ALTERED LEUKOCYTE SIGNALLING THRESHOLDS IN RHEUMATOID ARTHRITIS THROUGH CHANGES IN THE FUNCTION OF THE PROTEIN TYROSINE PHOSPHATASE PTPN22/LYP

By

Rachel Bayley

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Some words of wisdom……..

"I had so many things to do I just ended up doing none of them."

Anonymous (2013)

To Mom, Dad, Dave, Nan and especially my fiancé Nick

Abstract

Rheumatoid arthritis (RA) results from complex interactions between genetic and environmental risk factors. Two examples of these are the genetic variant PTPN22 R620W, a disease-associated form of the protein tyrosine phosphatase (PTP) Lyp and cigarette smoking (CS). Epidemiological studies have identified interactions between R620W and CS, but the biological mechanisms behind these interactions are unclear. Lyp is expressed by all leukocytes and changes in leukocyte function are implicated in the pathogenesis of RA. Thus the aim of this study was to characterise the effects of R620W and CS on leukocyte signalling, to determine possible mechanisms by which these factors could interact to promote the development of RA.

An assay to measure the specific activity of the Lyp phosphatase was developed. Healthy controls and RA patients were recruited and genotyped for the PTPN22 R620W variant. Following determination of genotype, neutrophils and CD4+ T cells were isolated and cell function assessed following cigarette smoke extract (CSE) treatment.

R620W in T lymphocytes increased Lyp phosphatase activity, decreased Lyp substrate phosphorylation and increased production of the pro-inflammatory cytokines IFN-γ and TNF-α. CSE treatment decreased T cell receptor signalling which was characterised by decreased PTP activity, decreased calcium (Ca^{2+}) flux and decreased cytokine production. R620W in neutrophils was associated with increased neutrophil activation and functions including Ca^{2+} flux, reactive oxygen species production and migration.

Overall these data suggest that R620W may facilitate RA development and persistence by promoting the generation of inflammatory T cells and by enhancing neutrophil activation and migration. CS may promote further signalling dysfunction by oxidising the proteins controlling leukocyte signalling. These separate pathways leading to altered Lyp function may act additively or synergistically to promote the immune disturbances which underpin the development of RA.

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Abbreviations

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AP	Alkaline Phosphatase
APC	Antigen Presenting Cell
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CCP	Cyclic Citrullinated Peptide
CFSE	Carboxyfluorescein diacetate succinimidyl ester
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette Smoke
CsA	Cyclosporin A
CSE	Cigarette Smoke Extract
DAG	Diacylglycerol
DC	Dendritic Cell
DHR	Dihydrorhodamine
DIFMU	6,8-difluoro-7-hydroxy-4-methylcoumarin
DiFMUP	6,8-Difluoro-4-Methylumbelliferyl Phosphate
DNA	Deoxyribo Nucleic Acid
DTNB	Dithio-bis 2-nitrobenzoic acid
DTT	Dithiothreitol
EBV	Epstein Barr Virus
ECL	Enhanced Chemoiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EULAR	European League Against Rheumatism
FACS	Fluorescent Assisted Cell Sorting
FCM	Flow Cytometry
FDP	Fluorescein Diphosphate
FI	Fluorescence Intensity
fMLP	N-formyl-Met-Leu-Phe
FPR	Formyl Peptide Receptor
GEF	Guanine Exchange Factor
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
GPS	Glutamine Penicillin Streptomycin
GSH	Glutathione
HBSS	Hanks Buffered Salt Solution
HIFCS	Heat Inactivated Foetal Calf Serum

HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
IAA	Iodoacetic acid
IAP	Iodoacetylpolyethylene oxide
ICAM	Intercellular Adhesion Molecule-1
IFN	Interferon
IL	Interleukin
IP	Immunoprecipitation
ITAM	Immunoreceptor Tyrosine-based Activating Motifs
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MHC	Major Histocompatibility Complex
MNC	Mononuclear Cell
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor Kappa B
NK cell	Natural Killer cell
OR	Odds Ratio
PADI	Peptidylarginine Deiminase
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCA	Perchloric Acid
PCR	Polymerase Chain Reaction
PCO	Protein carbonyl
PKC	Protein Kinase C
PLC	Phospholipase C
PM	Particulate Matter
PMN	Polymorphonuclear Leukocyte
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
ROS	Reactive Oxygen Species
RT	Room Temperature
SDS	Sodium dodecyl sulfate
SEM	Standard Error Mean
SF	Synovial Fluid

SFK	Src-Family Kinase
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
TB	Tuberculosis
TCEP	Tris (2-carboxyethyl) Phosphine
TCR	T Cell Receptor
TLR	Toll-like Receptor
TMB	Tetramethylbenzidine
TNF-α	Tumour Necrosis Factor Alpha
UK	United Kingdom
WB	Western Blotting
WHO	World Health Organisation

CHAPTER ONE: INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by chronic inflammation mostly seen in the diarthrodial joint, but with a significant systemic component. If left untreated, the condition results in significant joint damage mediated by inappropriate activation of infiltrating immune cells and resident synoviocytes. It is the most common form of inflammatory arthritis and is estimated to affect 1.16% of women and 0.44% of men in the United Kingdom (UK) (Symmons et al. 2002). The condition is three times more prevalent in women compared to men, however this difference between sexes is diminished with age (Ahlmen et al. 2010). Disease onset usually occurs between 30 and 50 years of age (Deane et al. 2010) and risk of disease increases with age (Ahlmen et al. 2010). The predominant clinical features of RA are joint pain, swelling, stiffness, loss of cartilage and bone and possibly deformity (Lee et al. 2001) eventually leading to disability and premature death, often from cardiovascular complications.

For the reasons outlined above, it is of importance to investigate the mechanisms promoting the development of RA. The work presented in this thesis focuses on the contribution of genes and environment to the disease process. This work was done by studying how risk factors alone and in combination modulate innate and adaptive immunity. Studies concentrated on how changes in the function of the protein tyrosine phosphatase (PTP) Lyp contribute to altered neutrophil and T cell function to facilitate the onset or perpetuation of RA. I investigated how expression of a commonly expressed genetic variant (R620W) of PTPN22/Lyp and the environmental risk factor of smoking directly affect the function of the Lyp protein and what the consequences of this are for neutrophil and T cell function. It was hypothesised that expression of R620W and cigarette smoking would synergise to increase T cell and neutrophil activation, which together could make a significant contribution to inflammation in RA. In order to set the hypothesis in the appropriate context the chapter that follows discusses the role of Lyp in the regulation of signalling networks in neutrophils and T cells, and how changes to these pathways may contribute to the immunopathogenesis of RA.

