and CEP-1

The peak SI values of REP-1-induced and CEP-1-induced PBMC proliferation detected in each of the 20 RA patients, 9 healthy subjects and 11 disease controls are displayed in Table 5.20.

Firstly I compared REP-1-induced PBMC proliferation with CEP-1-induced PBMC proliferation within each of the 3 groups. In the RA patient group, CEP-1 induced a significantly greater proportion of positive PBMC proliferative responses than REP-1 (95% versus 33%, P value = 0.0030, Chi-square). However, in both the healthy subject and disease control groups, CEP-1 and REP-1 induced comparable proportions of positive PBMC proliferative responses (CEP-1 versus REP-1 in healthy subject group and disease control group = 67% versus 56% and 100% versus 55% respectively, P values = 0.99 and 0.17 respectively, Chi-square).

In all 3 groups, the peak SI values of CEP-1-induced PBMC proliferation were significantly greater than those of REP-1-induced PBMC proliferation (mean ± SEM peak SI values of CEP-1 versus REP-1 of 10.2 ± 1.9 versus 2.1 ± 0.3 in RA patients, 4.9 ± 1.2 versus ± 2.8 ± 0.6 in healthy subjects and 21.4 ± 4.0 versus 2.1 ± 0.2 in disease controls, P values of 0.0015, 0.0440 and 0.0007 respectively, paired t test, Figure 5.3A, 5.3B & 5.3C respectively).

Secondly, I compared REP-1-induced and CEP-1-induced PBMC proliferation between the 3 groups, in terms of both the proportions of responders and the magnitudes of the responses. Although both the healthy subject and disease control groups showed greater proportions of positive REP-1-induced PBMC proliferative responses than the RA patient group (56% and 55% versus 33%), the differences were not statistically significant (P values of healthy subjects versus RA patients and disease controls versus RA patients were 0.87 and 0.87 respectively, Chi-square). In addition, the peak SI

values of REP-1-induced PBMC proliferation detected in the RA patient group were statistically similar to those detected in both the healthy subject group and the disease control group (median peak SI values of REP-1-induced PBMC proliferation were median = 1.6, IQR: 1.1-2.9 in RA patients, median = 2.0, IQR: 1.6-3.9 in healthy subjects and median = 2.0, IQR: 1.3-2.6 in disease controls, P values of RA patients versus healthy subjects and RA patients versus disease controls were 0.17 and 0.42 respectively, Mann Whitney, Figure 5.4A).

Figure 5.4B shows the peak SI values of CEP-1-induced PBMC proliferation for the three groups. Although the RA patient group showed a greater proportion of positive CEP-1-induced proliferative responses than the healthy subject group (95% versus 67%), the difference was not statistically significant (P value of RA patients versus healthy subjects = 0.38, Chi-square). Similarly, while there was a tend suggesting that the peak SI values of CEP-1-induced PBMC proliferation detected in the RA patients were higher than those detected in the healthy subjects (median peak SI values were median = 9.0, IQR: 3.6-13.3 in RA patients versus median = 5.7, IQR: 1.0-7.8 in healthy subjects), it did not reach statistical significance (P value = 0.06, Mann Whitney). However, the peak SI values of CEP-1-induced PBMC proliferation detected in the disease controls (median = 17.8, IQR: 9.6-33.7) were significantly greater than those of CEP-1-induced PBMC proliferation detected in the RA patients (P value = 0.01, Mann Whitney).

After analysing the proportions and the magnitudes of the proliferative responses to REP-1 and CEP-1 in the 3 subjects groups, I also explored whether the kinetics of the responses differed between the groups. Figure 5.5 shows the kinetics of the REP-1-induced and CEP-1-induced proliferative responses detected in the RA patients, healthy subjects and disease controls. In the RA patient group, peak levels of REP-1-induced proliferation were detected at days 7, 9 and 11 (n=2, 3 and 1 respectively, Figure 5.5A), while peak levels of CEP-1-induced proliferation were also detected at days 7, 9 and 11 (n=2, 8 and 9 respectively, Figure 5.5B). In the healthy subject group, peak levels of REP-1-induced proliferation were detected at days 5, 9 and 11 (n=1, 2 and 2 respectively, Figure 5.5C), while peak levels of CEP-1-induced proliferation were detected at days 9 and 11 (n=5 and 1, Figure 5.5D). In the disease control group, peak levels of REP-1-induced proliferation were detected at day 11 (n=6, Figure 5.5E), while peak levels of CEP-1-induced proliferation were detected at day 11 (Figure 5.5F). Thus,

while there were no clear differences between the kinetics of the proliferative responses to these peptides between RA patients and healthy subjects, the kinetics of CEP-1-induced proliferative responses in disease controls appeared to be delayed compared to RA patients.

5.2.3.2. Cytokine production responses to REP-1 and CEP-1

I first compared the levels of REP-1-induced cytokine production with those of CEP-1-induced cytokine production within each of the 3 subject groups. In the RA patient group, CEP-1 induced significantly higher levels of production of IL-10, IL-4, IL-6, TNF-α and IFN-γ from the PBMC than did REP-1 (mean ± SEM absolute pg/ml values of cytokine production induced by CEP-1 versus REP-1 were 30 ± 5 versus 2 ± 1 for IL-10, 25 ± 3 versus 3 ± 1 for IL-4, 10378 ± 666 versus 1514 ± 775 for IL-6, 838 ± 329 versus 44 ± 18 for TNF-α and 690 ± 269 versus 5 ± 5 for IFN-γ, P values of <0.0001, <0.0001, <0.0001, 0.04 and 0.03 respectively, paired t test, Figure 5.6A, 5.6C, 5.6D, 5.6E & 5.6F respectively). However, the levels of REP-1-induced and CEP-1-induced IL-17 production were comparable (mean ± SEM absolute pg/ml values of 32 ± 13 versus 1755 ± 1207 respectively, P value = 0.20, paired t test, Figure 5.6B).

In the healthy subject group, REP-1 and CEP-1 induced similar levels of IL-10, IL-17, IL-4, TNF-α and IFN-γ production (mean ± SEM absolute pg/ml values of cytokine production induced by REP-1 versus CEP-1 were 4 ± 3 versus 14 ± 3 for IL-10, 837 ± 666 versus 3141 ± 2142 for IL-17, 6 ± 4 versus 16 ± 4 for IL-4, 51 ± 43 versus 111 ± 45 for TNF-α and 62 ± 38 versus 155 ± 85 for IFN-γ, P values of 0.05, 0.36, 0.08, 0.37 and 0.30 respectively, paired t test, Figure 5.7A, 5.7B, 5.7C, 5.7E & 5.7F respectively). However, CEP-1 induced significantly higher levels of IL-6 production than did REP-1 (mean ± SEM absolute pg/ml values of 6978 ± 1569 versus 2456 ± 1593 respectively, P value = 0.03, paired t test, Figure 5.7D).

In the disease control group, CEP-1 induced significantly higher levels of production of IL-10, IL-4, IL-6 and IFN-γ than did REP-1 (mean ± SEM absolute pg/ml values of cytokine production induced by CEP-1 versus REP-1 were 22 ± 2 versus 3 ± 2 for IL-10, 31 ± 3 versus 7 ± 4 for IL-4, 9017 ± 849 versus 1920 ± 1111 for IL-6 and 307 ± 104 versus 4 ± 3 for IFN-γ, P values of 0.0002, 0.0007, 0.0011 and 0.02 respectively, paired t test, Figure 5.8A, 5.8C, 5.8D & 5.8F respectively). However, REP-1 and CEP-1 induced similar levels of IL-17 and TNF-α production (mean ± SEM absolute pg/ml

values of cytokine production induced by REP-1 versus CEP-1 were 621 ± 578 versus 1590 ± 1018 for IL-17 and 30 ± 18 versus 260 ± 106 for TNF-α, P values of 0.44 and 0.07 respectively, paired t test, Figure 5.8B & 5.8E respectively).

I also compared the levels of both REP-1 and CEP-1-induced cytokine production between each of the three subject groups. Figure 5.9 shows the levels of REP-1-induced IL-10, IL-17, IL-4, IL-6, TNF-α and IFN-γ production detected in the RA patients, healthy subjects and disease controls. While REP-1 induced similar levels of IL-10, IL-17, IL-4, IL-6 and TNF-α production in the RA patients as in the healthy subjects and disease controls (Figure 5.9A, 5.9B, 5.9C, 5.9D & 5.9E respectively), the levels of REP-1-induced IFN-γ production detected in the healthy subjects were significantly higher than those detected in the RA patients (median absolute pg/ml values in healthy subjects versus RA patients were median = 6, IQR: 0-162 versus median = 0, IQR: 0-0, P value = 0.02, Mann Whitney, Figure 5.9F).

Figure 5.10 shows the levels of CEP-1-induced IL-10, IL-17, IL-4, IL-6, TNF-α and IFN-γ production detected in the RA patients, healthy subjects and disease controls. The RA patients showed significantly higher levels of CEP-1-induced IL-10 production than the healthy subjects (median absolute pg/ml values in RA patients versus healthy subjects were median = 23, IQR: 16-40 versus median = 11, IQR: 8-18, P value = 0.01, Mann Whitney, Figure 5.10A). The RA patients showed significantly higher levels of CEP-1-induced IL-6 production than both the healthy subjects and the disease controls (median absolute pg/ml values were median = 10588, IQR: 10097-11474 in RA patients, median = 9348, IQR: 1939-9991 in healthy subjects and median = 9751, IQR: 9320-9902 in disease controls, P values of RA patients versus healthy subjects and RA patients versus disease controls were 0.01 and 0.01 respectively, Mann Whitney, Figure 5.10D). The RA patients also showed significantly higher levels of CEP-1-induced TNF-α production than both the healthy subjects and the disease controls (median absolute pg/ml values were median = 475, IQR: 387-685 in RA patients, median = 86, IQR: 18-122 in healthy subjects and median = 162, IQR: 92-270 in disease controls, P values of RA patients versus healthy subjects and RA patients versus disease controls were 0.0020 and 0.03 respectively, Mann Whitney, Figure 5.10E). The levels of CEP-1-induced IL-17, IL-4 and IFN-γ production detected in RA patients were comparable to those detected in both the healthy subjects and the disease controls (Figure 5.10B, 5.10C & 5.10F respectively).

5.2.4. The role of LPS contamination in CEP-1-induced responses

The experiments described in 5.2.3 showed that there were differences between the CEP-1-induced responses shown by each of the three subject groups. Firstly, the percentages of subjects in which positive CEP-1-induced PBMC proliferative responses were detected differed between the three subject groups, although these inter-group differences were not statistically significant. Secondly, the disease control group showed significantly higher levels of CEP-1-induced PBMC proliferation than the RA patient group. Thirdly, the RA patient group showed significantly higher levels of CEP-1-induced IL-10 production than the healthy subject group. Furthermore, the RA patient group showed significantly higher levels of CEP-1-induced IL-6 and TNF-α production than both the healthy subject and disease control groups. However, despite these differences, I had some concerns about the robustness of the CEP-1 data.

Of the first five healthy subjects that were tested with CEP-1, only two had shown positive CEP-1-induced PBMC proliferative responses (SI values of 4.3 and 5.7). In contrast, the initial six RA patients tested with CEP-1 had all shown positive CEP-1-induced PBMC proliferative responses (mean ± SEM SI value of CEP-1-induced PBMC proliferation was 15.6 ± 5.1). At this stage, the data strongly suggested that the frequency and magnitude of positive proliferative responses to CEP-1 were greater in the RA patients than in the healthy subjects. This prompted me to measure CEP-1-induced PBMC responses in disease controls to see how they would compare with those detected in RA patients and healthy subjects. I also continued testing more PBMC samples from RA patients and healthy subjects with CEP-1.

As was shown in Figure 5.3C, all eleven of the disease controls tested showed positive CEP-1-induced PBMC proliferative responses (mean ± SEM SI value of CEP-1-induced PBMC proliferation measuring 21.4 ± 4.0.) However, while further experiments with RA patient PBMC continued to show a very high incidence of CEP-1 responses in RA, a subsequent four healthy subjects tested all showed positive CEP-1-induced PBMC proliferative responses (mean ± SEM SI value of CEP-1-induced PBMC proliferation measuring 7.8 ± 1.1).

The observation of unusually high proportions of positive CEP-1-induced PBMC proliferative responses accompanied by high levels of IL-6 production in both RA patients and healthy subjects was similar to that observed when using DeglyAgg as an

antigenic stimulus. Therefore, I was concerned that the CEP-1 solution could be contaminated with LPS and indeed, when a LAL assay was performed on samples of the peptide solution bacterial endotoxin was detected (samples of REP-1 tested negative for endotoxin contamination, data not shown). At a concentration of 1mg/ml in RPMI 1640, the CEP-1 was shown to contain 1.6 EU/ml, while at a concentration of 10µg/ml it was found to contain 0.7EU/ml (data representative of two separate LAL assays). According to the literature accompanying the LAL assay kit, 10EU equate to 1ng of endotoxin, meaning that the 1mg/ml solution of CEP-1 contained 160pg/ml of endotoxin while the 10µg/ml solution of CEP-1 contained 70pg/ml of endotoxin. To determine whether 70pg/ml of LPS is sufficient to induce PBMC proliferation, I stimulated PBMC from a healthy volunteer with a range of concentrations of LPS (from 1µg/ml to 0.001pg/ml in serial 10-fold dilutions) and measured the resulting proliferation via 3HTdR incorporation. Interestingly, PBMC proliferation was only observed when using the top four concentrations of LPS (1µg/ml, 100ng/ml, 10ng/ml and 1ng/ml) while the other concentrations were insufficient to induce detectable proliferation (Figure 5.11). This observation suggested that the CEP-1 response was not driven by LPS directly.

However, it remains possible that LPS contamination was affecting the CEP-1 response indirectly. Therefore, I stimulated PBMC from a healthy subject with CEP-1, both with and without the presence of PMB, a compound that neutralises LPS (Cardoso *et al*, 2007). While the PBMC cultured with CEP-1 alone showed good proliferation, those cultured with CEP-1 and PMB did not (Figure 5.12A). In addition, cytokine production in response to CEP-1 was markedly reduced when PMB was present in the culture (Figure 5.12B). These data suggested that the CEP-1 response measured in my earlier experiments could have been influenced by LPS contamination. However, I also found that PMB could abrogate the responses to other stimuli in which LPS could not be detected via LAL assay (data not shown). Therefore, to further investigate the effect of LPS contamination on T-cell responses, I removed the contaminating LPS from the CEP-1 preparation to generate a 'clean' sample of CEP-1 using an endotoxin removal kit. Once I had obtained my sample of 'clean' CEP-1 solution (LAL assay confirmed that the 'clean' preparation contained 0EU/ml endotoxin), I performed an experiment in which PBMC from two subjects were stimulated with both the original and 'clean' CEP-1 samples, each at 10µg/ml. Figure 5.13A shows that in both cases, original CEP-1 preparation induced good proliferative responses (peak SI values of 4.6 and 15.2) yet

the corresponding 'clean' CEP-1 preparation did not (peak SI values of only 1.0 and 0.8). In both subjects, the levels of IL-17, IL-4 and TNF-α production detected in response to the 'clean' CEP-1 were also substantially lower than those detected in response to the original CEP-1 and indeed no IL-10, IL-6 or IFN-γ was detected in response to the 'clean' CEP-1 (Figure 5.13B). Taken together, these observations suggested that the CEP-1 response detected in my earlier experiments was substantially influenced by LPS contamination.

However, it was also possible that the LPS removal procedure had removed a substantial amount of the peptide. The manufacturers of the LPS removal kit stated that 5% of the protein/peptide would be lost during the endotoxin removal steps, and although I had adjusted the concentration of the 'clean' CEP-1 preparation accordingly for the PBMC assay, the removal process may actually have removed a greater proportion of the peptide. Indeed, when I performed a Bradford assay to measure the concentration of 'clean' CEP-1 preparation, I found that over 60% of the peptides was lost after the LPS removal procedure (a 'clean' sample that was expected to contain 5µg/ml of peptide was shown to contain only 1.9µg peptide). Therefore, the lack of responses to the 'clean' CEP-1 preparation could be a consequence of the use of suboptimal concentrations of the peptides, rather than the removal of contaminating LPS alone.

5.2.5. LPS-free REP-1-induced and LPS-free CEP-1-induced PBMC proliferation and cytokine production

In order to further explore the role that LPS contamination may have played in the CEP-1-induced proliferation and cytokine production responses described in 5.2.3, I obtained new, LPS-free preparations of REP-1 and CEP-1 and repeated some further REP-1 and CEP-1 experiments using these peptide preparations. Prior to use, samples of the new peptide preparations were tested with a LAL assay and both were found to be endotoxin-free. Thus, for my final set of experiments, I measured LPS-free REP-1-induced and LPS-free CEP-1-induced PBMC proliferation and cytokine production in eight RA patients and six healthy subjects (clinical details of the RA patients - RA 5.40 to RA 5.47 - are summarised in Table 2.6 in chapter 2). Tables 5.21 and 5.22 show the peak SI values of LPS-free REP-1-induced and LPS-free CEP-1-induced PBMC proliferation and the day 5, pg/ml minus background cytokine production values that were detected in the RA patients and healthy subjects respectively.

Firstly, I compared LPS-free REP-1-induced responses with LPS-free CEP-1-induced responses in each of the two subject groups. While only three of the eight RA patients showed positive proliferative or cytokine production responses to LPS-free REP-1 (Table 5.21A), five showed positive responses to LPS-free CEP-1 (Table 5.21B) although this difference was not significant (P value of 0.91, Chi-square). RA 5.41 responded to both peptides with PBMC proliferation alone, yet the magnitude of the response to LPS-free REP-1 was slightly greater than that of the response to LPS-free CEP-1 (SI values of 3.2 versus 2.1). RA 5.42 responded to LPS-free REP-1 with production of IL-17, IL-6 and TNF-α (9pg/ml, 756pg/ml and 50pg/ml respectively), yet responded to LPS-free CEP-1 with production of only IL-6 and TNF-α at reduced levels (24pg/ml and 3pg/ml respectively). RA 5.46 responded to LPS-free REP-1 with production of IL-6 alone (277pg/ml), yet responded to LPS-free CEP-1 with more potent IL-6 production (403pg/ml) as well as production of IL-10, IL-17, IL-4 and TNF-α (23pg/ml, 29pg/ml, 7pg/ml and 3022pg/ml). RA 5.45 and RA 5.47 only responded to LPS-free CEP-1, showing production of both IL-17 and IL-6 (5pg/ml and 8pg/ml respectively) or IL-6 alone (22pg/ml) respectively. Interestingly, no detectable IFN-γ production was observed in response to either LPS-free REP-1 or LPS-free CEP-1 in any of the RA patients and none showed detectable IL-10 or IL-4 production in response to LPS-free REP-1. Ultimately, the peak SI values of LPS-free REP-1-induced PBMC proliferation were comparable to those of LPS-free CEP-1-induced PBMC proliferation (mean ± SEM peak SI values of 1.5 ± 0.3 versus 1.2 ± 0.1 respectively, P value of 0.09, paired t test). Furthermore, the absolute values of LPS-free REP-1-induced IL-17, IL-6 and TNF-α production were comparable to those of LPS-free CEP-1-induced IL-17, IL-6 and TNF-α production (mean ± SEM absolute values of 1 ± 1 versus 4 ± 4 for IL-17, 129 ± 96 versus 57 ± 50 for IL-6 and 6 ± 6 versus 378 ± 378 for TNF-α, P values of 0.44, 0.48 and 0.36 respectively, paired t test).

Two of the six healthy subjects showed positive proliferative and/or cytokine production responses to LPS-free REP-1 (Table 5.22A), while four showed positive responses to LPS-free CEP-1 (Table 5.22B) although this difference was not significant (P value of 0.86, Chi-square). HS 5.30 responded to LPS-free REP-1 with PBMC proliferation (SI value of 2.9) and production of both IL-17 and IL-6 (16pg/ml and 52pg/ml respectively), yet responded to LPS-free CEP-1 with PBMC proliferation alone (SI value of 2.8). HS 5.32 responded to LPS-free REP-1 with production of IL-6 alone (13pg/ml), yet responded to LPS-free CEP-1 with more potent IL-6 production

(135pg/ml) as well as production of IL-17 and TNF-α (3pg/ml and 10pg/ml respectively). HS 5.31 and HS 5.34 only responded to LPS-free CEP-1, producing both IL-6 and TNF-α (9pg/ml and 3pg/ml respectively) or IL-6 alone (8pg/ml) respectively. Interestingly, no detectable IL-10, IL-4 or IFN-γ production was observed in response to either LPS-free REP-1 or LPS-free CEP-1 in any of the healthy subjects and none showed detectable TNF-α production in response to LPS-free REP-1. Ultimately, the peak SI values of LPS-free REP-1-induced PBMC proliferation were comparable to those of LPS-free CEP-1-induced PBMC proliferation (mean ± SEM peak SI values of 1.6 ± 0.3 versus 1.5 ± 0.3 respectively, P value of 0.06, paired t test). In addition, the absolute values of LPS-free REP-1-induced IL-17 and IL-6 production were comparable to those of LPS-free CEP-1-induced IL-17 and IL-6 production (mean ± SEM absolute values of 3 ± 3 versus 1 ± 1 for IL-17 and 11 ± 8 versus 25 ± 22 for IL-6, P values of 0.44 and 0.57 respectively, paired t test).

Secondly, I compared LPS-free REP-1-induced responses and LPS-free CEP-1-induced responses between each of the two subject groups. The proportions of subjects showing positive LPS-free REP-1-induced responses were comparable in both the RA patient group and the healthy subject group, as were the proportions of subjects showing positive LPS-free CEP-1-induced responses. In addition, the peak SI values of LPS-free REP-1-induced PBMC proliferation detected in both subject groups were comparable (median = 1.3, IQR: 1.1-1.5 in RA patients, median = 1.4, IQR: 1.2-2.0 in healthy subjects, P value of 0.60, Mann Whitney), as were the peak SI values of LPS-free CEP-1-induced PBMC proliferation (median = 1.2, IQR: 1.1-1.3 in RA patients, median = 1.2, IQR: 1.1-1.8 in healthy subjects, P value of 0.79, Mann Whitney). While none of the RA patients or healthy subjects showed detectable IL-10, IL-4 or IFN-γ production in response to LPS-free REP-1, detectable levels of IL-17 and IL-6 production were observed in both cohorts in response to this stimulus. However, while none of the healthy subjects showed detectable levels of REP-1-induced TNF-α production, one of the RA patients did. The median absolute values of LPS-free REP-1-induced IL-17 and IL-6 production were comparable between the RA patients and the healthy subjects (median = 0, IQR: 0-0 versus median = 0, IQR: 0-5 for IL-17 and median = 0, IQR: 0-208 versus median = 1, IQR: 0-23 for IL-6, P values of 0.69 and 0.89 respectively, Mann Whitney). While none of the RA patients or healthy subjects showed detectable IFN-γ production in response to LPS-free CEP-1, detectable levels of IL-17, IL-6 and TNF-α were observed in both cohorts in response to this stimulus. However, while none

of the healthy subjects showed any detectable levels of LPS-free CEP-1-induced IL-10 or IL-4 production, one of the RA patients did. The median absolute values of LPS-free CEP-1-induced IL-17, IL-6 and TNF-α production were comparable between the RA patients and the healthy subjects (median = 0, IQR: 0-4 versus median = 0, IQR: 0-1 for IL-17, median = 4, IQR: 0-23 versus median = 4, IQR: 0-40 for IL-6 and median = 0, IQR: 0-2 versus median = 0, IQR: 0-5 for TNF-α, P values of 0.42, 0.95 and 0.50 respectively, Mann Whitney).