1.1 The nature of rheumatoid arthritis

The term rheumatoid arthritis (RA) was first documented in the 1800's (Storey et al. 1994) at which time the condition was difficult to diagnose. At present to help guide the diagnostic process, the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria are used in the specific diagnosis of both established and early RA (Aletaha et al. 2010). Classification is based on a points system corresponding to a number of clinical measures including the number of symptomatic joints, duration of symptoms and the presence of antibodies and other protein markers in the blood.

RA is a heterogeneous disease with a wide variety of clinical features. However, chronic inflammation of the synovium is present in all cases. Using histological analysis, the predominant features and outcomes of this inflammation have been identified and include large numbers of infiltrating leukocytes, increased angiogenesis, a hyperplastic synovial lining and local proliferation of synoviocytes, resulting in the formation of invasive synovial tissue (Lee et al. 2001). More advanced techniques have allowed further characterization of the rheumatoid joint environment and have revealed the presence of many cytokines including tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). In addition, attractant chemokines and increased expression of some adhesion molecules have been observed, which are thought to promote the recruitment and retention of immune cells at the site of joint inflammation (Lee et al. 2001).

The amount of synovial fluid (SF) in the joints of RA patients is greatly increased when compared to a healthy joint, as a result of high numbers of infiltrating immune cells and a thickened synovial membrane (Firestein 2003). Some of the main cell types which contribute to this infiltrate are synoviocytes, macrophages, neutrophils, B cells and T cells (Scott et al. 2010). Pannus tissue comprising mostly of activated macrophages and fibroblast-like cells forms across articular joint surfaces and this leads to proteinase-dependent destruction of

the cartilage and bone matrix within the joint (Larbre et al. 1994). Fusion of bone ends occurs through formation of scar tissue at sites of damage as the body attempts to repair the damaged joint. This disease process is known as ankylosis, which is the cause of the characteristic joint deformity observed in RA (Stecher 1958).

1.2 Potential causes of RA

Although there are now sophisticated classification criteria (Aletaha et al. 2010), the aetiology of RA remains largely unexplained. A range of potential autoantigens have been linked to RA, which have been identified by reactive antibodies present in patient sera. These can be categorised as (1) joint-associated, such as proteoglycans and collagen type II and (2) non joint-associated, for example citrullinated proteins, glucose-6-phosphate isomerise and heat shock proteins (Corrigall et al. 2002). Antibodies against citrullinated proteins are of particular importance and the antigens are commonly produced by deimination of arginine residues by the enzyme peptidylarginine deiminase (PADI) (Yamada et al. 2005). They are usually identified by enzyme-linked immunosorbent assay (ELISA) using a surrogate cyclised citrullinated peptide (CCP).

Citrullination of proteins alters the antigenicity of self-peptides through the conversion of positively charged arginine residues to less charged citrulline, resulting in structural changes and protein unfolding. It has been shown that these altered peptides, neoepitopes, may bind with a higher affinity to DR4, which allows recognition of these self-peptides in an major histocompatibility (MHC) class II dependent manner (Hill et al. 2003). These neoepitopes can also be recognised by anti-citrullinated protein antibodies (ACPA), the most common autoantibodies specific to RA (Yamada et al. 2005) and the appearance of which can precede disease onset by up to ten years (Nielen et al. 2004). Additionally a genetic polymorphism in PADI4, an isoform of the enzyme PADI, has been associated with an increased risk of developing RA through an upregulation of PADI enzyme activity (Suzuki et

al. 2003). Overall, the presence of ACPA and increased activity of the enzyme PADI in the joint are likely to play a pivotal role in pathogenesis of RA.

Another protein modification implicated in RA pathogenesis is carbamylation (Trouw et al. 2013). Carbamylation occurs when lysine residues react with cyanate to form homocitrulline. This modification has a very similar structure to citrulline, however contains an extra methylene group. A large proportion of carbamylation has been suggested to occur during inflammatory processes, in particular the release of myeloperoxidase by neutrophils (Wang et al. 2007, Sirpal 2009). Myeloperoxidase is an enzyme which catalyses the conversion of thiocyanate to cyanate, providing a source of cyanate required for carbamylation reactions. Given the increased activity of neutrophils observed in the RA joint (Wright et al. 2010) this could favour the modification of self-peptides to alter their immunogenicity, similar to the process of citrullination. Furthermore, carbamylated proteins have been found in the joints of RA patients (Trouw et al. 2013) where they could be involved in T cell activation and production of autoantibodies (Mydel et al. 2010, Turunen et al. 2010). Indeed, autoantibodies which recognize carbamylated proteins containing homocitrulline have been observed in the sera of around 50% of RA patients and can be used as a predictor of more severe ACPA-negative disease (Shi et al. 2011). In summary, carbamylation may be one alternative mechanism by which the antigenicity of self-peptides is altered in ACPA-negative patients, rather than predominantly by citrullination in ACPA-positive patients.

Other autoantibodies have also been implicated in the disease such as Rheumatoid Factor (RF), an antibody directed against the Fc portion of IgG (Firestein 2003). The IgM form of RF is known to be secreted by B cells at the site of synovial inflammation in around 70% of RA patients, but has also been observed in other autoimmune conditions such as primary Sjögrens syndrome and systemic lupus erythematosus (SLE) as well as healthy ageing individuals (Nell et al. 2005). The role of RFs in pathology seems to stem from their ability to undergo affinity maturation, as RFs in healthy individuals are of low affinity and remain

polyreactive (Borretzen et al. 1997). Physiologically, RFs have important functions in the immune response including facilitating fixation of complement (Brown et al. 1982) and aiding the clearance of immune complexes (Van Snick et al. 1978). It is believed that if there is a high titre of RFs with increased affinity as in RA, these functions are modified and thus RFs become pathogenic.