5.3. Discussion

As discussed in chapter 4, care must be taken when interpreting data showing cytokine production in response to test stimuli. Some of the subjects tested in the experiments described in chapter 5 showed substantial levels of background production of IL-10, IL-17, IL-4 and IFN-γ (see Table 5.23 and Table 5.24 for RA patients and healthy subjects respectively), yet the absolute values of production of these cytokines in response to test peptides were generally very low (see Table 5.2-Table 5.8, Table 5.10-Table 5.17 and Table 5.21 and Table 5.22), therefore peptide-induced production of IL-10, IL-17, IL-4 and IFN-γ could potentially be over-interpreted. As mentioned in the previous chapter, if in a given subject the absolute value of test peptide-induced production of a particular cytokine is considerably less than the level of background production of the same cytokine, it is open to debate whether the absolute value of peptide-induced cytokine production is representative of a positive response, regardless of whether it exceeds the threshold level of detection for a positive response.

The experiments described in 5.2.1 and 5.2.2 showed that the peptides comprising peptide panels #1 and #2 respectively induced similar proportions of positive proliferative and/or cytokine production responses in both the RA patient groups and the healthy subject groups. Furthermore, for all 15 of the peptides tested in the experiments described in 5.2.1 and 5.2.2, there were no significant differences between the magnitudes of the proliferative and cytokine production responses that were elicited in the RA patient groups and those that were elicited in the healthy subject groups. In addition, the citrullinated peptides induced similar proportions of positive proliferative and or cytokine production responses as their non-citrullinated counterparts, and the magnitudes of the responses elicited by citrullinated peptides were comparable to those elicited by their corresponding non-citrullinated peptides. While responses were generally weak, the vast majority of the subjects did show positive responses to at least

one of the peptides in the panels. Indeed, substantial numbers of both RA patients and healthy subjects showed positive responses to three or more of the peptides in these peptide panels. However, the peptides that elicited responses and those that failed to elicit responses often varied between individuals. Interestingly, the phenotypes of the positive responses also differed between individuals but no clear trends were visible and the two subject groups could not be distinguished from each other on this basis, nor could responses to citrullinated peptides versus non-citrullinated peptides.

The data from the initial experiments described in 5.2.3 showed that in the RA patient group but not in the healthy subject or disease control groups, the citrullinated peptide CEP-1 induced a greater proportion of positive proliferative responses than its non-citrullinated counterpart REP-1. However, CEP-1 had induced positive responses in a substantial proportion of the healthy subjects and all of the disease controls and indeed the magnitudes of the CEP-1-induced proliferative and IL-17, IL-4 and IFN-γ production responses detected in the RA patient group did not differ significantly from those detected in the healthy subject group. Thus it was evident that CEP-1 was eliciting responses in large numbers of subjects, regardless of whether they were RA patients, healthy subjects or disease controls. Subsequent experiments indicated that the responses to CEP-1 detected in the initial experiments were likely to be the results of LPS contamination. Firstly, the CEP-1 preparation was found to contain LPS, and data from further experiments showed that neutralising the LPS in the CEP-1 using PMB as well as the removal of the LPS from the CEP-1 could abrogate or substantially dampen the CEP-1- responses. Additionally, when PBMC from a further eight RA patients and six healthy subjects were tested with new LPS-free REP-1 and CEP-1 preparations, I found no significant differences in the T-cell responses to CEP-1 or REP-1 between RA patients and healthy subjects with regards to the proportion of positive responses, the magnitude of the proliferative responses and also cytokine production. Such findings were in contrast to those when using the original, LPS-contaminated CEP-1 preparations.

However, different RA patients and healthy subjects were tested for T-cell responses to the new LPS-free preparation of CEP-1 therefore I could not completely exclude the possibility that the differences in the responses to the new batch of LPS-free CEP-1 versus the original LPS-contaminated batch were a consequence of different subjects having been tested. Nevertheless, taken collectively, the data from the experiments

described in 5.2.4 and 5.2.5 suggested that LPS contamination contributed substantially to the CEP-1 responses that were detected in the experiments described in 5.2.3. However, the precise role that the LPS had been playing in the CEP-1 responses was unclear.

It is possible that the CEP-1 responses detected in the experiments described in 5.2.3 could simply have been responses to contaminating LPS, which could have been inducing T-cell proliferation via two mechanisms. Firstly, the LPS could have been acting directly on T-cells via TLR4, as it has been reported that the recognition of LPS by human $CD8^+$ T-cells via TLR4 can elicit T-cell responses (Komai-Koma *et al*, 2009). Secondly, the LPS could have been acting on T-cells indirectly, activating antigen presenting cells via TLR4 ligation and causing them to secrete cytokines and/or express co-stimulatory molecules which in turn induce T-cell proliferation, as has been previously described (Tough *et al*, 1997 and Mattern *et al*, 1998). However, there are two arguments against these explanations. Firstly, the data from the LPS titration experiments described in 5.2.4 suggested that the level of LPS added to the cell cultures in the assays described in 5.2.3 was not sufficient to induce a detectable proliferative response. The 1mg/ml working solution of CEP-1 was also found to contain LPS at a concentration of 160pg/ml. When 10µl of the 1mg/ml working solution of CEP-1 were diluted in 1ml RPMI to produce the final concentration of 10µg/ml CEP-1 that was used in the assays, this solution was found to contain LPS at a concentration of 70pg/ml. Data from my LPS titration experiments showed that even a 100pg/ml concentration of LPS was incapable of inducing detectable proliferation, and this observation is similar to what has previously been reported (Tough *et al*, 1997 and Tulic *et al*, 2002). Secondly, there were significant differences between the magnitudes of the CEP-1-induced responses detected in each of the three subject groups. For example, the magnitudes of the CEP-1-induced proliferative responses detected in the disease control group were significantly greater than those detected in the RA patient and healthy subject groups. In addition, the magnitude of CEP-1-induced IL-10 production in both the RA patient and disease control groups was significantly greater than CEP-1-induced IL-10 production in the healthy subject group, while the magnitude of CEP-1-induced IL-4 production in the disease control group was significantly greater than CEP-1-induced IL-4 production in the healthy subject group. Furthermore, the magnitudes of CEP-1-induced IL-6 and TNF-α production in the RA patient group were significantly greater than CEP-1-induced IL-6 and TNF-α production in both the healthy subject and

disease control groups. If the CEP-1 response was purely a response to the LPS contaminant within the CEP-1 preparation, then all subjects should respond similarly with no significant differences between the three subject groups in the way that they respond to CEP-1, assuming equivalent sensitivities to LPS between the groups. One would not expect an LPS-specific response to differ between the different subject groups. However, there is evidence to suggest that the synovial macrophages of RA patients express more TLR4 than control macrophages and are therefore more sensitive to microbial products like LPS (Huang *et al*, 2007). Thus RA patient macrophages may produce more T-cell-activating cytokines in response to LPS stimulation than their healthy subject/disease control counterparts.

However, it is also possible that the LPS was acting as an adjuvant, enhancing the responses to the CEP-1 peptide by activating antigen-presenting cells and inducing expression of certain cytokines and co-stimulatory molecules which could in turn could have provided additional activation signals to $CD4^+$ T-cells recognising MHC class II:CEP-1 peptide complexes presented by the antigen-presenting cells. Thus the LPS may have simply been amplifying CEP-1 peptide-specific proliferative and cytokine production responses. It is possible that the addition of an adjuvant like LPS to the autoantigen-stimulated cultures is required for autoantigen-specific responses to be detected.

Peptide ID	Sequence	Antigen	Reference
1	SAVRA(Cit)SSVPGVR	Cit-Vimentin$_{65-77}$	1*
2	QDFTN(Cit)INKLKNS	Cit-Fibrinogen α–chain$_{80-92}$	2*
3	KIHA(Cit)EIFDS(Cit)GNPTVE	Cit-Enolase$_{5-21}$	3*
4	HSASQDGQDTI(Cit)GHPGSS	Cit-Filaggrin$_{2776-2793}$	4*
5	DSGH(Cit)GYSGSQASDNEGH	Cit-Filaggrin$_{3005-3022}$	
6	CVLLVATEGRVRVNSAYQDC	Aggrecan$_{84-103}$ with terminal cysteines	5*
7	CVLLVATEG(Cit)VRVNSAYQDC	Cit-Aggrecan$_{84-103}$ with terminal cysteines	
8	AGWLADQTVRYPI	Aggrecan$_{201-213}$	6* (non-cit peptides only)
9	AGWLADQTV(Cit)YPI	Cit-Aggrecan$_{201-213}$	
10	AGWLADRSVRYPI	Aggrecan$_{299-311}$	
11	AGWLAD(Cit)SVRYPI	Cit-Aggrecan$_{299-311}$	
12	GRSFTLASSETGVGA	HCgp39$_{262-276}$	7* (non-cit peptide only)
13	G(Cit)SFTLASSETGVGA	Cit-HCgp39$_{262-276}$	
14	VVLLVATEG(Cit)VRVNSAYQDK	N Cit-Aggrecan$_{84-103}$	5*
15	VVLLVATEGRV(Cit)VNSAYQDK	C Cit-Aggrecan$_{84-103}$	
REP-1	CKIHAREIFDSRGNPTVEC	Enolase$_{5-21}$ with terminal cysteines	3*
CEP-1	CKIHA(Cit)EIFDS(Cit)GNPTVEC	Cit-Enolase$_{5-21}$ with terminal cysteines	

Table 5.1. Details of peptide stimuli used in chapter 5. Peptides 1-7 comprised peptide panel #1, while peptides 8-15 comprised peptide panel #2. (Cit) = citrulline. Cit- = citrullinated. 1* = Hill *et al*, 2003, 2* = Hill *et al*, 2008, 3* = Lundberg *et al*, 2008, 4* = Fritsch *et al*, 2002, 5* = von Delwig *et al*, 2010, 6* = Boots *et al*, 1997, 7* = Verheijden *et al*, 1997. Peptide 14 (N citrullinated Aggrecan$_{84-103}$) was the citrullinated peptide tested in the experiments described by von Delwig *et al*, 2010.

A) RA patients

ID	Peptide 1-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	1.2	0	26	0	203	18	0
RA 5.2	13.4	0	0	0	164	31	29
RA 5.3	1.4	0	31	0	306	18	0
RA 5.4	-	-	-	-	-	-	-
RA 5.5	1.2	0	0	0	0	0	0
RA 5.6	1.2	0	0	0	0	0	9
RA 5.7	1.0	0	0	0	0	0	0
RA 5.8	1.0	0	0	0	0	0	0
RA 5.9	1.5	0	0	0	12	0	0
RA 5.10	1.6	0	0	0	0	0	0

B) Healthy subjects

ID	Peptide 1-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	1.2	0	0	0	0	0	0
HS 5.2	1.6	0	0	0	0	0	10
HS 5.3	1.5	0	0	0	0	0	0
HS 5.4	3.8	0	0	0	0	0	0
HS 5.5	3.0	0	246	0	698	35	7
HS 5.6	0.9	0	0	0	0	0	0
HS 5.7	1.2	9	15	0	1322	25	0
HS 5.8	1.6	0	0	0	0	0	0
HS 5.9	1.6	0	0	0	0	0	0
HS 5.10	1.3	0	0	0	0	0	0

Table 5.2. Peptide 1-induced PBMC proliferation and cytokine production detected in RA patients and healthy subjects. A & **B**, the peak SI values of peptide 1-induced PBMC proliferation and the day 5 absolute values of peptide 1-induced cytokine production detected in the RA patients and healthy subjects respectively. A dash (-) represents an untested value.

A) RA patients

ID	Peptide 2-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	1.1	0	0	0	0	0	10
RA 5.2	0.7	0	7	0	39	0	0
RA 5.3	1.5	0	0	0	0	0	0
RA 5.4	-	-	-	-	-	-	-
RA 5.5	1.2	0	0	0	14	3	0
RA 5.6	0.9	0	0	0	0	0	15
RA 5.7	0.9	0	0	0	0	0	8
RA 5.8	1.0	0	0	0	0	0	0
RA 5.9	1.3	0	358	0	4	0	0
RA 5.10	1.6	0	0	0	0	0	0

B) Healthy subjects

ID	Peptide 2-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	1.9	0	0	0	0	0	45
HS 5.2	1.2	0	0	0	0	0	0
HS 5.3	1.8	0	0	0	0	0	0
HS 5.4	5.7	0	359	0	0	4	0
HS 5.5	4.4	0	0	0	690	49	57
HS 5.6	0.8	0	0	0	3	0	0
HS 5.7	1.0	0	3	0	90	10	0
HS 5.8	1.5	0	0	0	0	0	0
HS 5.9	1.6	0	0	0	0	0	0
HS 5.10	1.3	0	27	0	88	3	0

Table 5.3. Peptide 2-induced PBMC proliferation and cytokine production detected in RA patients and healthy subjects. A & **B**, the peak SI values of peptide 2-induced PBMC proliferation and the day 5 absolute values of peptide 2-induced cytokine production detected in the RA patients and healthy subjects respectively. A dash (-) represents an untested value.

A) RA patients

ID	Peptide 3-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	1.1	0	31	0	0	0	14
RA 5.2	1.1	0	8	0	0	0	0
RA 5.3	2.0	0	0	0	0	0	0
RA 5.4	-	-	-	-	-	-	-
RA 5.5	1.1	0	0	0	0	0	0
RA 5.6	1.0	0	0	0	17	0	0
RA 5.7	1.0	0	0	0	4	0	0
RA 5.8	1.0	0	0	0	0	0	0
RA 5.9	1.5	3	163	0	5	0	0
RA 5.10	1.3	0	0	0	0	0	0

B) Healthy subjects

ID	Peptide 3-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	0.9	0	0	0	0	0	35
HS 5.2	1.2	0	0	0	0	0	0
HS 5.3	1.5	0	0	0	30	6	16
HS 5.4	2.2	0	0	0	0	0	0
HS 5.5	9.6	0	0	0	0	3	10
HS 5.6	0.7	0	0	0	9	0	0
HS 5.7	0.8	3	5	0	254	8	0
HS 5.8	1.8	0	0	0	0	0	0
HS 5.9	0.9	0	0	0	0	0	0
HS 5.10	1.2	0	0	0	0	0	0

Table 5.4. Peptide 3-induced PBMC proliferation and cytokine production detected in RA patients and healthy subjects. A & **B**, the peak SI values of peptide 3-induced PBMC proliferation and the day 5 absolute values of peptide 3-induced cytokine production detected in the RA patients and healthy subjects respectively. A dash (-) represents an untested value.

A) RA patients

ID	Peptide 4-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	-	-	-	-	-	-	-
RA 5.2	1.3	0	0	0	13	4	0
RA 5.3	1.3	0	4	0	12	12	0
RA 5.4	1.3	0	0	0	0	0	0
RA 5.5	1.0	0	0	0	0	0	11
RA 5.6	1.1	0	0	0	3	0	0
RA 5.7	0.9	0	0	0	0	0	16
RA 5.8	1.1	0	0	0	0	0	0
RA 5.9	1.2	0	0	0	46	10	0
RA 5.10	1.5	0	0	0	3	2	0

B) Healthy subjects

ID	Peptide 4-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	1.0	0	104	0	0	0	49
HS 5.2	1.1	0	0	0	0	0	31
HS 5.3	1.4	0	25	0	40	18	51
HS 5.4	3.6	4	1220	5	1817	31	57
HS 5.5	5.6	0	0	0	0	4	0
HS 5.6	0.7	0	0	0	0	0	0
HS 5.7	0.9	4	21	2	1310	20	0
HS 5.8	1.4	0	0	0	0	0	0
HS 5.9	2.1	0	0	0	0	0	0
HS 5.10	1.8	0	77	0	6	0	0

Table 5.5. Peptide 4-induced PBMC proliferation and cytokine production detected in RA patients and healthy subjects. A & **B**, the peak SI values of peptide 4-induced PBMC proliferation and the day 5 absolute values of peptide 4-induced cytokine production detected in the RA patients and healthy subjects respectively. A dash (-) represents an untested value.

A) RA patients

ID	Peptide 5-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	-	-	-	-	-	-	-
RA 5.2	0.8	0	0	0	0	0	0
RA 5.3	1.7	16	272	27	9692	297	0
RA 5.4	3.3	0	0	0	0	0	0
RA 5.5	1.2	0	0	0	0	0	19
RA 5.6	1.1	0	0	0	0	0	0
RA 5.7	1.0	0	0	0	0	0	22
RA 5.8	1.1	0	0	0	0	0	0
RA 5.9	1.2	0	3	0	0	0	0
RA 5.10	2.0	0	0	0	4	2	20

B) Healthy subjects

ID	Peptide 5-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	1.4	0	0	0	0	0	39
HS 5.2	1.3	0	0	0	5	3	33
HS 5.3	1.2	2	0	0	63	29	39
HS 5.4	1.5	0	0	0	0	0	0
HS 5.5	5.3	0	0	0	106	21	79
HS 5.6	0.7	0	0	0	0	3	0
HS 5.7	0.8	0	0	0	3	0	0
HS 5.8	2.0	0	0	0	0	0	0
HS 5.9	1.2	0	0	0	0	0	0
HS 5.10	1.3	0	0	0	3	0	0

Table 5.6. Peptide 5-induced PBMC proliferation and cytokine production detected in RA patients and healthy subjects. A & **B**, the peak SI values of peptide 5-induced PBMC proliferation and the day 5 absolute values of peptide 5-induced cytokine production detected in the RA patients and healthy subjects respectively. A dash (-) represents an untested value.

A) RA patients

ID	Peptide 6-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	-	-	-	-	-	-	-
RA 5.2	1.2	0	6	0	0	0	0
RA 5.3	2.2	0	3	0	0	0	0
RA 5.4	1.1	0	0	0	0	0	0
RA 5.5	1.2	0	0	0	0	0	9
RA 5.6	1.0	0	0	0	0	0	0
RA 5.7	0.8	0	0	0	3	0	0
RA 5.8	1.1	0	0	0	0	0	0
RA 5.9	3.0	0	7	0	13	2	0
RA 5.10	2.2	0	0	0	4	0	15

B) Healthy subjects

ID	Peptide 6-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	1.6	0	0	0	0	0	37
HS 5.2	1.6	0	3	0	20	0	29
HS 5.3	1.2	0	0	0	11	5	37
HS 5.4	5.1	0	0	0	0	0	0
HS 5.5	3.4	0	0	0	0	0	60
HS 5.6	1.5	0	87	0	21	14	0
HS 5.7	1.4	0	0	0	34	2	0
HS 5.8	1.4	0	0	0	0	0	0
HS 5.9	1.6	0	12	0	133	0	0
HS 5.10	1.4	0	0	0	0	0	0

Table 5.7. Peptide 6-induced PBMC proliferation and cytokine production detected in RA patients and healthy subjects. A & **B**, the peak SI values of peptide 6-induced PBMC proliferation and the day 5 absolute values of peptide 6-induced cytokine production detected in the RA patients and healthy subjects respectively. A dash (-) represents an untested value.

A) RA patients

ID	Peptide 7-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	-	-	-	-	-	-	-
RA 5.2	1.7	0	5	0	0	2	12
RA 5.3	2.4	0	14	0	0	0	0
RA 5.4	1.7	0	0	0	11	0	0
RA 5.5	1.1	0	0	0	0	0	0
RA 5.6	0.9	0	0	0	5	0	0
RA 5.7	0.7	0	0	0	5	0	0
RA 5.8	0.9	0	0	0	0	0	0
RA 5.9	1.4	0	5	0	0	0	0
RA 5.10	3.0	0	0	0	5	0	9

B) Healthy subjects

ID	Peptide 7-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	2.3	0	0	0	0	0	45
HS 5.2	1.3	0	11	0	468	3	44
HS 5.3	1.0	0	0	0	2	0	31
HS 5.4	1.4	0	0	0	0	0	0
HS 5.5	4.4	0	0	0	0	0	31
HS 5.6	1.0	0	0	0	4	0	0
HS 5.7	1.3	2	12	0	960	0	0
HS 5.8	2.0	0	5	0	31	0	0
HS 5.9	1.0	3	0	0	0	0	0
HS 5.10	4.6	5	3	0	41	0	0

Table 5.8. Peptide 7-induced PBMC proliferation and cytokine production detected in RA patients and healthy subjects. A & **B**, the peak SI values of peptide 7-induced PBMC proliferation and the day 5 absolute values of peptide 7-induced cytokine production detected in the RA patients and healthy subjects respectively. A dash (-) represents an untested value.

A) RA patients

ID	Positive PBMC proliferative and/or cytokine production response (Yes/No)						
	Peptide 1	**Peptide 2**	**Peptide 3**	**Peptide 4**	**Peptide 5**	**Peptide 6**	**Peptide 7**
RA 5.1	Yes	Yes	Yes	-	-	-	-
RA 5.2	Yes	Yes	Yes	Yes	No	Yes	Yes
RA 5.3	Yes	No	Yes	Yes	Yes	Yes	Yes
RA 5.4	-	-	-	No	Yes	No	Yes
RA 5.5	No	Yes	No	Yes	Yes	Yes	No
RA 5.6	Yes	Yes	Yes	Yes	No	No	Yes
RA 5.7	No	Yes	Yes	Yes	Yes	Yes	Yes
RA 5.8	No	No	No	No	No	No	No
RA 5.9	Yes	Yes	Yes	Yes	Yes	Yes	Yes
RA 5.10	No	No	No	Yes	Yes	Yes	Yes

B) Healthy subjects

ID	Positive PBMC proliferative and/or cytokine production response (Yes/No)						
	Peptide 1	**Peptide 2**	**Peptide 3**	**Peptide 4**	**Peptide 5**	**Peptide 6**	**Peptide 7**
HS 5.1	No	Yes	Yes	Yes	Yes	Yes	Yes
HS 5.2	Yes	No	No	Yes	Yes	Yes	Yes
HS 5.3	No	No	Yes	Yes	Yes	Yes	Yes
HS 5.4	Yes	Yes	Yes	Yes	No	Yes	No
HS 5.5	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HS 5.6	No	Yes	Yes	No	Yes	Yes	Yes
HS 5.7	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HS 5.8	No	No	No	No	Yes	No	Yes
HS 5.9	No	No	No	Yes	No	Yes	Yes
HS 5.10	No	Yes	No	Yes	Yes	No	Yes

Table 5.9. Responders and non-responders; peptide panel #1. A, how each of the RA patients responded to the peptides. A dash (-) represents an untested value. **B**, how each of the healthy subjects responded to the peptides.