There are a range of treatment options for RA, the most successful directed towards specific components of the disease. It is strongly recommended that treatment begins as soon as the diagnosis of RA is confirmed, as evidence indicates that early treatment is associated with a better disease outcome (Lard et al. 2001, Raza et al. 2006). To date some of the most effective drugs have been Methotrexate, Rituximab, Etanercept and Infliximab, with many studies illustrating the efficacy of these drugs in the treatment of RA (Emery 2006). Methotrexate was first used due to its effects on lymphocyte proliferation (Gubner et al. 1951). A number of other anti-inflammatory effects of Methotrexate have been reported since including immunosuppression via increased production of adenosine, generalised inhibition of leukocyte proliferation, induction of leukocyte apoptosis and decrease in cyclo-oxygenase and lipoxygenase product formation (Cutolo et al. 2001). Rituximab is a chimeric monoclonal antibody against the surface molecule CD20, found on B cells. The exact mechanism of action of the drug is unclear, but treatment has been shown to severely deplete peripheral B cells. Etanercept and Infliximab are anti-TNF agents which directly bind to TNF-α to reduce its bioavailability. This has anti-inflammatory effects and reduces the level of inflammation and joint destruction in RA patients (Emery 2006). Although new and improved treatments are always in development, research into the trigger for RA is vital to prevent development of the condition in the first place, as present treatments only relieve symptoms and do not often induce remission.

1.2.1 Genetic risk factors

It is evident that RA is strongly dependent on genetics, as disease concordance has been shown to be 15% in monozygotic twins and 4% in dizygotic twins (Cook et al. 1993). However it is also clear that genes alone do not account for all of the disease risk suggesting that other factors, perhaps environmental, play a pivotal role. The primary risk factor for RA is female gender, with as few as 25% of patients being male (Ahlmen et al. 2010). The gender bias is a feature of most autoimmune diseases and is believed to be due to the fact that immune responses are enhanced in females when compared to males. Features of this enhanced immune cell response include an increased CD4:CD8 T cell ratio and increased immunoglobulin levels, indicating a better response to antigen stimulation (Lichtman et al. 1967, Amadori et al. 1995). This is advantageous for responding to infectious challenge, however presents problems in later life in the context of inappropriate immune activation. These differences between males and females indicate a role for sex hormones in controlling the responses of the immune system, in particular the hormone oestrogen. Increased levels of oestrogen have been observed in the synovial fluid of RA patients, and oestrogen is known to increase the proliferation of synovial cells including synovial fibroblasts and macrophages, which could result in joint symptoms associated with RA (Castagnetta et al. 2003, Cutolo et al. 2004). There are further studies that support the role of hormones in RA susceptibility, for example male RA patients have been shown to have reduced levels of testosterone, DHEA and estrone, while levels of estradiol (a form of oestrogen) are significantly increased and correlate with levels of joint inflammation (Tengstrand et al. 2003).

In addition to gender bias, numerous genome wide association studies (GWAS) have identified specific genetic variants which predispose to RA (summarised in Table 1.1). In total, 101 RA risk loci have been identified in individuals of European ancestry (Eyre et al. 2012, Okada et al. 2014). The majority of these genes code for proteins which participate in the immune response. The two genes which show the highest association are (1) MHC class

II, which codes for the human leukocyte antigen (HLA) alleles involved in antigen recognition and (2) protein tyrosine phosphatase non-receptor type 22 (PTPN22), which codes for the protein tyrosine phosphatase Lyp involved in the regulation of signalling through immune cell receptors.

Locus	SNP ID	Candidate gene(s)	OR (95% CI)
6p21	rs6910071	HLA-DRB1	2.88 (2.73–3.03)
1p13	rs2476601	PTPN22	1.94 (1.81–2.08)
6q23	rs5029937	TNFAIP3	1.40 (1.24–1.58)
6q23	rs6920220	TNFAIP3	1.22 (1.16–1.29)
2q32	rs7574865	STAT4	1.16 (1.10–1.23)
9q33	rs3761847	TRAF1, C5	1.13 (1.08–1.18)
1p13	rs11586238	CD2, CD58	1.13 (1.07–1.19)
2p16	rs13031237	REL	1.13 (1.07–1.18)
1q23	rs12746613	FCGR2A	1.13 (1.06–1.21)

Table 1.1 Validated risk alleles for sero-positive rheumatoid arthritis in individuals of European ancestry. Established risk alleles presented in order of highest to lowest odds ratio of risk. Table adapted from **(Scott et al. 2011)**. Single nucleotide polymorphism (SNP), Odds ratio (OR).

1.2.1.1 MHC Class II, PTPN22 and RA susceptibility

MHC class II molecules are involved in antigen presentation to T cells in the periphery and during thymic selection. To allow the immune system to respond to antigen, MHC class II associates with intracellular peptides produced from an extracellular protein antigen to form a complex which is then presented to CD4+ T cells. Genes for MHC class II in humans are located on chromosome 6 in region p21, which contains sequences for the HLA alleles (Gregersen et al. 1987). HLADRB1 is a subgroup of these alleles, which contains what is commonly known as the 'shared epitope' as all the proteins produced by this gene region share a structurally similar motif in their extracellular domain (Gregersen et al. 1987). This shared epitope region comprising of a 5 amino acid (aa) motif can be found in the β1 domain of the HLA-DR molecule, a type of MHC class II surface receptor which is present in around 50% of all RA patients (Salvarani et al. 1998). The shared epitope is believed to contribute to RA by altering the interactions of antigen with the T cell receptor (TCR) either during T cell differentiation or in the periphery, to facilitate an inappropriate immune response.