A)

ID	Peptide 8-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	1.0	0	3	0	55	0	0
RA 5.12	1.5	0	0	0	0	0	75
RA 5.13	1.3	-	-	-	-	-	-
RA 5.14	1.3	0	7	0	0	0	0
RA 5.15	-	-	-	-	-	-	-
RA 5.16	-	-	-	-	-	-	-
RA 5.17	-	-	-	-	-	-	-
RA 5.18	-	-	-	-	-	-	-
RA 5.19	-	-	-	-	-	-	-

B)

ID	Peptide 9-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	1.0	0	29	0	1433	23	0
RA 5.12	1.2	0	0	0	0	0	5
RA 5.13	1.4	-	-	-	-	-	-
RA 5.14	1.2	2	135	5	5343	26	0
RA 5.15	1.1	0	2	0	0	0	0
RA 5.16	2.1	0	0	0	0	0	0
RA 5.17	0.9	0	0	0	0	39	11
RA 5.18	1.2	45	41	17	642	4599	0
RA 5.19	1.4	4	29	4	0	11	0

Table 5.10. Peptide 8-induced and peptide 9-induced PBMC proliferation and cytokine production detected in RA patients. A, the peak SI values of peptide 8-induced PBMC proliferation and the day 5 absolute values of peptide 8-induced cytokine production detected in the RA patients. **B**, the peak SI values of peptide 9-induced PBMC proliferation and the day 5 absolute values of peptide 9-induced cytokine production detected in the RA patients. In both tables, a dash (-) represents an untested value.

A)

ID	Peptide 8-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	0.9	0	0	0	0	0	0
HS 5.12	1.5	0	0	0	0	0	0
HS 5.13	3.2	0	0	0	0	0	0
HS 5.14	2.2	0	0	0	0	0	459
HS 5.15	1.1	0	0	0	0	2	0
HS 5.16	1.1	0	0	0	0	0	0
HS 5.17	-	-	-	-	-	-	-
HS 5.18	-	-	-	-	-	-	-
HS 5.19	-	-	-	-	-	-	-

B)

ID	Peptide 9-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	0.8	0	0	0	0	0	0
HS 5.12	1.2	0	0	0	11	0	0
HS 5.13	1.5	0	0	0	0	0	0
HS 5.14	5.4	0	943	0	1665	100	12
HS 5.15	0.8	0	0	0	0	0	0
HS 5.16	1.4	0	0	0	20	0	0
HS 5.17	2.9	0	0	0	3	0	5
HS 5.18	0.8	0	0	0	13	0	0
HS 5.19	1.6	0	0	0	0	0	0

Table 5.11. Peptide 8-induced and peptide 9-induced PBMC proliferation and cytokine production detected in healthy subjects. A, the peak SI values of peptide 8-induced PBMC proliferation and the day 5 absolute values of peptide 8-induced cytokine production detected in the healthy subjects. A dash (-) represents an untested value. **B**, the peak SI values of peptide 9-induced PBMC proliferation and the day 5 absolute values of peptide 9-induced cytokine production detected in the healthy subjects.

A)

ID	Peptide 10-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	2.6	0	0	0	22	0	0
RA 5.12	1.5	0	0	0	31	11	99
RA 5.13	1.4	-	-	-	-	-	-
RA 5.14	1.2	0	0	0	733	0	0
RA 5.15	-	-	-	-	-	-	-
RA 5.16	-	-	-	-	-	-	-
RA 5.17	-	-	-	-	-	-	-
RA 5.18	-	-	-	-	-	-	-
RA 5.19	-	-	-	-	-	-	-

B)

ID	Peptide 11-induced PBMC responses						
	3HTdRincorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	0.8	0	59	0	0	0	0
RA 5.12	1.2	0	0	0	0	0	87
RA 5.13	1.2	-	-	-	-	-	-
RA 5.14	1.2	0	0	0	509	9	0
RA 5.15	1.5	0	0	0	0	0	0
RA 5.16	1.5	0	0	0	0	0	0
RA 5.17	1.0	0	0	0	0	60	11
RA 5.18	1.6	33	3	13	0	2752	0
RA 5.19	1.3	3	22	3	0	0	7

Table 5.12. Peptide 10-induced and peptide 11-induced PBMC proliferation and cytokine production detected in RA patients. A, the peak SI values of peptide 10-induced PBMC proliferation and the day 5 absolute values of peptide 10-induced cytokine production detected in the RA patients. **B**, the peak SI values of peptide 11-induced PBMC proliferation and the day 5 absolute values of peptide 11-induced cytokine production detected in the RA patients. In both tables, a dash (-) represents an untested value.

A)

ID	Peptide 10-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	1.0	0	0	0	0	0	0
HS 5.12	1.2	0	14	0	484	7	0
HS 5.13	1.2	0	0	0	0	0	0
HS 5.14	4.1	17	0	3	0	23	130
HS 5.15	1.1	0	0	0	0	0	0
HS 5.16	2.6	0	2	0	271	0	0
HS 5.17	-	-	-	-	-	-	-
HS 5.18	-	-	-	-	-	-	-
HS 5.19	-	-	-	-	-	-	-

B)

ID	Peptide 11-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	0.9	0	0	0	0	0	0
HS 5.12	2.3	0	0	0	46	0	0
HS 5.13	3.3	0	0	0	0	0	0
HS 5.14	1.6	0	0	0	0	3	261
HS 5.15	1.1	0	0	0	0	0	0
HS 5.16	1.4	5	17	0	1057	33	0
HS 5.17	2.1	0	0	0	0	0	0
HS 5.18	1.0	0	0	0	0	0	0
HS 5.19	1.9	0	0	0	0	0	0

Table 5.13. Peptide 10-induced and peptide 11-induced PBMC proliferation and cytokine production detected in healthy subjects. A, the peak SI values of peptide 10-induced PBMC proliferation and the day 5 absolute values of peptide 10-induced cytokine production detected in the healthy subjects. A dash (-) represents an untested value. **B**, the peak SI values of peptide 11-induced PBMC proliferation and the day 5 absolute values of peptide 11-induced cytokine production detected in the healthy subjects.

A)

ID	Peptide 12-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	1.3	0	0	0	11	0	0
RA 5.12	1.0	0	0	0	0	13	99
RA 5.13	1.1	-	-	-	-	-	-
RA 5.14	-	-	-	-	-	-	-
RA 5.15	-	-	-	-	-	-	-
RA 5.16	-	-	-	-	-	-	-
RA 5.17	-	-	-	-	-	-	-
RA 5.18	-	-	-	-	-	-	-
RA 5.19	-	-	-	-	-	-	-

B)

ID	Peptide 13-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	1.0	0	0	0	0	0	0
RA 5.12	1.0	0	0	0	10	6	105
RA 5.13	1.2	0	0	0	0	0	0
RA 5.14	-	-	-	-	-	-	-
RA 5.15	1.4	0	0	0	0	0	0
RA 5.16	1.5	0	0	0	0	0	0
RA 5.17	-	-	-	-	-	-	-
RA 5.18	1.2	16	0	8	222	1117	0
RA 5.19	1.2	2	23	3	0	0	6

Table 5.14. Peptide 12-induced and peptide 13-induced PBMC proliferation and cytokine production detected in RA patients. A, the peak SI values of peptide 12-induced PBMC proliferation and the day 5 absolute values of peptide 12-induced cytokine production detected in the RA patients. **B**, the peak SI values of peptide 13-induced PBMC proliferation and the day 5 absolute values of peptide 13-induced cytokine production detected in the RA patients. In both tables, a dash (-) represents an untested value.

A)

ID	Peptide 12-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	0.8	0	0	0	0	0	6
HS 5.12	0.9	0	7	0	0	0	0
HS 5.13	1.5	0	0	0	0	0	20
HS 5.14	1.5	3	0	0	44	25	11
HS 5.15	0.9	0	0	0	0	0	0
HS 5.16	0.9	0	0	0	9	0	0
HS 5.17	-	-	-	-	-	-	-
HS 5.18	-	-	-	-	-	-	-
HS 5.19	-	-	-	-	-	-	-

B)

ID	Peptide 13-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	0.9	0	0	0	0	0	10
HS 5.12	1.4	0	6	0	0	0	0
HS 5.13	2.6	0	0	0	0	0	0
HS 5.14	1.8	0	0	0	0	0	7
HS 5.15	0.9	0	0	0	0	0	0
HS 5.16	1.2	3	4	0	681	13	0
HS 5.17	1.8	0	6	0	0	0	5
HS 5.18	1.1	3	0	0	6	0	0
HS 5.19	1.1	0	0	0	0	0	0

Table 5.15. Peptide 12-induced and peptide 13-induced PBMC proliferation and cytokine production detected in healthy subjects. A, the peak SI values of peptide 12-induced PBMC proliferation and the day 5 absolute values of peptide 12-induced cytokine production detected in the healthy subjects. A dash (-) represents an untested value. **B**, the peak SI values of peptide 13-induced PBMC proliferation and the day 5 absolute values of peptide 13-induced cytokine production detected in the healthy subjects.

A)

ID	Peptide 14-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	3.3	0	0	0	603	0	0
RA 5.12	1.1	0	0	0	3	7	104
RA 5.13	1.2	-	-	-	-	-	-
RA 5.14	1.3	0	24	0	254	0	0
RA 5.15	1.1	0	0	0	0	0	0
RA 5.16	1.4	0	0	0	0	0	0
RA 5.17	0.7	0	7	0	0	0	0
RA 5.18	1.1	17	13	7	516	2107	0
RA 5.19	0.9	0	0	0	0	0	0

B)

ID	Peptide 15-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.11	1.3	0	52	0	90	0	0
RA 5.12	1.2	0	0	0	0	0	104
RA 5.13	1.4	-	-	-	-	-	-
RA 5.14	1.1	0	145	3	3685	10	0
RA 5.15	1.2	0	0	0	0	0	0
RA 5.16	1.3	0	0	0	0	0	0
RA 5.17	1.1	0	0	0	0	0	12
RA 5.18	1.1	31	0	17	832	2925	0
RA 5.19	0.9	4	26	4	0	0	0

Table 5.16. Peptide 14-induced and peptide 15-induced PBMC proliferation and cytokine production detected in RA patients. A, the peak SI values of peptide 14-induced PBMC proliferation and the day 5 absolute values of peptide 14-induced cytokine production detected in the RA patients. **B**, the peak SI values of peptide 15-induced PBMC proliferation and the day 5 absolute values of peptide 15-induced cytokine production detected in the RA patients. In both tables, a dash (-) represents an untested value.

A)

ID	Peptide 14-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	1.3	0	0	0	0	0	0
HS 5.12	1.2	0	2	0	0	0	0
HS 5.13	3.0	0	0	0	0	0	0
HS 5.14	3.3	7	612	0	98	10	0
HS 5.15	0.9	0	0	0	0	0	0
HS 5.16	1.1	6	33	3	2670	33	0
HS 5.17	1.5	0	2	0	0	0	7
HS 5.18	1.8	9	0	0	0	0	0
HS 5.19	2.3	0	0	0	144	13	38

B)

ID	Peptide 15-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.11	1.1	0	0	0	424	0	0
HS 5.12	1.0	2	13	0	341	30	0
HS 5.13	1.5	0	0	0	0	0	0
HS 5.14	2.3	0	0	0	12	6	0
HS 5.15	0.9	0	0	0	10	0	0
HS 5.16	1.1	5	16	0	780	11	0
HS 5.17	2.3	0	18	0	0	0	6
HS 5.18	0.9	0	0	0	0	0	0
HS 5.19	1.5	0	0	0	0	0	0

Table 5.17. Peptide 14-induced and peptide 15-induced PBMC proliferation and cytokine production detected in healthy subjects. A, the peak SI values of peptide 14-induced PBMC proliferation and the day 5 absolute values of peptide 14-induced cytokine production detected in the healthy subjects. **B**, the peak SI values of peptide 15-induced PBMC proliferation and the day 5 absolute values of peptide 15-induced cytokine production detected in the healthy subjects.

ID	Positive PBMC proliferative and/or cytokine production response (Yes/No)							
	Peptide 8	**Peptide 9**	**Peptide 10**	**Peptide 11**	**Peptide 12**	**Peptide 13**	**Peptide 14**	**Peptide 15**
RA 5.11	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
RA 5.12	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
RA 5.13	No	No	No	No	No	No	No	No
RA 5.14	Yes	Yes	Yes	Yes	-	-	Yes	Yes
RA 5.15	-	Yes	-	No	-	No	No	No
RA 5.16	-	Yes	-	No	-	No	No	No
RA 5.17	-	Yes	-	Yes	-	-	Yes	Yes
RA 5.18	-	Yes	-	Yes	-	Yes	Yes	Yes
RA 5.19	-	Yes	-	Yes	-	Yes	No	Yes

Table 5.18. RA patient responders and non-responders; peptide panel #2. How each of the RA patients responded to the peptides. A dash (-) represents an untested value.

ID	Positive PBMC proliferative and/or cytokine production response (Yes/No)							
	Peptide 8	**Peptide 9**	**Peptide 10**	**Peptide 11**	**Peptide 12**	**Peptide 13**	**Peptide 14**	**Peptide 15**
HS 5.11	No	No	No	No	Yes	Yes	No	Yes
HS 5.12	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HS 5.13	Yes	No	No	Yes	Yes	Yes	Yes	No
HS 5.14	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HS 5.15	Yes	No	No	No	No	No	No	Yes
HS 5.16	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
HS 5.17	-	Yes	-	Yes	-	Yes	Yes	Yes
HS 5.18	-	Yes	-	No	-	Yes	Yes	No
HS 5.19	-	No	-	No	-	No	Yes	No

Table 5.19. Healthy subject responders and non-responders; peptide panel #2. How each of the healthy subjects responded to the peptides. A dash (-) represents an untested value.

RA patients			Healthy subjects			Disease controls		
ID	3HTdR incorporation SI value (peak)		ID	3HTdR incorporation SI value (peak)		ID	3HTdR incorporation SI value (peak)	
	REP-1	CEP-1		REP-1	CEP-1		REP-1	CEP-1
RA5.20	2.8	35.2	HS5.20	1.1	0.6	DC5.1	1.5	13.0
RA5.21	1.1	8.4	HS5.21	1.5	1.0	DC5.2	2.8	6.0
RA5.22	1.1	26.1	HS5.22	4.4	4.3	DC5.3	2.3	10.1
RA5.23	3.9	4.8	HS5.23	3.4	5.7	DC5.4	2.2	27.0
RA5.24	1.8	5.8	HS5.24	1.6	1.0	DC5.5	1.3	17.8
RA5.25	2.1	13.3	HS5.25	2.0	5.7	DC5.6	2.6	42.4
RA5.26	0.9	11.7	HS5.26	3.2	9.5	DC5.7	1.1	35.4
RA5.27	0.9	1.4	HS5.27	1.6	6.1	DC5.8	2.0	33.7
RA5.28	1.6	16.7	HS5.28	6.4	9.8	DC5.9	3.8	7.3
RA5.29	5.4	3.2				DC5.10	1.8	32.9
RA5.30	5.1	2.7				DC5.11	1.3	9.6
RA5.31	1.5	9.5						
RA5.32	1.8	11.0						
RA5.33	0.8	13.2						
RA5.34	0.8	3.2						
RA5.35	1.5	2.0						
RA5.36	3.1	5.8						
RA5.37	1.2	9.8						
RA5.38	-	4.6						
RA5.39	-	15.3						

Table 5.20. REP-1-induced and CEP-1-induced PBMC responses detected in RA patients, healthy subjects and disease controls. PBMC from 20 RA patients, 9 healthy subjects and 11 disease controls were cultured in triplicate with either medium alone, 5µg/ml PHA, 1µg/ml PPD, 10µg/ml REP-1 or 10µg/ml CEP-1. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. The table shows the peak SI values of both REP-1-induced and CEP-1-induced PBMC proliferation detected in the RA patients, healthy subjects and disease controls. A dash (-) represents an untested value.

A)

ID	'LPS-free' REP-1-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.40	1.5	0	0	0	0	0	0
RA 5.41	3.2	0	0	0	0	0	0
RA 5.42	1.5	0	9	0	756	50	0
RA 5.43	1.2	0	0	0	0	0	0
RA 5.44	1.4	0	0	0	0	0	0
RA 5.45	0.8	0	0	0	0	0	0
RA 5.46	1.2	0	0	0	277	0	0
RA 5.47	1.1	0	0	0	0	0	0

B)

ID	'LPS-free' CEP-1-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.40	1.3	0	0	0	0	0	0
RA 5.41	2.1	0	0	0	0	0	0
RA 5.42	1.2	0	0	0	24	3	0
RA 5.43	1.2	0	0	0	0	0	0
RA 5.44	1.2	0	0	0	0	0	0
RA 5.45	0.7	0	5	0	8	0	0
RA 5.46	1.2	23	29	7	403	3022	0
RA 5.47	1.0	0	0	0	22	0	0

Table 5.21. 'LPS-free' REP-1-induced and 'LPS-free' CEP-1-induced PBMC proliferation and cytokine production detected in RA patients. PBMC from 8 RA patients were cultured in triplicate with either medium alone, 5µg/ml PHA, 1µg/ml PPD, 10µg/ml LPS-free REP-1 or 10µg/ml LPS-free CEP-1. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of LPS-free REP-1-induced PBMC proliferation and the day 5 absolute values of LPS-free REP-1-induced cytokine production. **B**, the peak SI values of LPS-free CEP-1-induced PBMC proliferation and the day 5 absolute values of LPS-free CEP-1-induced cytokine production.

A)

ID	'LPS-free' REP-1-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.29	1.5	0	0	0	0	0	0
HS 5.30	2.9	0	16	0	52	0	0
HS 5.31	1.7	0	0	0	0	0	0
HS 5.32	1.2	0	0	0	13	0	0
HS 5.33	1.1	0	0	0	0	0	0
HS 5.34	1.3	0	0	0	0	0	0

B)

ID	'LPS-free' CEP-1-induced PBMC responses						
	3HTdR incorporation SI value (peak)	Cytokine production (day 5)					
		IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.29	1.2	0	0	0	0	0	0
HS 5.30	2.8	0	0	0	0	0	0
HS 5.31	1.5	0	0	0	9	3	0
HS 5.32	1.1	0	3	0	135	10	0
HS 5.33	1.2	0	0	0	0	0	0
HS 5.34	1.0	0	0	0	8	0	0

Table 5.22. 'LPS-free' REP-1-induced and 'LPS-free' CEP-1-induced PBMC proliferation and cytokine production detected in healthy subjects. PBMC from 6 healthy subjects were cultured in triplicate with either medium alone, 5µg/ml PHA, 1µg/ml PPD, 10µg/ml LPS-free REP-1 or 10µg/ml LPS-free CEP-1. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of LPS-free REP-1-induced PBMC proliferation and the day 5 absolute values of LPS-free REP-1-induced cytokine production. **B**, the peak SI values of LPS-free CEP-1-induced PBMC proliferation and the day 5 absolute values of LPS-free CEP-1-induced cytokine production.

ID	Background cytokine production in pg/ml (day 5)					
	IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
RA 5.1	0	0	0	198	4	0
RA 5.2	0	0	0	0	2	0
RA 5.3	0	0	0	675	6	0
RA 5.4	0	0	0	48	0	0
RA 5.5	0	0	0	342	32	0
RA 5.6	0	0	0	234	16	0
RA 5.7	8	47	15	186	76	0
RA 5.8	0	0	0	5896	89	0
RA 5.9	0	0	0	18	23	9
RA 5.10	0	0	0	27	8	0
RA 5.11	0	0	0	56	0	0
RA 5.12	0	0	0	34	12	0
RA 5.13	0	0	6	8954	103	12
RA 5.14	3	0	0	47	28	0
RA 5.15	0	0	0	23	6	0
RA 5.16	4	23	11	5	0	0
RA 5.17	0	0	0	56	34	0
RA 5.18	0	0	0	4524	57	0
RA 5.19	0	6	0	18	10	0
RA 5.40	0	0	0	2145	43	0
RA 5.41	3	0	0	9	15	0
RA 5.42	0	0	0	0	0	0
RA 5.43	0	0	0	41	8	0
RA 5.44	0	0	0	867	97	6
RA 5.45	0	0	0	54	36	0
RA 5.46	0	0	0	24	29	0
RA 5.47	0	0	0	38	17	0

Table 5.23. Background cytokine production detected in the RA patients tested for candidate RA autoantigen-derived peptide-induced cytokine production in the experiments described in chapter 5.

ID	Background cytokine production in pg/ml (day 5)					
	IL-10	IL-17	IL-4	IL-6	TNF-α	IFN-γ
HS 5.1	0	0	0	0	0	0
HS 5.2	0	0	0	29	12	0
HS 5.3	0	0	0	43	65	0
HS 5.4	0	0	0	214	29	0
HS 5.5	15	10	21	3245	56	18
HS 5.6	0	0	0	21	6	0
HS 5.7	0	0	8	153	17	0
HS 5.8	0	0	0	44	8	0
HS 5.9	0	0	0	387	32	0
HS 5.10	0	0	0	24	24	0
HS 5.11	0	0	0	27	52	0
HS 5.12	3	5	0	324	28	7
HS 5.13	0	0	0	0	0	0
HS 5.14	0	0	13	2879	43	0
HS 5.15	6	0	0	43	58	0
HS 5.16	0	0	0	62	87	0
HS 5.17	0	0	0	0	0	0
HS 5.18	0	0	0	27	9	0
HS 5.19	0	0	0	434	35	0
HS 5.29	9	6	32	5132	62	9
HS 5.30	0	0	0	76	94	0
HS 5.31	0	0	0	0	0	0
HS 5.32	0	0	0	32	84	0
HS 5.33	0	0	0	254	26	0
HS 5.34	0	0	0	120	10	0

Table 5.24. Background cytokine production detected in the healthy subjects tested for candidate RA autoantigen-derived peptide-induced cytokine production in the experiments described in chapter 5.