Until recently, very little was known about how self-peptides bound to HLA molecules specifically contribute to autoimmunity. In terms of RA, the strongest genetic association is located within the HLADR4 alleles, a sub group which is part of the HLADRB1 genes. The HLADRβ chain in particular contains a highly polymorphic region at the N-terminal which corresponds to positions 70-74 in the aa sequence (Viatte et al. 2013). Within this sequence a positively charged residue is present at position 71, which is believed to have a critical influence on the aa present in the P4 pocket of the antigen-binding groove, essential for stabilising interaction of the antigen with the TCR (Hammer et al. 1995). Alleles which contain this 70-74 conserved region are described as a shared susceptibility epitope. Aside from aa position 71, studies comparing RA patients and healthy controls have identified position 11, 13 and 74 to also be important in influencing RA susceptibility suggesting that these regions in particular allow the binding and presentation of self-peptides (Raychaudhuri

et al. 2012). In particular, the HLADRB1 alleles are most strongly associated with ACPA positive RA, leading to the suggestion that the HLA molecules can restrict or promote the binding and presentation of citrullinated autoantigens. In support of this, the crystal structure of the RA-associated HLADRB1*04:01 molecule bound to different antigens has recently been studied (Scally et al. 2013). This showed that the P4 pocket of HLADRB1*04:01 preferentially accommodates citrulline rather than arginine, providing a structural explanation as to why citrullinated autoantigens may preferentially bind and be presented in RA patients with this allele. In contrast, it was shown that the opposite was true of the RA-protective allele HLADRB1*04:02, which preferentially accommodated arginine rather than citrulline. Overall, these findings suggest that the association of the shared epitope with RA is due to changes in the ability of the HLADR4 molecules to bind citrulline, which is crucial in the pathogenesis of ACPA positive RA.

The PTPN22 gene encodes the protein Lyp, a PTP involved in the regulation of signalling through immune cell receptors. Lyp function is most well characterized in T cells where it is a negative regulator of TCR signal transduction (Hermiston et al. 2009). This is achieved mainly via Lyp dephosphorylating activating tyrosine residues of Src-family kinases such as Lck. Lyp is believed to have a similar negative regulatory role in other cell types, however further study is required to ascertain its specific function. A polymorphic variant of PTPN22 exists known as PTPN22 R620W, which has been widely associated with an increased risk of developing RA and other autoimmune diseases (Burn et al. 2011). The variant results from a single base substitution which confers a change in the encoded protein at position 620 from arginine to tryptophan, and has been shown to reduce the ability of Lyp to interact with its binding partner C terminal Src kinase (Csk) in T cells (Begovich et al. 2004, Bottini et al. 2004).

1.2.1.2 Other genes and RA susceptibility

Aside from genetic changes in MHC Class II and PTPN22, a large number of other genes have also been associated with an increased risk of RA, many of which are involved in regulating the immune response. For example, Tumour necrosis factor alpha inducible protein 3 (TNFAIP3) is a gene which encodes the ubiquitin-modifying enzyme A20, a negative regulator of responses to TNF-α, TLR and NOD signalling mediated by activation of the transcription factor NfκB (Coornaert et al. 2009). TNFAIP3 has an important role in regulating inflammation and mice lacking TNFAIP3 display increased inflammation in multiple organs including synovial joints (Lee et al. 2000). Polymorphisms in this gene have been associated with an increased risk of RA in humans and a number of other autoimmune diseases, for example SLE (Plenge et al. 2007, Thomson et al. 2007). There are few studies looking at the functional consequences of these disease-associated variants, however *in vitro* testing has suggested that expression of the disease-associated variants results in decreased TNFAIP3 mRNA and decreased A20 protein expression, which overall would result in increased inflammation (Adrianto et al. 2011).

Another important gene is signal transducer and activator of transcription 4 (STAT4) which is a gene encoding the STAT4 protein, a transcription factor which aids cytokine signalling between T cells and monocytes. These cytokines include type I interferon, IL-12 and IL-23 which stimulate T cell differentiation and monocyte activation (Watford et al. 2004). Polymorphisms in the STAT4 gene alter activation of these immune cells and have been associated with multiple autoimmune diseases including RA (Amos et al. 2006, Remmers et al. 2007). Like many genes, it is not known exactly how the polymorphism contributes to disease pathogenesis, however the STAT4 protein is expressed by lymphocytes, monocytes, NK cells, macrophages and DCs, indicating perhaps multiple effects in multiple cell types (Thierfelder et al. 1996, Fukao et al. 2001).

In addition to TNFAIP3, another gene which is important in TNF receptor signalling is TNF receptor-associated factor 1 (TRAF1). TRAF1 is located on chromosome 9 of the human genome and has been implicated as a susceptibility gene for RA. The TRAF1 gene encodes the protein TRAF1 which forms a heterodimeric complex with its associated protein TRAF2, to allow TNF-mediated activation of the signalling proteins MAPK8/JNK and the transcription factor NfκB (Carpentier et al. 1999). This results in the generation of anti-apoptotic signals mediated by TNF receptors and an overall increase in inflammatory responses. Another gene which is associated with the TRAF1 coding region is Complement component 5 (C5). This is vital for cell killing in many inflammatory processes and the cleavage of C5 generates the pro-inflammatory C5a and an important initiator of the membrane attack complex, C5b (Guo et al. 2005). The TRAF1/C5 genetic locus is known to contain many SNPs, many of which have been associated with an increased risk of RA (Zhu et al. 2011). SNPs in these two genes could contribute to disease by altering TNF and its associated inflammatory signalling pathways, for example by increasing TNF-mediated activation of NfκB and increasing C5a and C5b generation. Overall, this could increase levels of inflammation and could contribute to heightened TNF signalling and responses in RA.

As discussed, a large number of the polymorphisms associated with RA are located within genes involved in the immune response. This is hardly surprising given that a large number of changes to the immune system and inflammatory responses are present in RA. Two common themes of these genes is their involvement in T cell signalling as part of the adaptive immune response and the importance of the cytokine TNF. T cells have been thought to be important in the pathogenesis of RA for a long time, and targeting T cells as a treatment for RA has proven successful (Kremer et al. 2003). Similarly, the importance of TNF in RA pathogenesis has also been well documented, with one of the most successful treatments being anti-TNF therapy (Emery 2006). Based on these observations, it seems

reasonable that targeting TNF signalling pathways in multiple immune cell types including T cells would be advantageous in individuals with a genetically susceptible background.

1.2.2 Environmental risk factors

As mentioned previously, RA is a multi-factorial disease which is highly influenced by external factors present within the environment. Using analysis of heritability and twin studies, it has been estimated that genes account for approximately 60% of the risk of disease (Cook et al. 1993, MacGregor et al. 2000) attributing the remaining 40% to other environmental factors. Several environmental factors have been identified as significant contributors to RA including ageing, cigarette smoking (CS), lack of antioxidant intake, exposure to road traffic and previous infections (Liao et al. 2009). All these factors have been shown to modulate the responses of the immune system and some have specific effects on signalling through immune cell receptors.

Ageing occurs in almost all organisms and the risk of most human inflammatory conditions including rheumatic disease, increases with age (Burkle et al. 2007). The theory of inflammageing hypothesises that the features of ageing can be attributed to an imbalance between the immune systems inflammatory and anti-inflammatory processes, which induces a permanent low-grade inflammation referred to as inflammageing (Franceschi et al. 2007). This is of relevance to RA since the peak of disease onset is mid to late middle age, which leads to the suggestion that older immune cells could be ‘primed’ to exhibit a more aggressive inflammatory response when activated.

Studies on CS have illustrated that smokers have an increased risk of RA of at least 100% when compared to non-smokers, without even considering the presence of any other risk factors (Sugiyama et al. 2010). The components of CS are known to be potent activators of the immune system and associated inflammatory responses. Smoking introduces large numbers of reactive oxygen species (ROS) into the body via the lungs (Borgerding et al.

2005), which cause damage to cellular components mainly by oxidation. This is due to depletion of essential antioxidants required to maintain cell growth and repair and is illustrated by findings of decreased circulating antioxidants in the blood of even healthy smokers (Alberg 2002). CS also increases levels of cyanate, another reactive species known to be required for carbamylation reactions which modify proteins by converting lysine residues to homocitrulline (Wang et al. 2007). These processes of oxidation and carbamylation could be important mechanisms by which smoking alters the immunogenicity of self-peptides to promote RA.

Environmental exposure to certain infections has also been implicated as a potential trigger for RA. Several infectious agents including bacteria and viruses have been suggested, but at present the evidence for a specific causal infectious agent is lacking. The infection most well studied is Epstein-Barr virus (EBV), which has been estimated by the World Health Organisation (WHO) to have infected 95% of the adult population worldwide (www.who.int/en). It has been observed that the EBV-load in RA patients is around 10 fold higher than healthy individuals, and increased titres of antibodies to the virus are also present (Balandraud et al. 2004). These observations suggest differences in handling of the virus by the immune system and implicate EBV as a possible influencing factor for changes in the immune response observed in disease.

1.3 Immune cell signalling in RA

RA is a complex disorder which may result from an inability of the immune system to co-ordinate innate and adaptive immune responses, and identifying the cell type in which the initial change occurs is problematic. For example the first events in the development of RA, just as in most infections, could be activation of components of the innate immune system such as neutrophils, macrophages and dendritic cells (DCs) (Fearon et al. 1996, Nathan 2006). This may trigger unwanted adaptive immune responses involving B and T cells

through presentation of 'self' antigens (Firestein 2003). Alternatively, innate immune responses may be needed to support disease progression and cells such as macrophages may only become important later in the destructive phase of the disease (Mulherin et al. 1996). To date, numerous cell types have been implicated in the initiation of RA.

A popular model of disease development considers multiple pathogenic mechanisms that begin with activation of the innate immune system (Figure 1.1) (Firestein 2003). This model suggests that DCs, macrophages, fibroblasts and mast cells are activated via complement, immune complexes and Toll-like receptors (TLRs). This results in mass migration of immune cells to the synovium, directed by cytokine and chemokine gradients, providing an opportunity for adaptive immune responses involving B and T cells in susceptible individuals (Firestein 2003).

In the more advanced stages of RA, osteoclasts become activated and synoviocytes begin to invade the cartilage in a destructive manner. This model could accounts for 'flares' of severe symptoms seen in RA, suggesting that these are caused by activation of the innate and adaptive immune system in parallel. Using this model of disease progression it is clear T lymphocytes play a pivotal role in pathogenesis, but other innate immune cells and their associated inflammatory mediators could be involved early on. For example, large numbers of neutrophils can be found at the destructive interface between the pannus and cartilage and also in the SF of joints affected by RA (Mohr et al. 1981). If appropriately activated, these cells of the innate immune system can produce cytokines which contribute to the maintenance of inflammation in the joint, including IL-1 and TNF-α (Cassatella 1995). Activated neutrophils also have the potential to express MHC class II molecules, which could allow activation of T lymphocytes in the joint through presentation of antigen (Gosselin et al. 1993). Studies are being carried out to try and address these issues of initiating cell type, by studying cohorts of early RA patients to characterise immunological changes at specific stages of the disease (Raza et al. 2005).