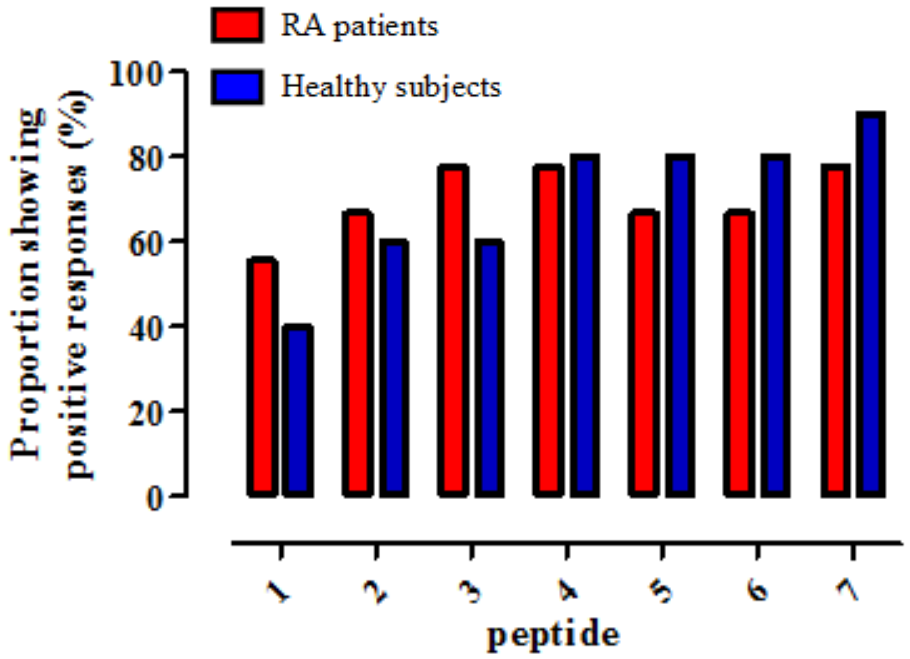

Figure 5.1. Proportions of positive responses to peptides 1-7 in RA patients versus healthy subjects. PBMC from 10 RA patients and 10 healthy subjects were cultured in triplicate with either medium alone, 5µg/ml PHA, 1µg/ml PPD or 10µg/ml each of peptides 1, 2, 3, 4, 5, 6 or 7. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. Graph shows the proportions of RA patients and healthy subjects showing positive PBMC proliferative and/or cytokine production responses to each of the 7 peptides comprising peptide panel #1.

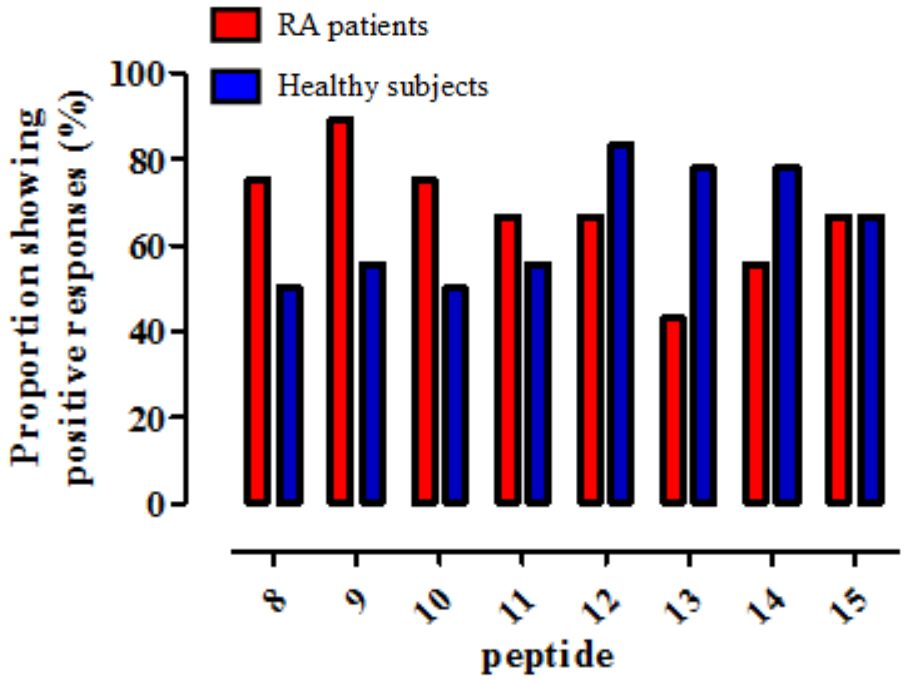

Figure 5.2. Proportions of positive responses to peptides 8-15 in RA patients versus healthy subjects. PBMC from 9 RA patients and 9 healthy subjects were cultured in triplicate with either medium alone, 5µg/ml PHA, 1µg/ml PPD or 10µg/ml each of peptides 8, 9, 10, 11, 12, 13, 14 or 15. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. Graph shows the proportions of RA patients and healthy subjects showing SI value-based positive PBMC proliferative and/or cytokine production responses to each of the 8 peptides comprising peptide panel #2.

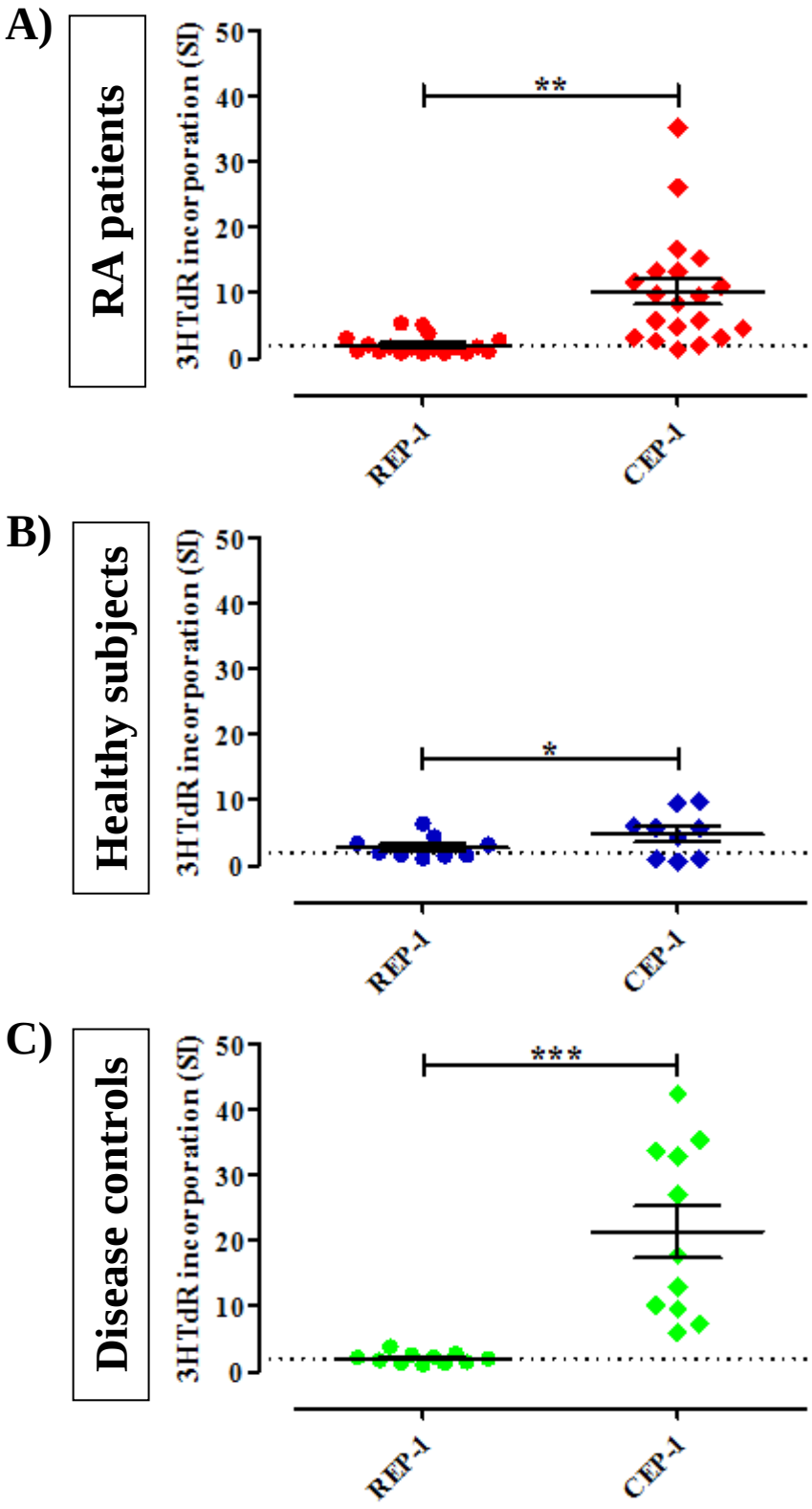

Figure 5.3. Comparing REP-1-induced PBMC proliferation with CEP-1-induced PBMC proliferation in RA patients, healthy subjects and disease controls. The peak SI values of REP-1-induced PBMC proliferation compared with those of CEP-1-induced PBMC proliferation in **A)** RA patients, **B)** healthy subjects and **C)** disease controls. In all graphs, the dotted horizontal line represents the threshold level for a positive response, while the bold horizontal line with error bars in each data set represents the mean value and SEM.

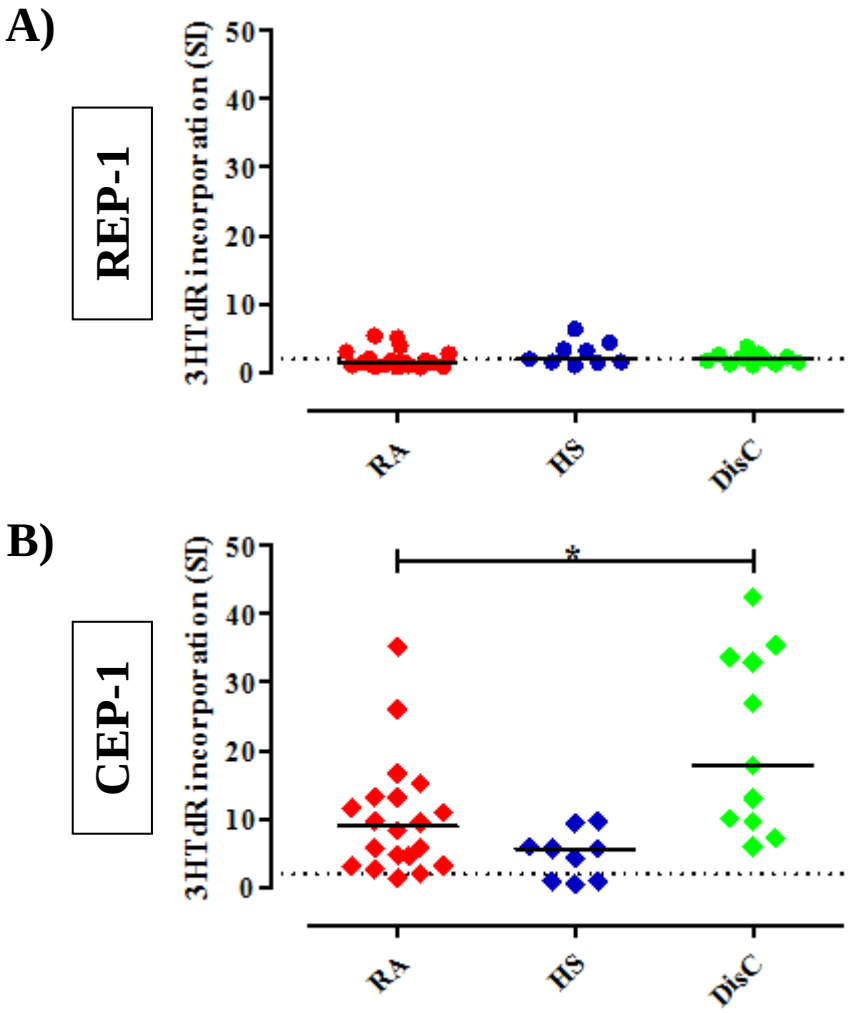

Figure 5.4. Comparing REP-1-induced PBMC proliferation and CEP-1-induced PBMC proliferation in RA patients (RA), healthy subjects (HS) and disease controls (DisC). A compares the peak SI values of REP-1-induced PBMC proliferation detected in each of the 3 subject groups. **B** compares the peak SI values of CEP-1-induced PBMC proliferation detected in each of the 3 subject groups. In each graph, the dotted horizontal line represents the threshold level for a positive response, while the bold horizontal line in each data set represents the median value.

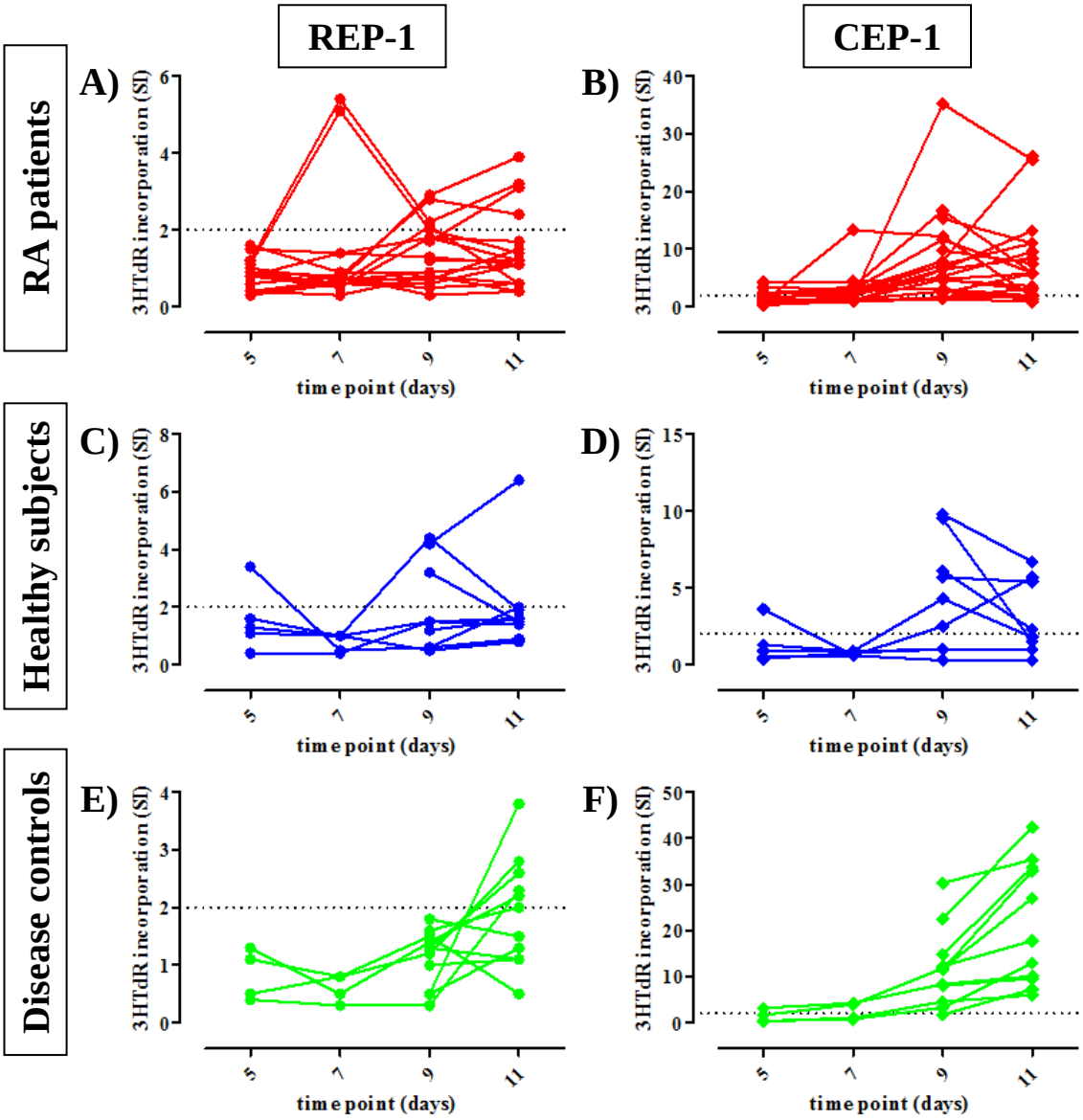

Figure 5.5. Kinetics of the REP-1-induced and CEP-1-induced proliferative responses. The kinetics of the REP-1-induced and CEP-1-induced PBMC proliferative responses detected in the RA patients are shown in **A** & **B** respectively. The kinetics of the REP-1-induced and CEP-1-induced PBMC proliferative responses detected in the healthy subjects are shown in **C** & **D** respectively. The kinetics of the REP-1-induced and CEP-1-induced PBMC proliferative responses detected in the disease controls are shown in **E** & **F** respectively. In each graph, the dotted horizontal line represents the threshold level for a positive response, and each data point represents an individual SI value.

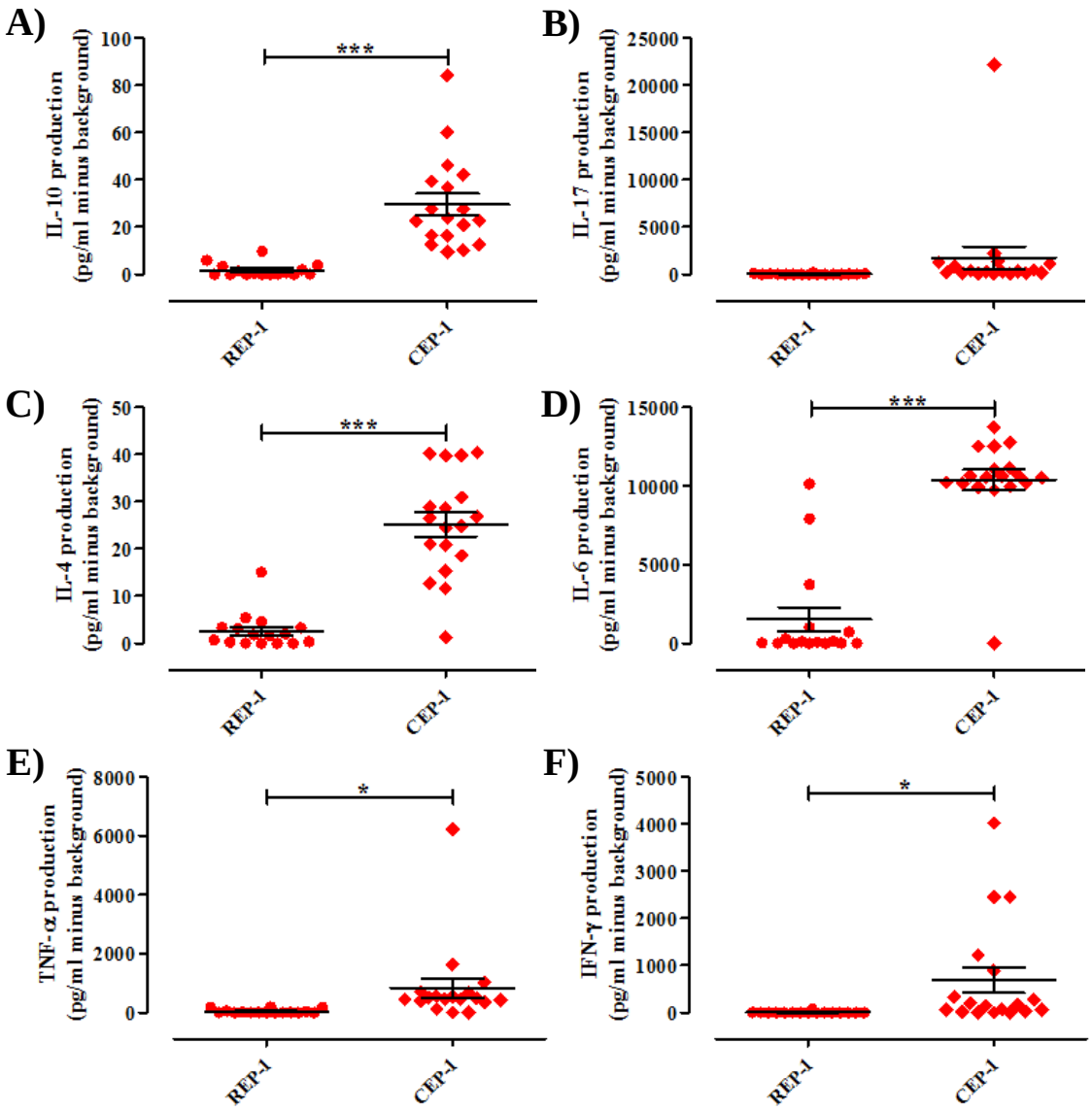

Figure 5.6. Comparing REP-1-induced cytokine production with CEP-1-induced cytokine production in RA patients. A, **B**, **C**, **D**, **E** & **F** show the day 5 absolute values of both REP-1-induced and CEP-1-induced IL-10, IL-17, IL-4, IL-6, TNF-α & IFN-γ production respectively that were detected within the RA patient group. In all graphs, the bold horizontal line with error bars in each data set represents the mean value and SEM.

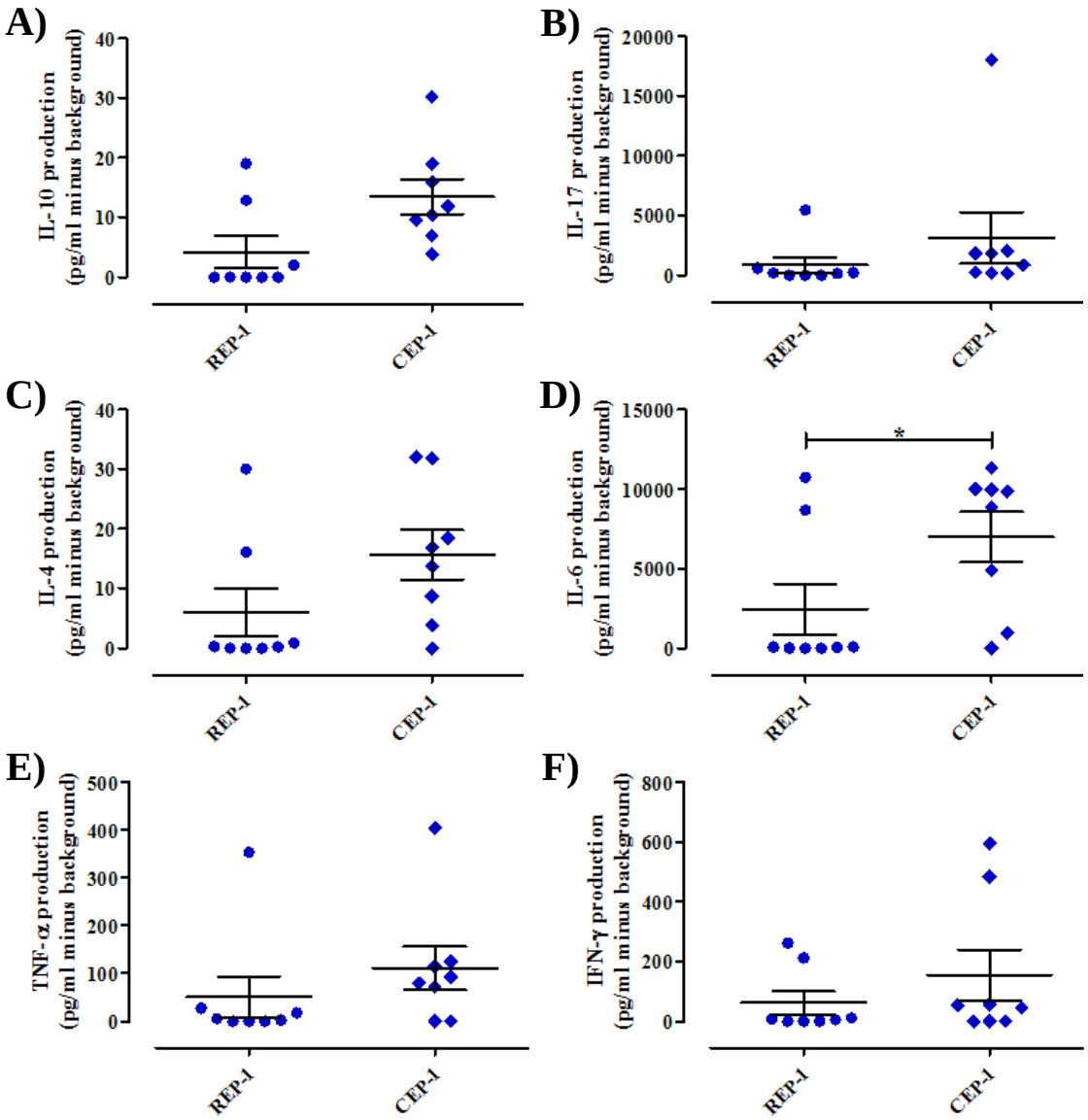

Figure 5.7. Comparing REP-1-induced cytokine production with CEP-1-induced cytokine production in healthy subjects. A, **B**, **C**, **D**, **E** & **F** show the day 5 absolute values of both REP-1-induced and CEP-1-induced IL-10, IL-17, IL-4, IL-6, TNF-α & IFN-γ production respectively that were detected within the healthy control group. In all graphs, the bold horizontal line with error bars in each data set represents the mean value and SEM.

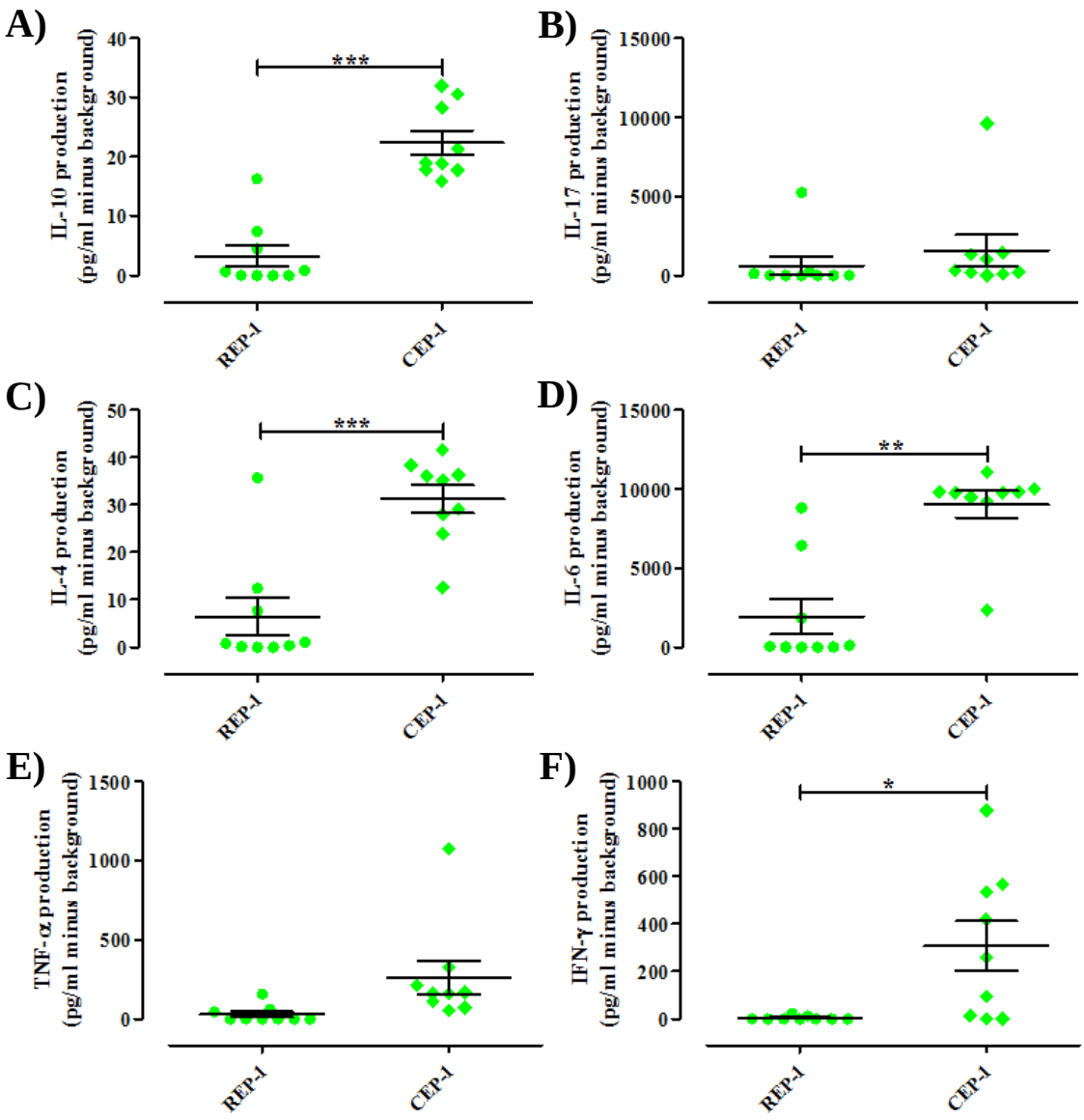

Figure 5.8. Comparing REP-1-induced cytokine production with CEP-1-induced cytokine production in disease controls. A, **B**, **C**, **D**, **E** & **F** show the day 5 absolute values of both REP-1-induced and CEP-1-induced IL-10, IL-17, IL-4, IL-6, TNF-α & IFN-γ production respectively that were detected within the disease control group. In all graphs, the bold horizontal line with error bars in each data set represents the mean value and SEM.

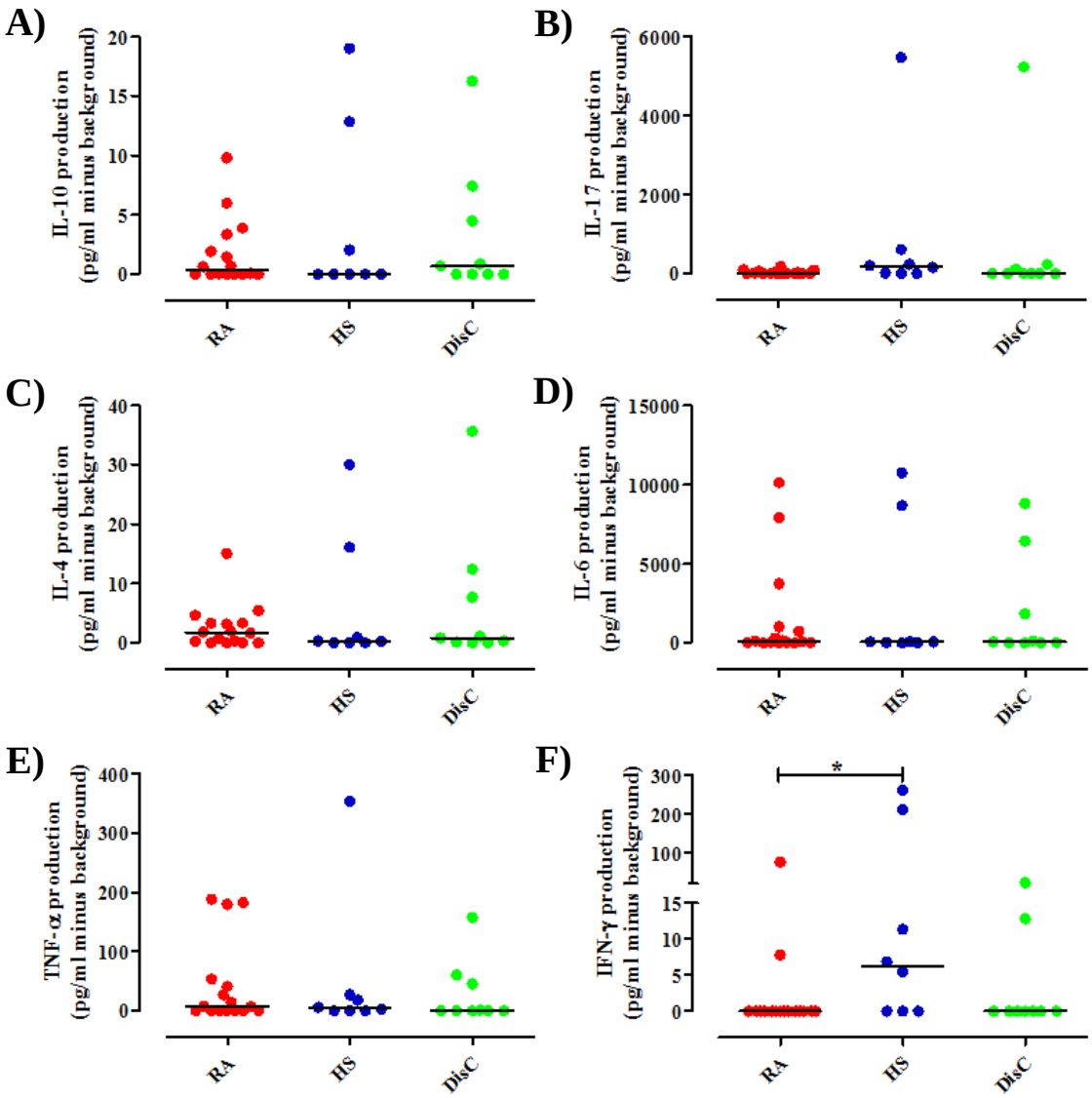

Figure 5.9. Comparing REP-1-induced cytokine production in RA patients (RA), healthy subjects (HS) and disease controls (DisC). A, **B**, **C**, **D**, **E** & **F** show the day 5 absolute values of REP-1-induced IL-10, IL-17, IL-4, IL-6, TNF-α & IFN-γ production respectively that were detected in each of the 3 subject groups. In all graphs, the bold horizontal line in each data set represents the median value.

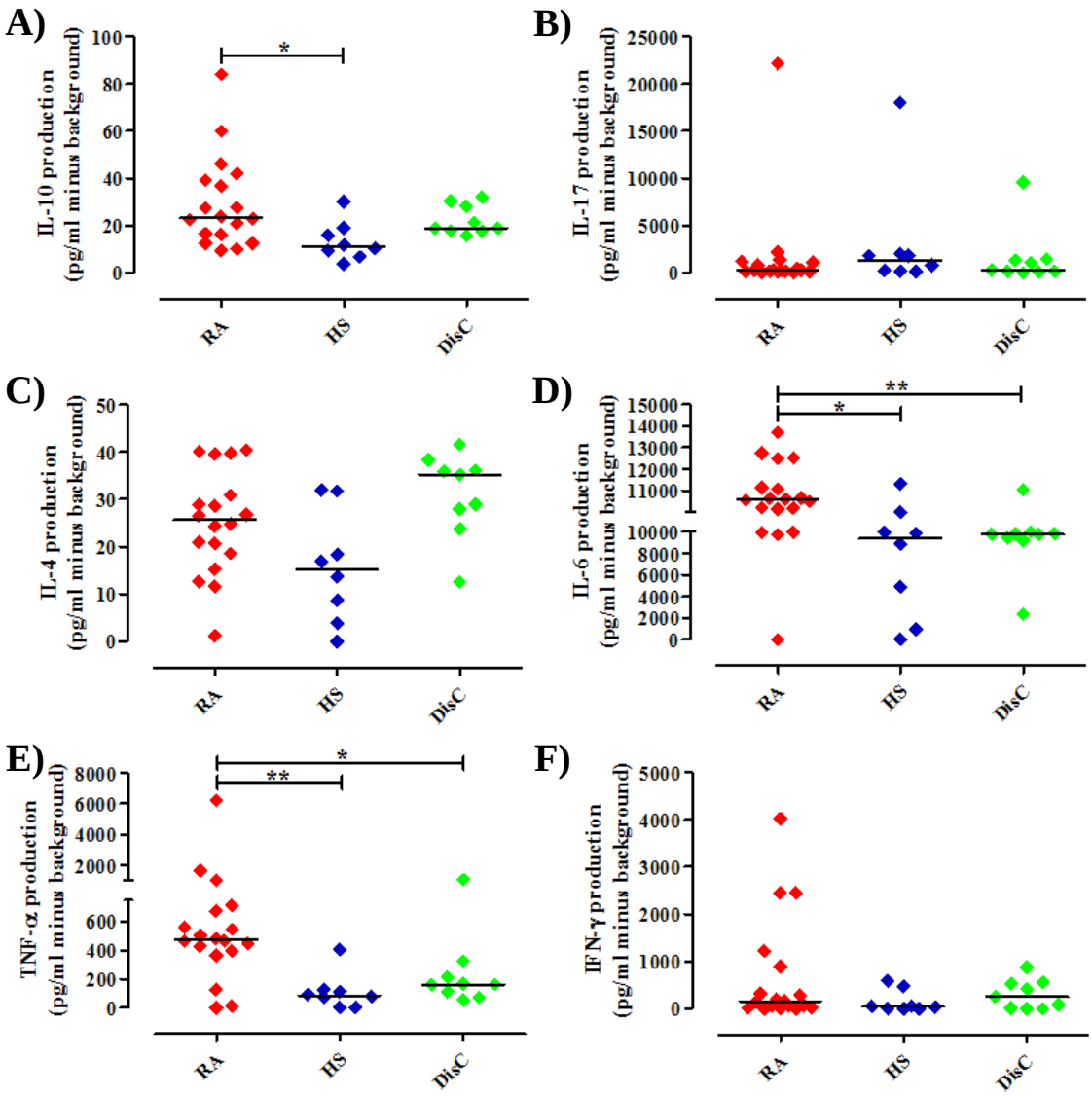

Figure 5.10. Comparing CEP-1-induced cytokine production in RA patients (RA), healthy subjects (HS) and disease controls (DisC). A, **B**, **C**, **D**, **E** & **F** show the day 5 absolute values of CEP-1-induced IL-10, IL-17, IL-4, IL-6, TNF-α & IFN-γ production respectively that were detected in each of the 3 subject groups. In all graphs, the bold horizontal line in each data set represents the median value.

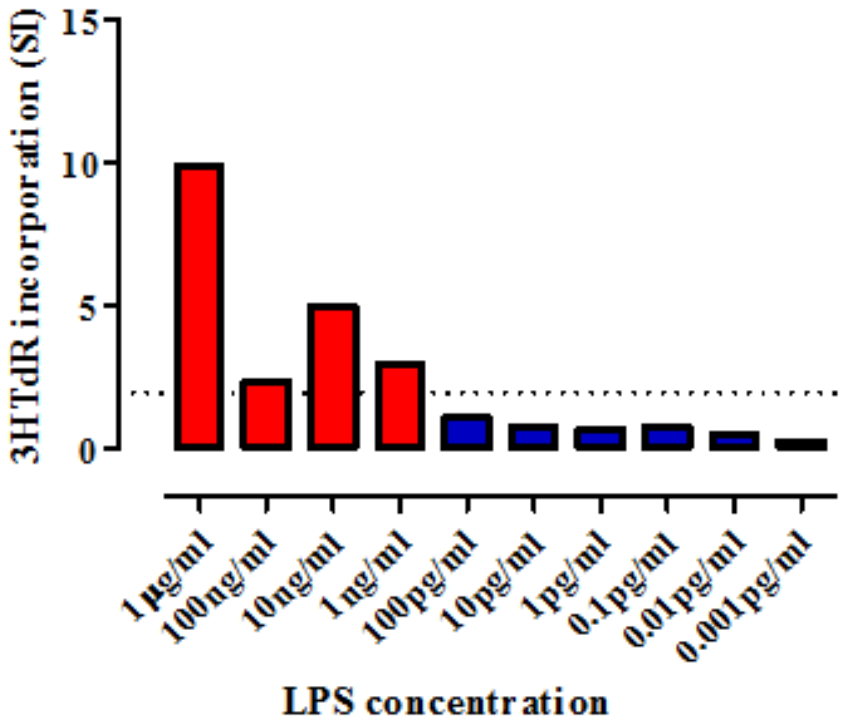

Figure 5.11. LPS-induced PBMC proliferation. PBMC from 3 healthy subjects were cultured in triplicate with either medium alone, 5µg/ml PHA, 1µg/ml PPD or a range of concentrations of LPS, and proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation. The graph shows representative data from a single subject showing the peak SI values of PBMC proliferation detected in response to the varying concentrations of LPS. The dotted horizontal line represents the threshold level for a positive response.

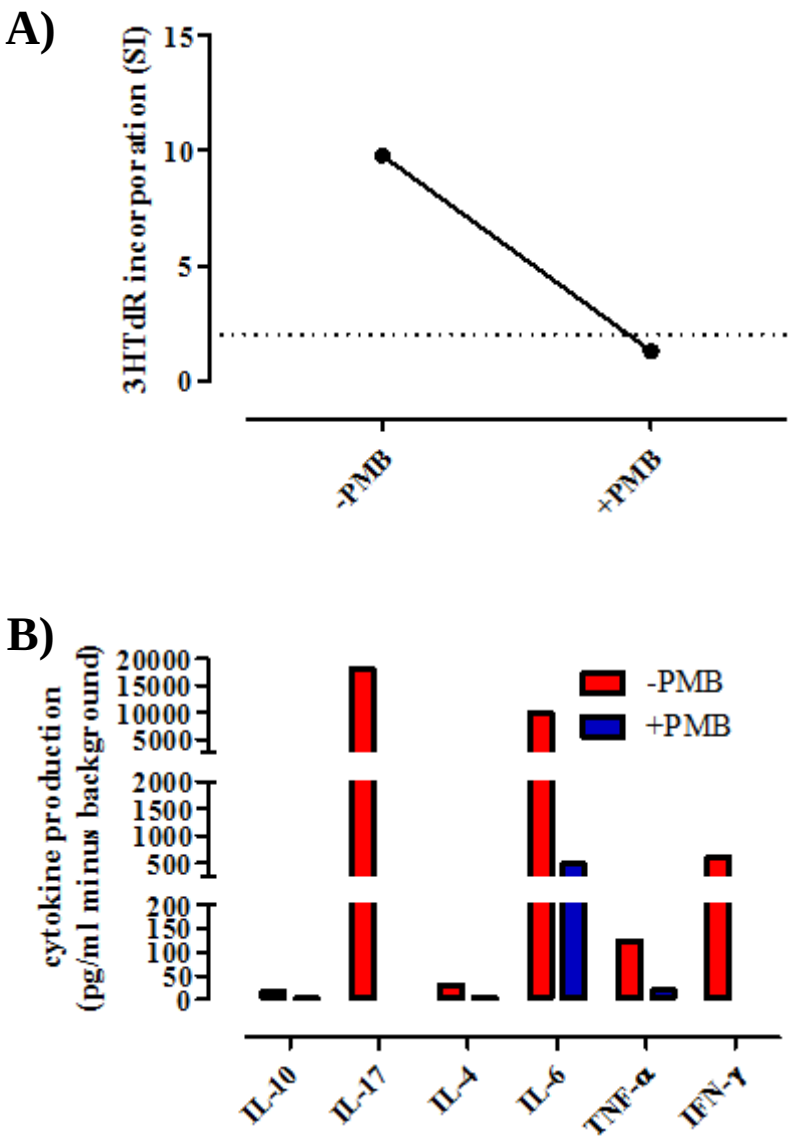

Figure 5.12. The effect of polymyxin B on the CEP-1 response. PBMC from a healthy subject were cultured in triplicate with either medium alone or 10µg/ml CEP-1, with or without the presence of PMB (+PMB or –PMB respectively). Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of CEP-1-induced PBMC proliferation detected with and without the presence of PMB. The dotted horizontal line represents the threshold level for a positive response. **B**, the day 5 absolute values of CEP-1-induced cytokine production detected with and without the presence of PMB.

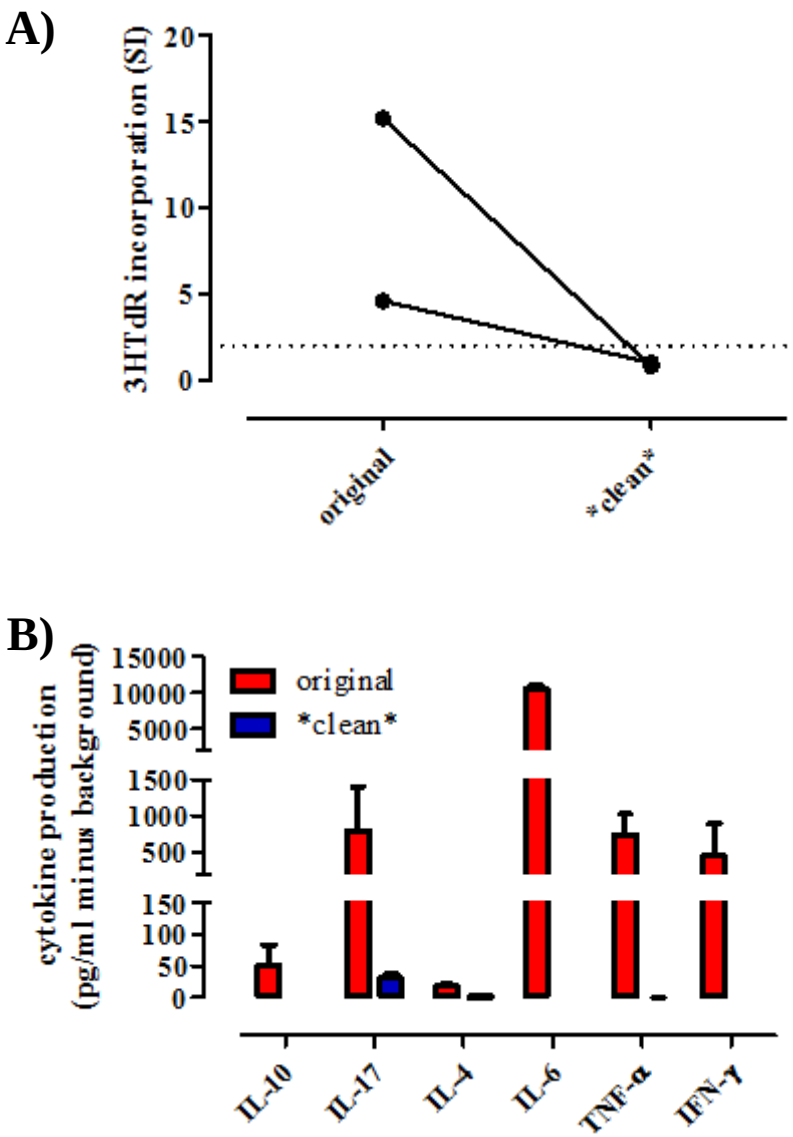

Figure 5.13. Comparing the immunogenicities of original CEP-1 and *clean* CEP-1. PBMC from 2 healthy subjects were cultured in triplicate with either medium alone, 10µg/ml original CEP-1 or 10µg/ml *clean* CEP-1. Proliferation was assessed after 3, 5, 7, 9 & 11 days via 3HTdR incorporation and cytokine levels in cell culture supernatant samples harvested at day 5 were assessed via ECL multiplex and ELISA. **A**, the peak SI values of PBMC proliferation detected in response to original CEP-1 and *clean* CEP-1 in each subject. The dotted horizontal line represents the threshold level for a positive response. **B**, the mean day 5 absolute values of cytokine production detected in response to original CEP-1 and *clean* CEP-1 (the vertical bars in each data set represent SEM).