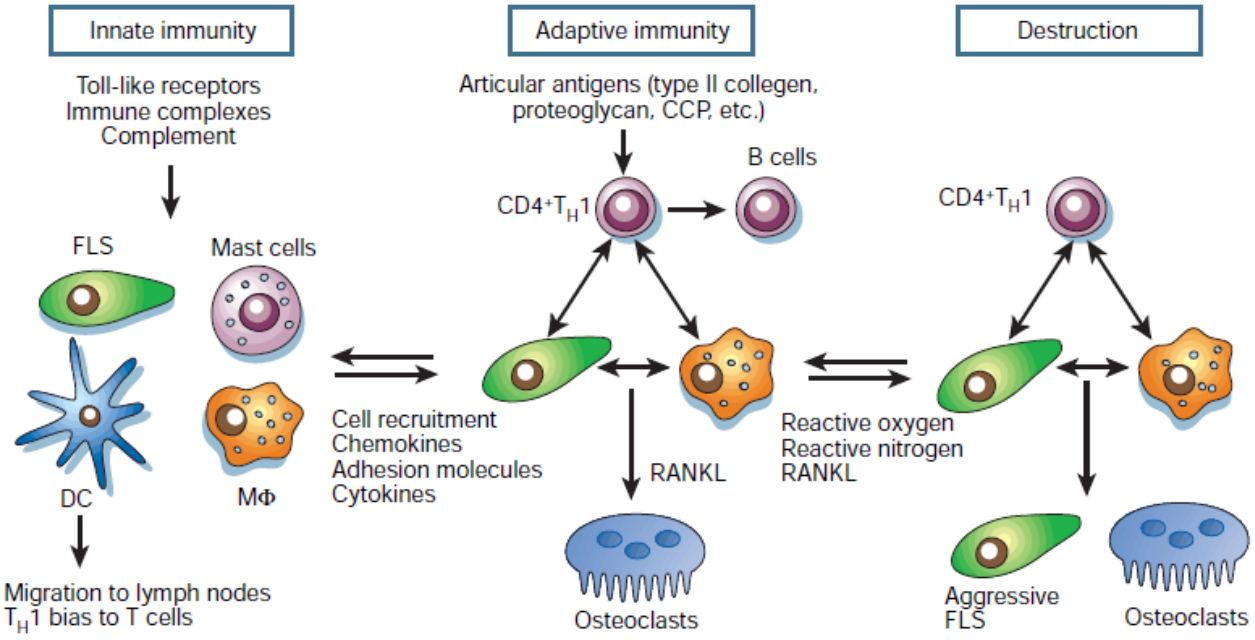


Figure 1.1 A proposed model implicating multiple pathogenic mechanisms and cell types in the development of rheumatoid arthritis. Figure taken from (Firestein 2003). Abbreviations: DC, dendritic cell; CCP, cyclic citrullinated peptide; FLS, fibroblast-like synoviocyte; MΦ, macrophage.

1.3.1 Fibroblast-like synoviocytes

Fibroblast-like synoviocytes (FLS) are also known as Type B cells and are located within the intimal lining layer of the joint. They are present in a thin layer only 2-3 cells thick which is comprised of approximately half FLS and half macrophage-like synovial cells, which are also known as Type A cells (Bartok et al. 2010). In RA, the intimal lining layer of the joint greatly increases in cellularity to a layer of 10-20 cells thick due to expansion of both Type A and Type B cells. It has been suggested Type B cells (FLS) are activated by pro-inflammatory cytokines, chemokines and growth factors produced by Type A cells (Bartok et al. 2010). Activation of FLS results in production of mediators such as prostanoids, IL-6 and matrix metallo-proteinases (MMPs) which can contribute to joint inflammation and destruction (Noss et al. 2008). Due to this highly activated state, the FLS take on an invasive phenotype which results in destruction of the joint structures, in particular cartilage (Firestein 2003).

1.3.2 Osteoclasts

Osteoclasts are essential for bone shaping and remodelling and are able to carry out these functions using a proton/protein pump to solubilise calcium (Ca^{2+}) and matrix degrading enzymes such as cathepsins and MMPs (Blair et al. 1989, Schett 2007). Osteoclasts normally create resorption pits at the surface of bones before the arrival of osteoblasts which lay down a new bone matrix. However, pathological osteoclast function can also occur and in RA is triggered by local joint inflammation eventually resulting in bone erosion (Bromley et al. 1984). A key mechanism driving the inappropriate function osteoclasts in the inflamed joint is thought to be through the production of IL-17 and receptor activator of NfκB ligand (RANKL) by B and T cells infiltrating the joint (Shigeyama et al. 2000, Sato et al. 2006).

1.3.3 Dendritic cells

DCs are the major antigen presenting cells of the immune system and have a range of functions including priming of naïve T cells, enhancing the function of NK cells and also playing a role in central and peripheral tolerance (Kitamura et al. 1999). There are three main mechanisms by which DCs could be involved in RA pathogenesis, (1) priming of autoimmune responses in lymphoid organs, (2) production of inflammatory mediators and (3) processing and presentation of self-antigens in the synovium (Lutzky et al. 2007). Migration of DC precursors into secondary lymphoid organs is known to occur in the absence of an inflammatory insult and is believed to have a role in the induction of tolerance (Kurts et al. 1997, Steptoe et al. 2005). However, migration and stimulation of DC induces activation and maturation which could then activate lymphocytes to produce pro-inflammatory cytokines (Sallusto et al. 1999). DCs themselves can also produce pro-inflammatory cytokines including IL-1, IL-6, IL-12, IL-23 and TNF-α (Blanco et al. 2008), which could activate other immune cells infiltrating the synovium and directly contribute to an inflammatory environment. Furthermore, DCs are known to be present in the inflamed joints of RA patients (Thomas et al. 1994) and could be involved in presenting autoantigens such as citrullinated proteins to prime lymphocyte responses.

1.3.4 Macrophages

Macrophages are a key cell type involved in innate immune responses and are derived from the differentiation of monocytes following entry into tissues. They are broadly classified into two types, M1 macrophages which are pro-inflammatory and M2 macrophages which are anti-inflammatory (Martinez et al. 2009). Macrophages can be found in large numbers in the rheumatoid synovium and display a highly activated phenotype characterised by production of cytokines, high expression of MHC class II molecules and increased production of chemokines and chemoattractants (Yanni et al. 1994, Huang et al. 2007). Furthermore it has

been found that numbers of macrophages in the joint correlate with levels of inflammation (Tak et al. 1997) suggesting macrophages are important in initiating or maintaining inflammation in RA. This could be through the ability of macrophages to interact with other cell types in the joint including resident cells (fibroblasts) and infiltrating cells (T lymphocytes). Also, TNF-α is a key cytokine in the pathogenesis of RA and the largest producers of this cytokine in the joint are macrophages (Feldmann et al. 1996). Lastly, macrophages have been implicated as an important cell type mediating joint destruction due to their ability to produce proteases which damage the joint architecture (Tetlow et al. 1993).

1.3.5 Neutrophils

Neutrophils are important in innate immunity to infection, and are one of the first cell types to arrive at a site of infection. However, many of the mechanisms used by neutrophils to combat infection can also cause significant tissue damage if neutrophil activation is prolonged or enhanced by certain stimuli. For example, one mechanism by which neutrophils defend against infection is through the production of ROS (Babior 1999). In small quantities these reactive molecules play an essential role in infection control, however an excess of ROS due to increased neutrophil activation can cause damage to cellular proteins and joint components (Hitchon et al. 2004). Neutrophils also produce proteinases such as neutrophil elastase which allow them to quickly destroy invading pathogens. However, these proteinases have been implicated in the pathogenesis of inflammatory diseases such as RA as they also cause damage to host tissues (Pronai et al. 1991). In the affected joints of RA patients, neutrophils are found in large numbers, exhibit resistance to death and show increased effector functions, suggesting that neutrophils are of importance to the disease pathology (Weissmann et al. 1984, Wright et al. 2010).