Chapter 6. Discussion

6.1. Introduction

At the start of this study, I had three main aims, the first of which was to optimise an experimental system suitable for detecting candidate RA autoantigen-specific proliferative and cytokine production responses. In the experiments described in chapter 3, I showed that the use of 5% AS-supplemented culture medium enabled the most sensitive detection of antigen-specific proliferative and cytokine production T-cell responses. The use of 10% FBS-supplemented culture medium was associated with high levels of background proliferation and cytokine production that could interfere with detection of antigen-specific T-cell responses and to a lesser extent even mitogenic responses. 5% HABS-supplemented culture medium was not capable of supporting cells for the prolonged culture periods that are necessary for antigen-specific T-cell responses to be manifested. These experiments also demonstrated that using the 3HTdR incorporation assay to measure proliferation within populations of un-separated PBMC gives an accurate assessment of T-cell proliferation within the PBMC.

6.1.1. *Qualitative and quantitative comparisons of candidate RA autoantigen-specific T-cell responses in RA patients and healthy subjects*

My second aim was to compare, both in terms of quality and quantity, candidate RA autoantigen-specific T-cell responses in RA patients and healthy subjects in order to determine whether such responses are heightened in the former. My postulates were as follows:

i) Candidate RA autoantigen-specific T-cell responses are more prevalent in RA patients than in healthy subjects.
ii) Candidate RA autoantigen-specific T-cell responses of RA patients are qualitatively different from those of healthy subjects, the former being pro-inflammatory in nature and the latter being immunosuppressive.

In the experiments described in chapters 4 and 5, I measured proliferation and cytokine production in response to several whole protein and peptide candidate RA autoantigens in RA patient, healthy subject and disease control groups. The experiments described in chapter 4 showed that T-cell responses to the whole protein of the candidate RA autoantigens HCgp39, CII, GlyAgg and DeglyAgg were detected in similar proportions

of RA patients and healthy subjects. In addition, the magnitudes of the proliferative and cytokine production responses detected in response to the candidate RA autoantigens were comparable between both groups. Furthermore, the kinetics of the candidate autoantigen-induced proliferative responses were similar in both RA patients and healthy subjects, with peak proliferation being observed mostly at later time-points. Overall, both the quantity and the quality of T-cell responses against these whole autoantigen proteins were similar between RA patients and healthy subjects. The experiments described in chapter 5 showed that T-cell responses to peptides derived from the candidate RA autoantigens HCgp39, aggrecan, α-enolase, fibrinogen, filaggrin and vimentin were also detected in comparable proportions of RA patients as healthy subjects. As was observed in the experiments with the whole protein autoantigens, the magnitudes of the responses detected in the RA patients were similar to those detected in the healthy subjects and there was no difference in the quality and quantity of the T-cell responses against these peptides between RA patients and healthy subjects. Ultimately, with both whole protein and peptide forms of the candidate autoantigens, proliferative responses to these stimuli were generally very weak. Taken together, the weak magnitudes and delayed manifestations of the candidate RA autoantigen-induced proliferative responses are suggestive of primary responses of naïve T-cells (Berard & Tough, 2002 and Davies *et al*, 2002).

6.1.2. *Exploring the role of citrullinated candidate RA autoantigens*

My third aim was to explore whether the citrullination of candidate autoantigens enhances their immunogenicity in RA patients. My initial postulates regarding T-cell responses to citrullinated candidate RA autoantigens in RA patients and healthy subjects were as follows:

i) T-cell responses to citrullinated candidate RA autoantigens are more frequent than T-cell responses to uncitrullinated candidate RA autoantigens, but only in RA patients and not in healthy subjects.

ii) The T-cell responses elicited by citrullinated candidate RA autoantigens are qualitatively different from those elicited by their uncitrullinated counterparts, with the former being more pro-inflammatory in nature than the latter.

In the experiments described in chapter 5 I measured proliferative and cytokine production responses of PBMC from RA patients, healthy subjects and disease controls to the citrullinated and uncitrullinated analogues of a panel of candidate RA autoantigen-derived peptides. The experiments described in 5.2.1 and 5.2.2 showed that citrullinated candidate RA autoantigen-derived peptides were no more immunogenic than their uncitrullinated counterparts in either RA patients or healthy subjects. Both citrullinated and uncitrullinated versions of the same peptides induced similar proportions of positive responses in both groups. Furthermore, the magnitudes and cytokine profiles of the responses were also comparable between those induced by citrullinated and uncitrullinated peptide analogues in both groups. This differs from what was shown in studies by von Delwig *et al* (2010) and more recently Law *et al* (2012), where phenotypically distinct, citrulline-specific responses were observed in RA patients but not healthy subjects. The former study showed that while the native form of aggrecan$_{84\text{-}103}$ failed to induce proliferation in both RA patients and healthy subjects, N citrullinated aggrecan$_{84\text{-}103}$ induced PBMC proliferation detectable in ~60% of the RA patients but none of the healthy subjects. In addition, in the RA patients but not the healthy subjects, the magnitudes of the proliferative responses induced by the citrullinated peptide were significantly greater than those induced by the native peptide. Furthermore, the citrulline-specific response observed in the RA patients was accompanied by IL-17 production and an induction of IL-17^{+} CD4^{+} T-cells (von Delwig *et al*, 2010). While the very low SI values of PBMC proliferation in response to both native and citrullinated forms of several candidate RA autoantigen-derived peptides in both RA patients and healthy subjects that were reported by Law *et al* may support my observations described in 5.2.1 and 5.2.3, the group showed that in RA patients but not healthy subjects, the magnitudes of the proliferative responses induced by a citrullinated aggrecan peptide were significantly greater than those induced by its native counterpart. In addition, the group showed that the citrullinated form of the aggrecan peptide induced significantly greater levels of IL-10 and TNF-α in the RA patients than in the healthy subjects, while a similar trend for IL-17 production in response to the citrullinated peptides of aggrecan and fibrinogen was also observed (Law *et al*, 2012).

The results from the experiments described in 5.2.3 however were more consistent with my hypotheses. Firstly, the citrullinated form of the α-enolase$_{5\text{-}21}$ peptide with terminal cysteines (CEP-1) elicited a significantly greater proportion of positive proliferative responses than its uncitrullinated counterpart (REP-1) in the RA patient group but not in

either of the control groups. Secondly, in the RA patient group, the levels of proliferation and of IL-17, IL-4, IL-6, TNF-α and IFN-γ production detected in response to the citrullinated peptide were significantly greater than those detected in response to the uncitrullinated peptide, and while a similar observation was made in the disease control group, this was not the case in the healthy subject group. In addition, the levels of production of the pro-inflammatory cytokines IL-6 and TNF-α detected in response to the citrullinated form of the peptide were significantly greater in the RA patient group than in either of the control groups. Furthermore, the levels of production of the immunosuppressive cytokine IL-10 detected in response to the citrullinated form of the peptide were significantly greater than in the healthy subject group. Ultimately however, the experiments described in 5.2.4 suggested that the responses to the citrullinated form of the enolase peptide were potentially being influenced by contaminating LPS within the CEP-1 preparations. Indeed the experiments with LPS-free preparations of the peptide described in 5.2.5 showed that the citrullinated form of the peptide induced similar proportions of T-cell proliferation and cytokine production responses as its uncitrullinated counterpart in both the RA patient group and the healthy subject group and the T-cell responses against these peptides were similar in the RA patient and healthy control groups.

However, it is debatable whether the responses to the initial, LPS-contaminated batch of CEP-1 are simply indirect, bystander responses to LPS, as there were substantial differences in the T-cell responses to 'LPS-contaminated CEP-1' from the three study groups. As mentioned in the discussion section of chapter 5, it is possible that the macrophages of RA patients, because of increased expression of TLR4 compared to those of controls (Huang *et al*, 2007), are more sensitive to LPS than control macrophages. Thus LPS may induce secretion of greater levels of cytokines and expression of greater numbers of T-cell co-stimulatory molecules in RA patients than in controls which may account for the increased cytokine profiles and proliferative responses of the former. Alternatively, the LPS could simply have been acting as an adjuvant, amplifying/enhancing responses to the CEP-1 by inducing the expression of co-stimulatory molecules and cytokines by antigen-presenting cells so that they were capable of activating T-cells recognising MHC class II:CEP-1 peptide complexes that the former expressed on their surfaces. LPS contamination may potentially explain some of the discrepant data on autoantigen-specific T-cell responses in RA reported in the literature. The underlying mechanisms of LPS-'induced' amplification of the T-cell

responses against autoantigens in my experimental system require further investigation. Furthermore, whether LPS contamination also amplifies T-cell responses against non-self antigens is an interesting question to be pursued in future experiments.

6.2. Limitations of the study

I initially set out hoping only to recruit early RA patients with no prior history of immunomodulatory therapy, anticipating that autoantigen-specific T-cell responses, if present, would be more readily detected in such patients. However, as the tables of clinical details show (Tables 2.1-2.6 in chapter 2), the majority of the RA patients tested in this study have long-standing RA and/or a history of immunomodulatory therapy. Indeed, two thirds of the RA patients had been diagnosed with RA for at least 2 years at the time of recruitment, many of these had much longer disease duration, and all of these patients were receiving immunomodulatory therapy. Furthermore, of those RA patients only recently diagnosed at the time of recruitment (i.e. within 2 years of the time of recruitment), only 16% were not receiving immunomodulatory therapy. As previously mentioned, it is possible that measuring autoantigen-specific T-cell responses in RA patients with established disease and/or a history of immunomodulatory therapy may bias against the detection of such responses. In addition, information on whether the RA patients tested were using glucocorticoids was unavailable and it is possible that the use of corticosteroids may influence T-cell responses *in vitro*.

It is possible that the AS used as a culture medium supplement in the experiments described in this thesis may have contained significant levels of certain cytokines which could have influenced the data obtained from cytokine production assays performed on candidate autoantigen-stimulated PBMC cultured in AS-supplemented medium. While I measured cytokine production in cultures of un-stimulated PBMC that were cultured in AS-supplemented medium, I did not determine whether the AS itself contained substantial levels of any of the cytokines that I assayed for.

In the experiments described in chapter 3, I did not measure the sensitivities of the 3HTdR incorporation and CFSE dilution assays, and thus the frequencies of antigen-specific T-cells that may have been detectable with each of the assays were not determined. Although this would have been a difficult task, it could perhaps have been

achieved by spiking T-cell populations with differing proportions of T-cells from a clone of a known specificity.

Whenever possible I tested subjects with more than one candidate autoantigen but sometimes this was not feasible because of the large numbers of PBMC required and the numbers of PBMC obtained from some of the clinical samples were low. As a result, 27 of the RA patients studied were only screened for T-cell responses to a single autoantigen. Since it is possible that different autoantigens are involved in disease pathogenesis in different patients or at different stages of the disease, then if patients were tested with only a single candidate autoantigen, this may also have biased against the successful detection of autoantigen-specific T-cell responses in these patients.

While cell culture supernatant samples had been collected from the bulk culture experiments described in 4.2.2, these supernatants were not analysed for cytokine production therefore I only have proliferation data from these experiments. As mentioned in chapter 4, I only measured cytokine production in supernatant samples from the triplicate culture experiments as proliferative responses detected in these experiments were generally more frequent and more potent when using this approach as opposed to the bulk culture approach. It is possible that cytokine data from the bulk culture experiments may be more informative, although there is no obvious theoretical argument to suggest that this will be the case.

In the vast majority of cases, I did not see statistical differences between the autoantigen-specific T-cell responses of the RA groups and those of the control groups. While it is possible that the qualities and quantities of autoantigen-specific T-cell responses do not differ between RA patients and healthy subjects *in vivo*, it is also possible that I did not observe any significant differences between the different groups due to small sample size. With the levels of intra-group variability, it is possible that using a larger sample size may have revealed statistically significant differences in autoantigen-specific T-cell responses between RA patients and healthy subjects. However, if that is the case, it would suggest that such differences in T-cell responses are likely to be small and the clinical significance of such small differences is uncertain. Furthermore, to repeat the experiments with a substantially bigger sample size is beyond the scope of my PhD study due to time and financial constraints.

Another potential limitation is that the HLA genotypes of the subjects used in this study were not known. While SE-positive HLA-DR molecules are prevalent amongst seropositive RA patients, it is possible that some of the patients tested in this study were SE-negative. Furthermore, the proportion of healthy controls that carry the SE genotype is unclear but it is expected to be lower than in RA groups. Several of the peptides tested in the experiments described in chapter 5 were selected because previous studies had shown them to bind certain RA-associated HLA-DR molecules with high affinity. For example, the citrullinated form of the 65-77 peptide of vimentin was reported by Hill *et al* (2003) to bind only the SE-positive HLA-DR molecules DR1(DRB1*0101), DR4(DRB1*0401) and DR4(DRB1*0404), while the 201-213 and 299-311 peptides of aggrecan were shown to bind well to DR4(DRB1*0401) molecules but not to DR4(DRB1*0404) molecules (Boots *et al*, 1997). Therefore it could have been the case that these peptides were not being presented *in vitro* because the antigen-presenting cells lacked the correct subtype(s) of HLA-DR molecules. This could explain the lack of T-cell responses against these peptides.

For the experiments described in 5.2.5 in which subjects were tested for responses to LPS-free preparations of REP-1 and CEP-1, new groups of RA patients and healthy subjects were recruited who were not tested for reactivity to the original preparations of the peptides. Therefore this was not an optimal comparison of these two peptide preparations.

Because my experimental system involved measuring autoantigen-induced proliferation in cultures of whole PBMC, it is important to consider the effect that the autologous mixed lymphocyte reaction (AMLR) may have had on the interpretation of these proliferative responses. The AMLR is a phenomenon observed *in vitro* in which T-cells, predominantly of the $CD4^+$ subset, proliferate during culture with autologous non-T-cells as a result of TCAR stimulation by MHC-encoded molecules expressed on the surfaces of the latter (Smolen *et al*, 1981). The AMLR has been shown to demonstrate both immunological memory and specificity (Weksler & Kozak, 1977), and is thought to reflect the *in vivo* interactions of T-cells and non-T-cells that may be involved in the regulation of immune responses (Scheinecker *et al*, 1998). Indeed, abnormal AMLRs have been reported in RA and other autoimmune diseases (Pope *et al*, 1984; Miyasaka *et al*, 1980 and Takada *et al*, 1985). When measuring proliferation in unstimulated cultures of PBMC, I was effectively measuring the AMLR, and these cpm readings

were used to calculate the SI values of proliferation observed in autoantigen-stimulated PBMC cultures. Because the autoantigen-induced proliferative responses detected in this study were generally very weak, they would therefore have been more susceptible to influence by the AMLR.

6.3. Strengths of the study

Despite the aforementioned, the study has a number of strengths. For example, multiple candidate RA autoantigens in both whole protein and peptide forms were tested, and I measured both proliferation and the production of a range of selected cytokines (six in total) in response to these stimuli. In the majority of cases, proliferation was measured in conjunction with cytokine production. In addition, a variety of different approaches (un-separated PBMC in both bulk and triplicate cultures and mo-DC:T-cell co-cultures) and different assays (both CFSE dilution and 3HTdR incorporation assays to measure proliferation) were studied. Furthermore, large numbers of subjects within both RA patient and control groups were studied, and in many cases each subject was tested with several different autoantigens.

In the experiments described in chapter 4, I measured T-cell responses to whole protein candidate RA autoantigens; HCgp39, CII, GlyAgg and DeglyAgg. Table 6.1 summarises the approaches and assays used and the numbers of subjects tested with each of the four autoantigens in the experiments described in the various sections of chapter 4. In the experiments described in 4.2.3, substantial proportions of subjects from both the RA patient group (50%) and the healthy subject group (69%) were tested with two or more of the autoantigens. Table 6.2 shows the numbers of subjects tested with various combinations of the four autoantigens. Overall, in the experiments described in chapter 4, a total of fifty RA patients and twenty seven healthy subjects were tested with various candidate RA autoantigens.

In the experiments described in chapter 5, I measured T-cell responses to candidate autoantigen-derived peptides, and tested both non-citrullinated and citrullinated peptides of HCgp39, aggrecan, vimentin, fibrinogen, filaggrin and enolase. All experiments utilised triplicate culture approach. Table 6.3 shows the numbers of subjects tested with the various candidate autoantigen-derived peptides in the experiments described in the various sections of chapter 5. Overall, in the experiments described in chapter 5, a total of forty seven RA patients, thirty four healthy subjects and eleven disease controls were tested with various candidate RA autoantigen-derived peptides.

Thus, in the entire study, I tested a total of ninety seven RA patients, sixty one healthy subjects and eleven disease controls for candidate RA autoantigen-specific T-cell responses. Considering the range of candidate RA autoantigens tested and assays/readouts used to detect the T-cell responses, I believe that this body of work has made a substantial contribution to the existing literature on the role of candidate RA autoantigen-specific T-cell responses in RA.

6.4. Future work

- To obtain further samples from the subjects studied in order to determine their HLA-DR genotypes.
- Instead of assessing T-cell responses to individual candidate autoantigens, it may be more efficient to screen for the presence of autoantigen-specific T-cell responses using pooled autoantigen approaches. Alternatively, addition of an adjuvant (such as cytokines or co-stimulation) in the cell culture may enable the detection of autoantigen-specific T-cell responses that are otherwise undetectable when using the experimental approaches described in this study.
- To dissect the underlying mechanisms of LPS-induced amplification of autoantigen-stimulated T-cell responses.
- T-cells from the inflamed joints or draining lymph nodes could also be used instead of peripheral blood T-cells, as auto-reactive T-cells involved in RA pathogenesis may be selectively recruited to the disease sites and therefore not detected in the peripheral blood. However, obtaining sufficient numbers of T-cells from synovial tissues or draining lymph nodes can be challenging, especially with the improved medical treatments available for RA patients.
- Multimeric, MHC class II:autoantigen-derived peptide 'tetramers/multimers' could be used to identify the presence of autoantigen-specific $CD4^+$ T-cells, as has been previously described (Kotzin *et al*, 2000; Huang *et al*, 2004; Svendsen *et al*, 2004; Davis *et al*, 2011 and Massilamany *et al*, 2011). However, different MHC class II:autoantigen-derived peptide tetramers are needed for RA patients with different HLA-DR genotypes and also for different peptides being studied. Therefore, such an approach is expensive and unless there are dominant autoantigenic peptides involved, its usefulness in studying autoantigen-specific T-cell responses is likely to be limited.

6.5. Conclusions

In conclusion, the whole protein candidate RA autoantigens or their peptide derivatives (with or without citrullination) elicited similar T-cell responses in RA patients, healthy subjects and disease controls. Furthermore, I found no evidence of an RA-specific increase in T-cell responses against citrullinated autoantigens. Finally, LPS contamination, even at low concentrations, could amplify autoantigen-specific T-cell responses. Therefore, until the underlying mechanisms of how LPS augments autoantigen-specific T-cell responses are clarified, preparations of antigenic stimuli for use in assessing T-cell responses should be checked for potential LPS contamination. Future experiments to assess autoantigen-specific T-cell responses should focus on further optimisation of experimental protocol.

	Total number of subjects tested	Culture approach	Assay(s) used	Autoantigens (numbers of subjects tested)			
				HCgp39	CII	GlyAgg	DeglyAgg
4.2.1	15xRA	Bulk culture	CFSE dilution proliferation assay	15xRA	-	-	-
4.2.2	9xRA 8xHS	Bulk culture	3HTdR incorporation proliferation assay	9xRA 8xHS		4xRA 4xHS	-
4.2.3	24xRA 16xHS	Triplicate culture	3HTdR incorporation proliferation assay	10xRA 8xHS	7xRA 8xHS	9xRA 10xHS	12xRA 13xHS
			ECL multiplex & ELISA cytokine production assays	6xRA 6xHS	4xRA 6xHS	5xRA 8xHS	8xRA 8xHS
4.2.4	2xRA 3xHS	T-cell & mo-DC co-culture	3HTdR incorporation proliferation assay	2xRA 3xHS			

Table 6.1. Numbers of subjects tested with each of the four whole protein candidate autoantigens and the approaches and assays used in the experiments described in the various sections of Chapter 4. A dash (-) signifies untested.

Combinations of 2 or more autoantigens tested in 4.2.3	**Number of RA patients tested (out of 24)**	**Number of healthy subjects tested (out of 16)**
HCgp39 + CII	4	1
HCgp39 + GlyAgg	2	-
HCgp39 + DeglyAgg	-	-
CII + GlyAgg	-	-
CII + DeglyAgg	-	-
GlyAgg + DeglyAgg	4	3
HCgp39 + CII + GlyAgg	-	-
HCgp39 + CII + DeglyAgg	1	2
HCgp39 + GlyAgg + DeglyAgg	-	-
CII + GlyAgg + DeglyAgg	1	-
HCgp39 + CII + GlyAgg + DeglyAgg	-	5
Proportion & percentage of total subjects tested in 4.2.3 that were tested with 2 or more autoantigens	12 out of 24 = 50%	11 out of 16 = 69%

Table 6.2. Numbers of subjects tested with the various combinations of 2 or more of the four whole protein candidate autoantigens in the experiments described in 4.2.3. A dash (-) signifies untested.

	Total number of subjects tested	Assay(s) used	Autoantigens (numbers of subjects tested)					
			Peptide panel #1	Peptide panel #2	REP-1	CEP-1	LPS-free REP-1	LPS-free CEP-1
5.2.1	10xRA 10xHS	3HTdR incorporation assay	10xRA 10xHS	-	-	-	-	-
		ECL multiplex & ELISA cytokine production assays		-	-	-	-	-
5.2.2	9xRA 9xHS	3HTdR incorporation assay	-	9xRA 9xHS	-	-	-	-
		ECL multiplex & ELISA cytokine production assays	-	8xRA 9xHS	-	-	-	-
5.2.3	20xRA 9xHS 11x DisC	3HTdR incorporation assay	-	-	18xRA 9xHS 11x DisC	20xRA 9xHS 11x DisC	-	-
		ECL multiplex & ELISA cytokine production assays	-	-			-	-
5.2.5	8xRA 6xHS	3HTdR incorporation assay	-	-	-	-	8xRA 6xHS	
		ECL multiplex & ELISA cytokine production assays	-	-	-	-		

Table 6.3. Numbers of subjects tested with each of the various candidate autoantigen-derived peptides in the experiments described in the various sections of Chapter 5. A dash (-) signifies untested.

References

Adorini, L. & Nagy, Z.A. 1990. 'Peptide competition for antigen presentation'. *Immunology Today*, Vol. 11, No. 1, pp. 21-24.

Aletaha, D. *et al*. 2010. '2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative'. *Arthritis & Rheumatism*, Vol. 62, No. 9, pp. 2569-2581.

Annunziato, F. *et al*. 2002. 'Phenotype, localisation, and mechanism of suppression of $CD4^+$ $CD25^+$ human thymocytes'. *The Journal of Experimental Medicine*, Vol. 196, No. 3, pp. 379-387.

Asano, M., Toda, M., Sakaguchi, N. & Sakaguchi, S. 1996. 'Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation'. *The Journal of Experimental Medicine*, Vol. 184, No. 2, pp. 387-396.

Asquith, D.L., Miller, A.M., McInnes, I.B. & Liew, F.Y. 2009. 'Animal models of rheumatoid arthritis'. *European Journal of Immunology*, Vol. 39, No. 8, pp. 2040-2044.

Auger, I. *et al*. 2005. 'Influence of HLA-DR genes on the production of rheumatoid arthritis-specific autoantibodies to citrullinated fibrinogen'. *Arthritis & Rheumatism*, Vol. 52, No. 11, pp. 3424-3432.

Baecher-Allan, C., Brown, J.A., Freeman, G.J. & Hafler, D.A. 2001. '$CD4^+$ $CD25^{high}$ regulatory cells in human peripheral blood'. *The Journal of Immunology*, Vol. 167, No. 3, pp. 1245-1253.

Banerjee, S., Webber, C. & Poole, A.R. 1992. 'The induction of arthritis in mice by the cartilage proteoglycan aggrecan: roles of $CD4^+$ and $CD8^+$ T cells'. *Cellular Immunology*, Vol. 144, No. 2, pp. 347-357.

Begovich, A.B. *et al*. 2004. 'A Missense Single-Nucleotide Polymorphism in a Gene Encoding a Protein Tyrosine Phosphatase (*PTPN22*) Is Associated with Rheumatoid Arthritis'. *The American Journal of Human Genetics*, Vol. 75, No. 2, pp. 330-337.

Berard, M. & Tough, D.F. 2002. 'Qualitative differences between naïve and memory T-cells'. *Immunology*, Vol. 106, No. 1, pp. 127-138.

Bläss, S. *et al*. 1995. 'Novel 68 kDa autoantigen detected by rheumatoid arthritis specific antibodies'. *Annals of the Rheumatic Diseases*, Vol. 54, No. 5, pp. 355-360.

Bläss, S. *et al*. 2001. 'The stress protein BiP is overexpressed and is a major B and T cell target in rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 44, No. 4, pp. 761-771.

Bodman-Smith, M.D. *et al*. 2004. 'Antibody response to the human stress protein BiP in rheumatoid arthritis'. *Rheumatology*, Vol. 43, No. 10, pp. 1283-1287.

Boots, A.M.H. *et al*. 1997. 'Selection of self-reactive peptides within human aggrecan by use of a HLA-DRB1*0401 peptide binding motif'. *Journal of Autoimmunity*, Vol. 10, No. 6, pp. 569-578.

Bottini, N. *et al*. 2004. 'A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes'. *Nature Genetics*, Vol. 36, pp. 337-338.

Bowes, J. & Barton, A. 2008. 'Recent advances in the genetics of RA susceptibility'. *Rheumatology*, Vol. 47, No. 4, pp. 399-402.

Boyle, W.J., Simonet, W.S. & Lacey, D.L. 2003. 'Osteoclast differentiation and activation'. *Nature*, Vol. 423, pp. 337-342.

Bugatti, S., Codullo, V., Caporali, R. & Montecucco, C. 2007. 'B cells in rheumatoid arthritis'. *Autoimmunity Reviews*, Vol. 7, No. 2, pp. 137-142.

Cambridge, G. *et al*. 2003. 'Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 48, No. 8, pp. 2146-2154.

Cao, D., Malmström, V., Baecher-Allan, C., Hafler, D., Klareskog, L. & Trollmo, C. 2003. 'Isolation and functional characterisation of regulatory $CD25^{bright}$ $CD4^{+}$ T cells from the target organ of patients with rheumatoid arthritis'. *European Journal of Immunology*, Vol. 33, No. 1, pp. 215-223.

Cardoso, L.S. *et al*. 2007. 'Polymyxin B as inhibitor of LPS contamination of *Schistosoma mansoni* recombinant proteins in human cytokine analysis'. *Microbial Cell Factories*, Vol. 6, No. 1.

Chabaud, M. *et al*. 2001. 'IL-17 derived from juxta-articular bone and synovium contributes to joint degradation in rheumatoid arthritis'. *Arthritis Research*, Vol. 3, No. 3, pp. 168-177.

Chu, C.Q., Field, M., Feldmann, M. & Maini, R.N. 1991. 'Localisation of Tumour Necrosis Factor α in Synovial Tissues and at the Cartilage-Pannus Junction in Patients with Rheumatoid Arthritis'. *Arthritis & Rheumatism*, Vol. 34, No. 9, pp. 1125-1132.

Chu, C.Q., Field, M., Allard, S., Abney, E., Feldmann, M. & Maini, R.N. 1992. 'Detection Of Cytokines At The Cartilage/Pannus Junction In Patients With Rheumatoid Arthritis: Implications For The Role Of Cytokines In Cartilage Destruction And Repair'. *Rheumatology*, Vol. 31, No. 10, pp. 653-661.

Cohen, S.B. *et al*. 2006. 'Rituximab for rheumatoid arthritis refractory to anti-tumour necrosis factor therapy: Results of a multi-centre, randomised, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks'. *Arthritis & Rheumatism*, Vol. 54, No. 9, pp. 2793-2806.

Colin, E.M. *et al*. 2010. '1,25-dihydroxyvitamin D_3 modulates Th17 polarisation and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 62, No.1, pp. 132-142.

Cook, A.D. *et al*. 1996. 'Antibodies to type II collagen in early rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 39, No. 10, pp. 1720-1727.

Cope, A.P. *et al*. 1999. 'T cell responses to a human cartilage autoantigen in the context of rheumatoid arthritis-associated and non-associated HLA-DR4 alleles'. *Arthritis & Rheumatism*, Vol. 42, No. 7, pp. 1497-1507.

Croft, M., Bradley, L.M. & Swain, S.L. 1994. 'Naïve versus memory CD4 T-cell responses to antigen. Memory cells are less dependant on accessory cell co-stimulation and can respond to many antigen-presenting cell types including resting B-cells'. *The Journal of Immunology*, Vol. 152, No. 6, pp. 2675-2685.

Davies, M.L. *et al*, 2002. 'Candidate T-cell epitopes of the human La/SSB autoantigen'. *Arthritis & Rheumatism*, Vol. 46, No. 1, pp. 209-214.

Davis, M.M., Altman, J.D. & Newell, E.W. 2011. 'Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis'. *Nature Reviews Immunology*, Vol. 11, pp. 551-558.

de Jong, H. *et al*. 2010. 'Cartilage proteoglycan aggrecan epitopes include proinflammatory autoreactive T-cell responses in rheumatoid arthritis and osteoarthritis'. *Annals of the Rheumatic Diseases*, Vol. 69, No. 1, pp. 255-262.

Despres, N., Boire, G., Lopez-Longo, F.J. & Menard, H-A. 1994. 'The Sa system: a novel antigen-antibody system specific for rheumatoid arthritis'. *Journal of Rheumatology*, Vol. 24, No. 6, pp. 1027-1033.

De Vita, S. *et al*. 2002. 'Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis: Evidence for a pathogenic role of B cells'. *Arthritis & Rheumatism*, Vol. 46, No. 8, pp. 2029-2033.

de Vries-Bouwstra, J.K. *et al*. 2008. 'Progression of joint damage in early rheumatoid arthritis: Association with HLA-DRB1, rheumatoid factor, and anti-citrullinated protein antibodies in relation to different treatment strategies'. *Arthritis & Rheumatism*, Vol. 58, No. 5, pp. 1293-1298.

Ditzel, H.J. 2004. 'The K/BxN mouse: a model of human inflammatory arthritis'. *Trends in Molecular Medicine*, Vol. 10, No. 1, pp. 40-45.

Edwards, J.C.W. & Cambridge, G. 2001. 'Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes'. *Rheumatology*, Vol. 40, No. 2, pp. 205-211.

Edwards, J.C.W. *et al*. 2004. 'Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis'. *The New England Journal of Medicine*, Vol. 350, No. 25, pp. 2572-2581.

Ehrenstein, M.R. *et al*. 2004. 'Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNF-α therapy'. *The Journal of Experimental Medicine*, Vol. 200, No. 3, pp. 277-285.

El-Gabalawy, H.S. & Wilkins, J.A. 2004. 'Anti-Sa antibodies: prognostic and pathogenetic significance to rheumatoid arthritis '. *Arthritis Research & Therapy*, Vol. 6, No. 2, pp. 86-89.

Feldmann, M., Brennan, F.M. & Maini, R.N. 1996. 'Role of cytokines in rheumatoid arthritis'. *Annual Review of Immunology*, Vol. 14, No. 1, pp. 397-440.

Finck, B.K., Linsley, P.S. & Wofsy, D. 1994. 'Treatment of murine lupus with CTLA4Ig'. *Science*, Vol. 265, No. 5176, pp. 1225-1227.

Finckh, A. *et al*. 2007. 'B cell depletion may be more effective than switching to an alternative anti-tumour necrosis factor agent in rheumatoid arthritis patients with inadequate response to anti-tumour necrosis factor agents'. *Arthritis & Rheumatism*, Vol. 56, No. 5, pp. 1417-1423.

Firestein, G.S. 2003. 'Evolving concepts of rheumatoid arthritis'. *Nature*, Vol. 423, pp. 356-361.

Firestein, G.S. & Zvaifler, N.J. 1987. 'Peripheral blood and synovial fluid monocyte activation in inflammatory arthritis. ii. Low levels of synovial fluid and synovial tissue interferon suggest that γ-interferon is not the primary macrophage activating factor'. *Arthritis & Rheumatism*, Vol. 30, No. 8, pp. 864-871.

Fossiez, F. *et al*. 1996. 'T cell interleukin-17 induces stromal cells to produce proinflammatory and haematopoetic cytokines'. *The Journal of Experimental Medicine*, Vol. 183, No. 6, pp. 2593-2603.

Freeman, G.J. *et al*, 2000. 'Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation'. *The Journal of Experimental Medicine*, Vol. 192, No. 7, pp. 1027-1034.

Fritsch, R. *et al*. 2002. 'Characterisation of autoreactive T cells to the autoantigens heterogenous nuclear ribonucleoprotein A2 (RA33) and filaggrin in patients with rheumatoid arthritis'. *The Journal of Immunology*, Vol. 169, No. 2, pp. 1068-1076.

Genovese, M.C. *et al*. 2005. 'Abatacept for rheumatoid arthritis refractory to tumour necrosis factor α inhibition'. *The New England Journal of Medicine*, Vol. 353, No. 11, pp. 1114-1123.

Genovese, M.C. *et al*. 2008. 'Efficacy and safety of the selective co-stimulation modulator abatacept following 2 years of treatment in patients with rheumatoid arthritis and inadequate response to anti-tumour necrosis factor therapy'. *Annals of the Rheumatic Diseases*, Vol. 67, No. 4, pp. 547-554.

Girbal-Neuhauser, E. *et al*. 1999. 'The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues'. *The Journal of Immunology*, Vol. 162, No. 1, pp. 585-594.

Glant, T.T., Mikecz, K., Arzoumanian, A. & Poole, A.R. 1987. 'Proteoglycan-induced arthritis in balb/c mice'. *Arthritis & Rheumatism*, Vol. 30, No. 2, pp. 201-212.

Goldbach-Mansky, R. *et al*. 2000. 'Rheumatoid arthritis associated autoantibodies in patients with synovitis of recent onset'. *Arthritis Research*, Vol. 2, No. 3, pp. 236-243.

Goodstone, N.J. *et al*. 1996. 'Cellular immunity to cartilage aggrecan core protein in patients with rheumatoid arthritis and non-arthritic controls'. *Annals of the Rheumatic Diseases*, Vol. 55, No. 1, pp. 40-46.

Gorman, J.D. *et al*. 2004. 'Impact of shared epitope genotype and ethnicity on erosive disease: A meta-analysis of 3,240 rheumatoid arthritis patients'. *Arthritis & Rheumatism*, Vol. 50, No. 2, pp. 400-412.

Greenwald, R.J. *et al*, 2001. 'CTLA-4 regulates induction of anergy in vivo'. *Immunity*, Vol. 14, No. 2, pp. 145-155.

Gregersen, P.K., Silver, J. & Winchester, R.J. 1987. 'The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 30, No. 11, pp. 1205-1213.

Guerassimov, A. *et al*. 1998. 'Cellular immunity to the G1 domain of cartilage proteoglycan aggrecan is enhanced in patients with rheumatoid arthritis but only after removal of keratin sulphate'. *Arthritis & Rheumatism*, Vol. 41, No. 6, pp. 1019-1025.

Hakala, B.E., White, C. & Recklies, A.D. 1993. 'Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family'. *The Journal of Biological Chemistry*, Vol. 268, No. 34, pp. 25803-25810.

Hale, J.S. & Fink, P.J. 2010. 'T-cell receptor revision: friend or foe?' *Immunology*, Vol. 129, No. 4, pp. 467-473.

Hammer, J. *et al*. 1995. 'Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association'. *The Journal of Experimental Medicine*, Vol. 181, No. 5, pp. 1847-1855.

Hammerling, G.J. *et al*, 1991. 'Non-deletional mechanisms of peripheral and central tolerance: studies with transgenic mice with tissue-specific expression of a foreign MHC class I antigen'. *Immunological Reviews*, Vol. 122, No. 1, pp. 47-67.

Hassfeld, W. *et al*. 1995. 'Autoimmune response to the spliceosome'. *Arthritis & Rheumatism*, Vol. 38, No. 6, pp. 777-785.

Hill, J.A. *et al*. 2003. 'Cutting edge: The conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule'. *The Journal of Immunology*, Vol. 171, No. 2, pp. 538-541.

Hill, J.A. *et al*. 2008. 'Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice'. *The Journal of Experimental Medicine*, Vol. 205, No. 4, pp. 967-979.

Hinks, A. *et al*. 2005. 'Association between the *PTPN22* gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: Further support that *PTPN22* is an autoimmunity gene'. *Arthritis & Rheumatism*, Vol. 52, No. 6, pp. 1694-1699.

Hirota, K. *et al* #1. 2007. 'T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17^{+} Th cells that cause autoimmune arthritis'. *The Journal of Experimental Medicine*, Vol. 204, No. 1, pp. 41-47.

Hirota, K. *et al* #2. 2007. 'Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model'. *The Journal of Experimental Medicine*, Vol. 204, No. 12, pp. 2803-2812.

Hogquist, K.A., Baldwin, T.A. & Jameson, S.C. 2005. 'Central tolerance: learning self-control in the thymus'. *Nature Reviews Immunology*, Vol. 5, pp. 772-782.

Huang, J.C., Vestberg, M., Minguela, A., Holmdahl, R. & Ward, E.S. 2004. 'Analysis of autoreactive T cells associated with murine collagen-induced arthritis using peptide-MHC multimers'. *International Immunology*, Vol. 16, No. 2, pp. 283-293.

Huang, Q., Ma, Y., Adebayo, A. & Pope, R.M. 2007. 'Increased macrophage activation mediated through toll-like receptors in rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 56, No. 7, pp. 2192-2201.

Hueber, W., Hassfeld, W., Smolen, J.S. & Steiner, G. 1999. 'Sensitivity and specificity of anti-Sa autoantibodies for rheumatoid arthritis'. *Rheumatology*, Vol. 38, No. 2, pp. 155-159.

Iannone, F., Corrigall, V.M., Kingsley, G.H. & Panayi, G.S. 1994. 'Evidence for the continuous recruitment and activation of T cells into the joints of patients with rheumatoid arthritis'. *European Journal of Immunology*, Vol. 24, No. 11, pp. 2706-2713.

Isaacs, J.D. 2008. 'Therapeutic T-cell manipulation in rheumatoid arthritis: past, present and future'. *Rheumatology*, Vol. 47, No. 10, pp. 1461-1468.

Isaacs, J.D. & Moreland, L.W. 2011. 'Fast Facts: Rheumatoid Arthritis'. Health Press, second edition.

Johansen, J.S., Jensen, H.S. & Price, P.A. 1993. 'A new biochemical marker for joint injury. Analysis of YKL-40 in serum and synovial fluid'. *Rheumatology*, Vol. 32, No. 11, pp. 949-955.

Jovanovich, D.V. *et al*. 1998. 'IL-17 stimulates the production and expression of proinflammatory cytokines, IL-β and TNF-α, by human macrophages'. *The Journal of Immunology*, Vol. 160, No. 7, pp. 3513-3521.

Kallberg, H. *et al*. 2007. 'Gene-gene and gene-environmental interactions involving HLA-DRB1, *PTPN22* and smoking in two subsets of rheumatoid arthritis'. *The American Journal of Human Genetics*, Vol. 80, No. 5, pp. 867-875.

Kiani, C., Chen, L., Wu, Y.J., Yee, A.J. & Yang, B.B. 2002. 'Structure and function of aggrecan'. *Cell Research*, Vol. 12, No. 1, pp. 19-32.

Kim, H.Y. *et al*. 1999. 'Enhanced T cell proliferative response to type II collagen and synthetic peptide CII (255-274) in patients with rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 42, No. 10, pp. 2085-2093.

Kinloch, A. *et al*. 2005. 'Identification of citrullinated α-enolase as a candidate autoantigen in rheumatoid arthritis'. *Arthritis Research & Therapy*, Vol. 7, No. 6, pp. 1421-1429.

Kirwan, J.R. 1995. 'The effect of glucocorticoids on joint destruction in rheumatoid arthritis'. *The New England Journal of Medicine*, Vol. 333, No. 3, pp. 142-147.

Klareskog, L. *et al*. 2006. 'A new model for an aetiology of rheumatoid arthritis: Smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination'. *Arthritis & Rheumatism*, Vol. 54, No. 1, pp. 38-46.

Knijff-Dutmer, E. *et al*. 2002. 'Rheumatoid factor measured by fluoroimmunoassay: a responsive measure of rheumatoid arthritis disease activity that is associated with joint damage'. *Annals of the Rheumatic Diseases*, Vol. 61, No. 7, pp. 603-607.

Komai-Koma, M., Gilchrist, D.S. & Xu, D. 2009. 'Direct recognition of LPS by human but not murine $CD8^+$ T cells via TLR4 complex'. *European Journal of Immunology*, Vol. 39, No. 6, pp. 1564-1572.

Korganow, A.S. *et al*, 1999. 'From systemic T-cell self-reactivity to organ-specific autoimmune disease via immunoglobulins'. *Immunity*, Vol. 10, No. 4, pp. 451-461.

Kotake, S. *et al*. 1999. 'IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis'. *The Journal of Clinical Investigation*, Vol. 103, No. 9, pp. 1345-1352.

Kotzin, B.L. *et al*. 2000. 'Use of soluble peptide-DR4 tetramers to detect synovial T cells specific for cartilage antigens in patients with rheumatoid arthritis'. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 97, No. 1, pp. 291-296.

Kouskoff, V. *et al*, 1996. 'Organ-specific disease provoked by systemic autoimmunity'. *Cell*, Vol. 87, No. 5, pp. 811-822.

Kremer, J.M. *et al*. 2003. 'Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig'. *The New England Journal of Medicine*, Vol. 349, No. 20, pp. 1907-1915.

Kremer, J.M. *et al*. 2005. 'Treatment of rheumatoid arthritis with the selective co-stimulation modulator abatacept: twelve-month results of a phase iib, double-blind, randomised, placebo-controlled trial'. *Arthritis & Rheumatism*, Vol. 52, No. 8, pp. 2263-2271.

Kremer, J.M. *et al*. 2006. 'Effects of abatacept in patients with methotrexate-resistant active rheumatoid arthritis: a randomised trial'. *Annals of Internal Medicine*, Vol. 144, No. 12, pp. 865-876.

Kremer, J.M. *et al*. 2008. 'Results of a two-year follow-up study of patients with rheumatoid arthritis who received a combination of abatacept and methotrexate'. *Arthritis & Rheumatism*, Vol. 58, No. 4, pp. 953-963.

Langrish, C.L. *et al*. 2005. 'IL-23 drives a pathogenic T cell population that induces autoimmune inflammation'. *The Journal of Experimental Medicine*, Vol. 201, No. 2, pp. 233-240.

Law, S.C. *et al*, 2012. 'T cell autoreactivity to citrullinated autoantigenic peptides in rheumatoid arthritis patients carrying HLA-DRB1 shared epitope alleles'. *Arthritis Research & Therapy*, Vol. 14, No. 3, pp. 118-129.

Lawson, C.A. *et al*. 2006. 'Early rheumatoid arthritis is associated with a deficit in the $CD4^{+}$ $CD25^{high}$ regulatory T cell population in peripheral blood'. *Rheumatology*, Vol. 45, No. 10, pp. 1210-1217.

Lei, C. *et al*. 2005. 'Association of the CTLA-4 gene with rheumatoid arthritis in Chinese Han population'. *European Journal of Human Genetics*, Vol. 13, pp. 823-828.

Leroux, J-V. *et al*. 1992. 'Characterisation of proteoglycan-reactive T cell lines and hybridomas from mice with proteoglycan-induced arthritis'. *The Journal of Immunology*, Vol. 148, No. 7, pp. 2090-2096.