1.3.6 B lymphocytes

B lymphocytes are responsible for antibody production in response to invading pathogens, but are also the source of autoantibodies characteristic of autoimmune diseases such as RA. Autoantibodies of particular importance in RA are RF and ACPA, which have been shown to activate complement and promote the formation of immune complexes in the joint (Vaughan et al. 1975, Firestein 2003). Aside from autoantibody production, B lymphocytes are thought to contribute to RA pathogenesis through their interactions with T lymphocytes (Silverman et al. 2003). This could be by expression of costimulatory molecules which promote T lymphocyte activation or by the presentation of antigen to autoreactive T lymphocytes. In support of this, it has been shown that sustained T lymphocyte activation in the RA synovium is dependent on the presence of B lymphocytes (Takemura et al. 2001). In addition, B lymphocytes are a source of cytokines in the joint which support the survival of other immune cells. In particular, B lymphocytes found in the SF of RA patients have been shown to express RANKL, a key cytokine involved in driving joint destruction through activation of osteoclasts (Yeo et al. 2011).

1.3.7 CD4+ T lymphocytes

CD4+ T cells are important in adaptive immune responses and are activated by antigens associated with MHC class II binding to their TCR. This process is associated with interactions of costimulatory molecules such as CD80/CD86 expressed on the surface of APCs and alterations in these interactions between T cells and APCs have been associated with autoimmune diseases including RA (Zikherman et al. 2009). The strongest evidence for alterations in antigen recognition by CD4+ T cells in RA comes from genetic studies on the shared epitope, the strongest genetic risk factor associated with RA (Gregersen et al. 1987). It is thought antigen presentation to T cells can occur in the synovium or at extra-articular sites and the skewing of T cells to a Th1 phenotype is a key step in disease progression

(Firestein 2003). T cell specificity is also thought to be of importance and it has been shown that some T cell specificities preferentially migrate to the synovium via their increased ability to respond to chemokines present within synovial tissue (Shadidi et al. 2002).

1.4 T cell signalling and activation

In order for T cells to differentiate and proliferate they must be activated by the antigen/MHC complex binding to their TCR, as seen in Figure 1.2. The TCR signalling cascade begins when a peptide antigen bound to MHC I (CD8+ T cells) or II (CD4+ T cells) engages with the αβ chains of the TCR (Zikherman et al. 2009). Further signal transmission is dependent on phosphorylation of immunoreceptor tyrosine-based activating motifs (ITAMs) found within the δ, γ, ε, and ζ chains of CD3 and the TCR. These interactions are stabilised by binding of T cell co-receptors CD4/CD8 to the non-variable regions of the MHC component. This triggers ligation of CD4/CD8 and the kinase Lck, the predominant activating Src-family kinase in T cells (Hermiston et al. 2009). CD4/CD8 clustering induces transphosphorylation of Lck and signalling continues through phosphorylation of ITAMs situated within the CD3 and TCRζ chains of the TCR. Phosphorylated ITAMs contain SH2 domains, which are then used to recruit Syk-family kinases, such as Zap-70 (Zikherman et al. 2009). Zap-70 is phosphorylated by the kinase Lck, which in turn triggers an autophosphorylation mechanism to fully activate the kinase. A number of other kinases are also activated by phosphorylation including the Tec kinases, (Tec, Bmx, Itk and Rlk/Txk) of which Itk and Rlk/Txk are exclusively expressed by T cells and are important for calcium (Ca^{2+}) release downstream of the TCR (Takesono et al. 2002). Further signalling occurs when activated Zap-70 phosphorylates adaptor molecules SLP76 and LAT, which form a scaffold to assist assembly of numerous molecules including phospholipase C (PLC) γ1. Zap-70 is also involved in the activation of Vav1, an atypical guanine exchange factor which plays a key role in the formation of the immune synapse formed between the T cell and APC, through its ability to rearrange the T cell cytoskeleton (Swat et al. 2005). The final stages of TCR signalling

involve phosphorylation of mitogen-activated protein kinases (MAPKs), transactivation of the IL-2 gene and an increase in free Ca^{2+} within the cytosol (Zikherman et al. 2009).