Leroux, J-V. *et al*. 1996. 'Immunity to the G1 globular domain of the cartilage proteoglycan aggrecan can induce inflammatory erosive polyarthritis and spondylitis in BALB/c mice but immunity to G1 is inhibited by covalently bound keratin sulphate in vitro and in vivo'. *The Journal of Clinical Investigation*, Vol. 97, No. 3, pp. 621-632.

Li, J. *et al*. 2000. 'RANK is the intrinsic haematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism'. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 97, No. 4, pp. 1566-1571.

Li, N.L. *et al*. 2000. 'Isolation and characteristics of autoreactive T cells specific to aggrecan G1 domain from rheumatoid arthritis patients'. *Cell Research*, No. 10, pp. 39-49.

Londei, M. *et al*. 1989. 'Persistence of collagen type II-specific T-cell clones in the synovial membrane of a patient with rheumatoid arthritis'. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 86, No. 2, pp. 636-640.

Lundberg, K. *et al*. 2008. 'Antibodies to citrullinated α-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase'. *Arthritis & Rheumatism*, Vol. 58, No. 10, pp. 3009-3019.

Mannering, S.I. *et al*, 2003. 'A sensitive method for detecting proliferation of rare autoantigen-specific human T-cells'. *Journal of Immunological Methods*, Vol. 283, pp. 173-183.

Massilamany, C., Upadhyaya, B., Gangaplara, A., Kuszynski, C. & Reddy, J. 2011. 'Detection of autoreactive CD4 T cells using major histocompatibility complex class II dextramers'. *BMC Immunology*, Vol. 12, No. 40, pp. 1-14.

Masson-Bessiere, C. *et al*. 2000. 'In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgG than in synovial fluid and serum'. *Clinical & Experimental Immunology*, Vol. 119, No. 3, pp. 544-552.

Masson-Bessiere, C. *et al*. 2001. 'The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the α- and β-chains of fibrin'. *The Journal of Immunology*, Vol. 166, No. 6, pp. 4177-4184.

Mattern, T., Flad, H-D., Brade, L., Rietschel, E.T. & Ulmer, A.J. 1998. 'Stimulation of human T lymphocytes by LPS is MHC unrestricted, but strongly dependant on B7 interactions'. *The Journal of Immunology*, Vol. 160, No. 7, pp. 3412-3418.

McGargill, M.A., Derbinski, J.M. & Hogquist, K.A. 2000. 'Receptor editing in developing T-cells'. *Nature Immunology*, Vol. 1, pp. 336-341.

McInnes, I.B., Al-Mughales, J., Field, M., Leung, B.P., Huang, F.P., Dixon, R., Sturrock, R.D., Wilkinson, P.C. & Liew, F.Y. 1996. 'The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis'. *Nature Medicine*, 2, pp. 175-182.

McInnes, I.B., Leung, B.P., Sturrock, R.D., Field, M. & Liew, F.Y. 1997. 'Interleukin-15 mediates T-cell-dependant regulation of tumour necrosis factor-α production in rheumatoid arthritis'. *Nature Medicine*, 3, pp. 189-195.

McInnes, I.B. & Schett, G. 2007. 'Cytokines in the pathogenesis of rheumatoid arthritis'. *Nature Reviews Immunology*, Vol. 7, pp. 429-442.

Mewar, D. & Wilson, A.G. 2006. 'Autoantibodies in rheumatoid arthritis: a review'. *Biomedicine & Pharmacotherapy*, Vol. 60, No. 10, pp. 648-655.

Michou, L. *et al*. 2007. 'Linkage proof for *PTPN22*, a rheumatoid arthritis susceptibility gene and a human autoimmunity gene'. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 104, No. 5, pp. 1649-1654.

Mikecz, K., Glant, T.T., Buzás, E. & Poole, A.R. 1990. 'Proteoglycan-induced polyarthritis and spondylitis adoptively transferred to naïve (non-immunised) BALB/c mice'. *Arthritis & Rheumatism*, Vol. 33, No. 6, pp. 866-876.

Miyasaka, N. *et al*, 1980. 'Decreased autologous mixed lymphocyte reaction in Sjögren's syndrome'. *Journal of Clinical Investigation*, Vol. 66, No. 5, pp. 928-933.

Morgan, M.E. *et al*. 2003. 'CD25$^+$ cell depletion hastens the onset of severe disease in collagen-induced arthritis'. *Arthritis & Rheumatism*, Vol. 48, No. 5, pp. 1452-1460.

Möttönen, M., Heikkinen, J., Mustonen, L., Isomäki, P., Luukkainen, R. & Lassila, O. 2005. 'CD4$^+$ CD25$^+$ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis'. *Clinical & Experimental Immunology*, Vol. 140, No. 2, pp. 360-367.

Mueller, D.L. 2010. 'Mechanisms maintaining peripheral tolerance'. *Nature Immunology*, Vol. 11, No. 1, pp. 21-27.

Nakae, S. *et al* #1. 2003. 'IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist'. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 100, No. 10, pp. 5986-5990.

Nakae, S. *et al* #2. 2003. 'Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice'. *The Journal of Immunology*, Vol. 171, No. 11, pp. 6173-6177.

Ng, W.F. *et al*. 2001. 'Human CD4$^+$ CD25$^+$ cells: a naturally occurring population of regulatory T cells'. *Blood*, Vol. 98, No. 9, pp. 2736-2744.

Nyirkos, P. & Golds, E.E. 1990. 'Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period'. *Biochemical Journal*, Vol. 269, No. 1, pp. 265-268.

Orozco, G. *et al*. 2005. 'Association of a functional single-nucleotide polymorphism of *PTPN22*, encoding lymphoid protein phosphatase, with rheumatoid arthritis and systemic lupus erythematosus'. *Arthritis & Rheumatism*, Vol. 52, No. 1, pp. 219-224.

Padyukov, L., Silva, C., Stolt, P., Alfredsson, L. & Klareskog, L. 2004. 'A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 50, No. 10, pp. 3085-3092.

Palmer, E. 2003. 'Negative selection – clearing out the bad apples from the T-cell repertoire'. *Nature Reviews Immunology*, Vol. 3, No. 5, pp. 383-391.

Panayi, G.S. 2005. 'B cells: a fundamental role in the pathogenesis of rheumatoid arthritis?' *Rheumatology*, Vol. 44, No. 2, pp. ii3-ii7.

Park, S.H. *et al*. 2001. 'Shift toward T helper 1 cytokines by type II collagen-reactive T cells in patients with rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 44, No. 3, pp. 561-569.

Pettit, A.R., Walsh, N.C., Manning, C., Goldring, S.R. & Gravallese, E.M. 2006. 'RANKL protein is expressed at the pannus-bone interface at sites of articular bone erosion in rheumatoid arthritis'. *Rheumatology*, Vol. 45, No. 9, pp. 1068-1076.

Pope, R.M. *et al*, 1984. 'Characterisation of the defective autologous mixed lymphocyte response in rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 27, No. 11, pp. 1234-1244.

Pratt, A.G., Isaacs, J.D. & Mattey, D.L. 2009. 'Current concepts in the pathogenesis of early rheumatoid arthritis'. *Best Practice & Research Clinical Rheumatology*, Vol. 23, No. 1, pp. 37-48.

Rantapää-Dahlqvist, S. *et al*. 2003. 'Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 48, No. 10, pp. 2741-2749.

Reviron, D. *et al*. 2001. 'Influence of shared epitope-negative HLA-DRB1 alleles on genetic susceptibility to rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 44, No. 3, pp. 535-540.

Ria, F. *et al*. 2008. 'Collagen-specific T cell repertoire in blood and synovial fluid varies with disease activity in early rheumatoid arthritis'. *Arthritis Research & Therapy*, Vol. 10, No. 6, pp. 135-153.

Rosloniec, E.F. *et al*. 1997. 'An HLA-DR1 transgene confers susceptibility to collagen-induced arthritis elicited with human type II collagen'. *The Journal of Experimental Medicine*, Vol. 185, No. 6, pp. 1113-1122.

Rosloniec, E.F. *et al*. 1998. 'Induction of autoimmune arthritis in HLA-DR4 (DRB1*0401) transgenic mice by immunisation with human and bovine type II collagen'. *The Journal of Immunology*, Vol. 160, No. 6, pp. 2573-2578.

Sakaguchi, N. *et al*, 2003. 'Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice'. *Nature*, Vol. 426, pp. 454-460.

Sakaguchi, S. *et al*. 2001. 'Immunological tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumour immunity, and transplantation tolerance'. *Immunological Reviews*, Vol. 182, No. 1, pp. 18-32.

Sakaguchi, S. 2005. 'Naturally arising Foxp3-expressing $CD25^+$ $CD4^+$ regulatory T-cells in immunological tolerance to self and non-self'. *Nature Immunology*, Vol. 6, No. 4, pp. 345-352.

Sakaguchi, S. *et al*, 2006. 'SKG mice, a monogenic model of autoimmune arthritis due to altered signal transduction in T-cells'. *The Hereditary Basis of Rheumatic Diseases*, pp. 147-159.

Saruhan-Direskeneli, G. 1998. 'Shared epitope 'homozygosity' is strongly associated with rheumatoid arthritis in Turkey'. *Rheumatology*, Vol. 37, No. 10, pp. 1126-1128.

Saulot, V. *et al*. 2002. 'Presence of autoantibodies to the glycolytic enzyme α-enolase in sera from patients with early rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 46, No. 5, pp. 1196-1201.

Scheinecker, C. *et al*, 1998. 'Initiation of the autologous mixed lymphocyte reaction requires the expression of co-stimulatory molecules B7-1 and B7-2 on human peripheral blood dendritic cells'. *The Journal of Immunology*, Vol. 161, No. 8, pp. 3966-3973.

Schellekens, G.A. *et al*. 1998. 'Citrulline is an essential constituent of antigenic determinants recognised by rheumatoid arthritis-specific autoantibodies'. *The Journal of Clinical Investigation*, Vol. 101, No. 1, pp. 273-281.

Schellekens, G.A. *et al*. 2000. 'The diagnostic properties of rheumatoid arthritis antibodies recognising a cyclic citrullinated peptide'. *Arthritis & Rheumatism*, Vol. 43, No. 1, pp. 155-163.

Sebbag, M. *et al*. 1995. 'The antiperinuclear factor and the so-called antikeratin antibodies are the same rheumatoid arthritis-specific autoantibodies'. *The Journal of Clinical Investigation*, Vol. 95, No. 6, pp. 2672-2679.

Shaw, T., Quan, J. & Totoritis, M.C. 2003. 'B cell therapy for rheumatoid arthritis: the rituximab (anti-CD20) experience'. *Annals of the Rheumatic Diseases*, Vol. 62, Suppl. 2, pp. ii55-ii59.

Shen, H., Goodall, J.C. & Hill Gaston, J.S. 2009. 'Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 60, No. 6, pp. 1647-1656.

Shi, J. *et al*. 2011. 'Autoantibodies recognising carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage'. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 108, No. 42, pp. 17372-17377.

Silverman, G.J. & Weisman, S. 2003. 'Rituximab therapy and autoimmune disorders: Prospects for anti-B cell therapy'. *Arthritis & Rheumatism*, Vol. 48, No. 6, pp. 1484-1492.

Simon, M. *et al*. 1993. 'The cytokeratin filament-aggregating protein filaggrin is the target of the so-called 'antikeratin antibodies', autoantibodies specific for rheumatoid arthritis'. *The Journal of Clinical Investigation*, Vol. 92, No. 3, pp. 1387-1393.

Simonet, W.S. *et al*. 1997. 'Osteoprotegerin: a novel secreted protein involved in the regulation of bone density'. *Cell*, Vol. 89, No. 2, pp. 309-319.

Skriner, K. *et al*. 1997. 'Anti-A2/RA33 autoantibodies are directed to the RNA binding region of the A2 protein of the heterogenous nuclear ribonucleoprotein complex. Differential epitope recognition in rheumatoid arthritis, systemic lupus erythematosus, and mixed connective tissue disease'. *The Journal of Clinical Investigation*, Vol. 100, No. 1, pp. 127-135.

Smolen, J.S. *et al*, 1981. 'Responder cells in the human autologous mixed lymphocyte reaction'. *The Journal of Clinical Investigation*, Vol. 68, No. 6, pp. 1601-1604.

Snowden, N., Reynolds, I., Morgan, K. & Holt, L. 1997. 'T cell responses to human type II collagen in patients with rheumatoid arthritis and healthy controls'. *Arthritis & Rheumatism*, Vol. 40, No. 7, pp. 1210-1218.

Stebbings, R. *et al*. 2007. 'Cytokine storm in the phase 1 trial of monoclonal antibody TGN1412: better understanding of the causes to improve preclinical testing of immunotherapeutics'. *The Journal of Immunology*, Vol. 179, No. 5, pp. 3325-3331.

Steiner, G. & Smolen, J. 2002. 'Autoantibodies in rheumatoid arthritis and their clinical significance'. *Arthritis Research*, Vol. 4, Suppl. 2, pp. S1-S5.

Steiner, G. 2006. 'Autoantibodies in rheumatoid arthritis'. *Autoimmunity*, Vol. 18, No. 7, pp. 8-10.

Strand, V., Kimberly, R. & Isaacs, J.D. 2007. 'Biologic therapies in rheumatology: lessons learned, future directions'. *Nature Reviews Drug Discovery*, 6, pp. 75-92.

Suntharalingham, G. *et al*. 2006. 'Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412'. *The New England Journal of Medicine*, Vol. 355, No. 10, pp. 1018-1028.

Suri-Payer, E., Amar, A.Z., Thornton, A.M. & Shevach, E.M. 1998. '$CD4^+$ $CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells'. *The Journal of Immunology*, Vol. 160, No. 3, pp. 1212-1218.

Svendsen, P. *et al*. 2004. 'Tracking of proinflammatory collagen-specific T cells in early and late collagen-induced arthritis in humanised mice'. *The Journal of Immunology*, Vol. 173, No. 11, pp. 7037-7045.

Svensson, Jirholt, Holmdahl & Jansson. 1998. 'B cell-deficient mice do not develop type II collagen-induced arthritis (CIA)'. *Clinical & Experimental Immunology*, Vol. 111, No. 3, pp. 521-526.

Takada, S. *et al*, 1985. 'Abnormalities in autologous mixed lymphocyte reaction-activated immunologic processes in systemic lupus erythematosus and their possible correction by IL-2'. *European Journal of Immunology*, Vol. 15, No. 3, pp. 262-267.

Takemura, S. *et al*. 2001. 'T cell activation in rheumatoid synovium is B cell dependant'. *The Journal of Immunology*, Vol. 167, No. 8, pp. 4710-4718.

Terato, K. *et al*. 1990. 'Specificity of antibodies to type II collagen in rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 33, No. 10, pp. 1493-1500.

Tough, D.F., Sun, S. & Sprent, J. 1997. 'T cell stimulation in vivo by lipopolysaccharide (LPS)'. *The Journal of Experimental Medicine*, Vol. 185, No. 12, pp. 2089-2094.

Trentham, D.E., Townes, A.S. & Kang, A.H. 1977. 'Autoimmunity to type II collage: An experimental model of arthritis'. *The Journal of Experimental Medicine*, Vol. 146, No. 3, pp. 857-868.

Tsark, E.C. *et al*. 2002. 'Differential MHC class II-mediated presentation of rheumatoid arthritis autoantigens by human dendritic cells and macrophages'. *The Journal of Immunology*, Vol. 169, No. 11, pp. 6625-6633.

Tulic, M.K., Manoukian, J.J., Eidelman, D.H. & Hamid, Q. 2002. 'T-cell proliferation induced by local application of LPS in the nasal mucosa of nonatopic children'. *The Journal of Allergy and Clinical Immunology*, Vol. 110, No. 5, pp. 771-776.

van Bilsen, J.H.M. *et al*. 2004. 'Functional regulatory immune responses against human cartilage glycoprotein-39 in health vs. proinflammatory responses in rheumatoid arthritis'. *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 101, No. 49, pp. 17180-17185.

van Boxel, J.A. & Paget, S.A. 1975. 'Predominantly T-cell infiltrate in Rheumatoid Synovial Membranes'. *The New England Journal of Medicine,* 293, pp. 517-520.

van Gaalen, F.A. *et al*. 2004. 'Association between HLA class II genes and autoantibodies to cyclic citrullinated peptides (CCPs) influences the severity of rheumatoid arthritis'. *Arthritis & Rheumatism,* Vol. 50, No. 7, pp. 2113-2121.

van Gaalen, F.A., Ioan-Facsinay, A., Huizinga, T.W.J. & Toes, R.E.M. 2005. 'The devil in the details: the emerging role of anticitrulline autoimmunity in rheumatoid arthritis'. *The Journal of Immunology,* Vol. 175, No. 9, pp. 5575-5580.

van Hamburg, J.P. *et al*. 2011. 'Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production'. *Arthritis & Rheumatism,* Vol. 63, No. 1, pp. 73-83.

van Venrooij, W.J. & Pruijn, G.J.M. 2000. 'Citrullination: a small change for a protein with great consequences for rheumatoid arthritis'. *Arthritis Research & Therapy,* Vol. 2, No. 4, pp. 249-251.

Verheijden, G.F.M. *et al*. 1997. 'Human cartilage glycoprotein-39 as a candidate autoantigen in rheumatoid arthritis'. *Arthritis & Rheumatism,* Vol. 40, No. 6, pp. 1115-1125.

Villasenor, J., Benoist, C. & Mathis, D. 2005. 'AIRE and APECED: molecular insights into an autoimmune disease'. *Immunological Reviews,* Vol. 204, No. 1, pp. 156-164.

Volck, B. *et al*. 2001. 'Studies on YKL-40 in knee joints of patients with rheumatoid arthritis and osteoarthritis. Involvement of YKL-40 in the joint pathology'. *Osteoarthritis & Cartilage,* Vol. 9, No. 3, pp. 203-214.

von Delwig, A., Locke, J., Robinson, J.H. & Ng, W.F. 2010. 'Response of Th17 cells to a citrullinated arthritogenic aggrecan peptide in patients with rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 62, No. 1, pp. 143-149.

Vos, K. *et al* #1. 2000. 'Raised human cartilage glycoprotein-39 plasma levels in patients with rheumatoid arthritis and other inflammatory conditions'. *Annals of the Rheumatic Diseases*, Vol. 59, No. 7, pp. 544-548.

Vos, K. *et al* #2. 2000. 'Cellular immune response to human cartilage glycoprotein-39 (HCgp-39)-derived peptides in rheumatoid arthritis and other inflammatory conditions'. *Rheumatology*, Vol. 39, No. 12, pp. 1326-1331.

Vossenaar, E.R. *et al*. 2004. 'Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin'. *Arthritis Research & Therapy*, Vol. 6, No. 2, pp. 142-150.

Vossenaar, E.R., Zendman, A.J.W. & van Venrooij, W.J. 2004. 'Citrullination, a possible functional link between susceptibility genes and rheumatoid arthritis'. *Arthritis Research & Therapy*, Vol. 6, No. 1, pp. 1-5.

Vossenaar, E.R. & van Venrooij, W.J. 2004. 'Citrullinated proteins: sparks that may ignite the fire in rheumatoid arthritis'. *Arthritis Research & Therapy*, Vol. 6, No. 3, pp. 107-111.

Walker, L.S.K. & Abbas, A.K. 2002. 'The enemy within: keeping self-reactive T-cells at bay in the periphery'. *Nature Reviews Immunology*, Vol. 2, pp. 11-19.

Walunas, T.L., Bakker, C.Y. & Bluestone, J.A. 1996. 'CTLA-4 ligation blocks CD28-dependant T-cell activation'. *The Journal of Experimental Medicine*, Vol. 183, No. 6, pp. 2541-2550.

Webb, L.M.C., Walmsley, M.J. & Feldmann, M. 1996. 'Prevention and amelioration of collagen-induced arthritis by blockade of the CD28 co-stimulatory pathway: requirement for both B7-1 and B7-2'. *European Journal of Immunology*, Vol. 26, No. 10, pp. 2320-2328.

Weinblatt, M. *et al*. 2006. 'Safety of the selective co-stimulation modulator abatacept in rheumatoid arthritis patients receiving background biologic and non-biologic disease-modifying anti-rheumatic drugs: a one-year randomised, placebo-controlled study'. *Arthritis & Rheumatism*, Vol. 54, No. 9, pp. 2807-2816.

Weksler, M.E. & Kozak, R. 1977. 'Lymphocyte transformation induced by autologous cells. V. Generation of immunological memory and specificity during the autologous mixed lymphocyte reaction'. *The Journal of Experimental Medicine*, Vol. 146, No. 6, pp. 1833-1838.

Wessels, J.A.M. *et al*. 2006. 'Relationship between genetic variants in the adenosine pathway and outcome of methotrexate treatment in patients with recent-onset rheumatoid arthritis'. *Arthritis & Rheumatism*, Vol. 54, No. 9, pp. 2830-2839.

Weyand, C.M., McCarthy, T.G. & Goronzy, J.J. 1995. 'Correlation between disease phenotype and genetic heterogeneity in rheumatoid arthritis'. *The Journal of Clinical Investigation*, Vol. 95, No. 5, pp. 2120-2126.

Weyand, C.M. & Goronzy, J.J. 2003. 'Ectopic germinal centre formation in rheumatoid synovitis'. *Annals of the New York Academy of Sciences*, Vol. 987, Immune mechanisms and disease, pp. 140-149.

Wong, P. & Pamer, E.G. 2001. 'Cutting Edge: Antigen-independent CD8 T-cell proliferation'. *The Journal of Immunology*, Vol. 166, No. 10, pp. 5864-5868.

Wooley, P.H., Luthra, H.S., Stuart, J.M. & David, C.S. 1981. 'Type II collagen-induced arthritis in mice. 1. Major histocompatibility complex 1 (1 region) linkage and antibody correlates'. *The Journal of Experimental Medicine*, Vol. 154, No. 3, pp. 688-700.

Yeo, L. *et al*. 2011. 'Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis'. *Annals of the Rheumatic Diseases*, Vol. 70, No. 11, pp. 2022-2028.

Yoo, T.J. *et al*. 1988. 'Induction of arthritis in monkeys by immunisation with type II collagen'. *The Journal of Experimental Medicine*, Vol. 168, No. 2, pp. 777-782.

Zhang, Y. *et al*. 1998. ‘Arthritis induced by proteoglycan aggrecan G1 domain in BALB/c mice’. *The Journal of Clinical Investigation*, Vol. 101, No. 8, pp. 1678-1686.

Ziolkowska, M. *et al*. 2000. ‘High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 via cyclosporine A-sensitive mechanism’. *The Journal of Immunology*, Vol. 164, No. 5, pp. 2832-2838.

Zou, J. *et al*. 2003. ‘Predominant cellular immune response to the cartilage autoantigenic G1 aggrecan in ankylosing spondylitis and rheumatoid arthritis’. *Rheumatology*, Vol. 42, No. 7, pp. 846-855.