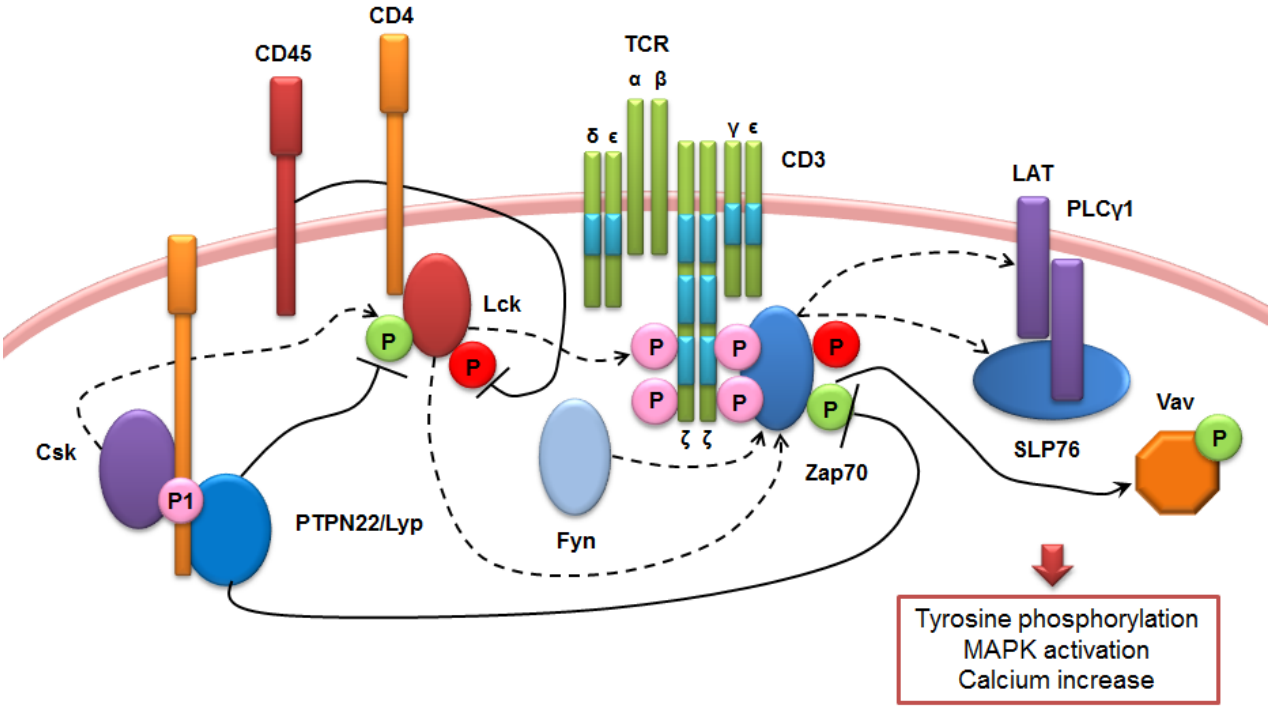


Figure 1.2 Schematic representation of some of the interactions of major protein tyrosine kinases and protein tyrosine phosphatases involved in T cell receptor signalling. Dashed lines represent phosphorylation events and solid lines represent dephosphorylation events. Solid arrows represent protein recruitment. Blue bars represent CD3 immunoreceptor tyrosine-based activating (ITAM) domains, not all are shown. Not all known interactions are depicted. MAPK, mitogen-activated protein kinase; PLCγ1, P, phosphotyrosine (green represents an activating residue, and red represents an inhibitory residue); Phospholipase C γ1; TCR, T cell antigen receptor.

1.4.1 Regulation of TCR signalling

TCR triggering is a tightly controlled process and down regulation of signalling through the receptor is equally as important as its efficient activation. Reversible tyrosine phosphorylation is an important mechanism used by T cells (Figure 1.2) and other immune cells to set the threshold for signalling through their receptors. Two classes of proteins are involved, (1) protein tyrosine kinases (PTKs) which act to phosphorylate tyrosine residues and (2) protein tyrosine phosphatases (PTPs) which act to dephosphorylate tyrosine residues. There is much more known about the function of PTKs compared with PTPs, but often the role of PTPs is more complex than simply to reverse the effects of PTKs.

Lck and Fyn are the predominant Src-family kinases (SFKs) expressed by T lymphocytes throughout their development and lifespan (Cooke et al. 1989, Thomas et al. 1997). Lck is primarily located near the plasma membrane, whereas Fyn is associated with mitotic and centrosomal structures. This difference in location is likely to be due to differences in the unique domain of each respective kinase. The unique domain of Lck is known to be a mediator of the interactions between Lck and CD4/CD8 co-receptors in TCR signal transduction. Lck activation is essential for Ca^{2+} release downstream of TCR activation, as T cells lacking Lck are unable to flux Ca^{2+} in response to anti-CD3 stimulation (Straus et al. 1992). Fyn is believed to have a role in regulating the duration of TCR engagement, cytoskeletal re-arrangement and adhesion. The duration of TCR engagement is an important factor which determines if a T cell will commit to effector functions such as proliferation and IL-2 production which are required for induction of memory responses. While Lck is important in TCR triggering, Fyn is known to regulate antigen responses and to be involved in memory T cell generation (Davidson et al. 2007). Fyn is known to oppose some of the activating functions of Lck, for example CD8 T cells lacking Fyn produce increased amounts of IL-2 following TCR activation (Filby et al. 2007). This suggests Fyn is a negative regulator of some T cell effector functions and is important in preventing over-activation of T cells.

As well as PTKs which phosphorylate tyrosine residues, T cells also express a number of PTPs which dephosphorylate tyrosine residues. There are three PTPs that regulate signalling through the TCR, namely CD148, CD45 and PTPN22/Lyp (Hermiston et al. 2009). There is most known about the function of CD45 which has a positive regulatory role. Upregulation of signalling through the TCR is induced when CD45 dephosphorylates tyrosine residue 505 of Lck, priming the kinase for activation. In contrast the Lyp phosphatase is important in negative regulation, as it dephosphorylates tyrosine residue 394 of Lck which inactivates the kinase. The ability of Lyp to carry out its function is thought to be dependent upon its association and dissociation with its binding partner Csk (Hermiston et al. 2009, Vang et al. 2012). Lastly, CD148 is thought to have a role in negative regulation via dephosphorylation of PLCγ-1 and LAT, substrates downstream of Lck (Hermiston et al. 2009).

1.5 Neutrophil signalling and activation

Recruitment and activation of neutrophils is one of the first events to occur at the site of an inflammatory insult. Stimulants which attract neutrophils can be bacterially-derived including lipopolysaccharide (LPS) and formylated proteins, or cytokines and chemoattractants such as IL-17 and TNF-α (Zhang et al. 2010). Once neutrophils have entered the tissue via transendothelial migration mediated by β2 integrins and Intercellular Adhesion Molecule-1 (ICAM), they can carry out effector functions following appropriate receptor engagement (Ley et al. 2007).

Signalling mediated by formylated proteins is most well characterized and is initiated by bacterially derived formylated proteins binding to formyl peptide receptors (FPRs), a type of classical G-protein-coupled receptor found on the neutrophil surface (Wittmann et al. 2002). The FPR contains seven hydrophobic segments connected by hydrophilic domains, and there are a number potential phosphorylation sites present within the receptor structure (Liu

et al. 2004). Following receptor engagement a conformational change occurs to enable FPR interaction with G proteins and the exchange of GDP to GTP binding within the α subunit. This triggers further signalling to generate diacylglycerol (DAG) through activation of phospholipase C β, which induces the activation of protein kinase C (PKC) and inositol-1,4,5-trisphosphate (Niggli 2003). This process causes Ca^{2+} release from intracellular stores and later by an influx of Ca^{2+} across the plasma membrane. Also downstream of G protein signalling is activation of the MAPK cascade, PTPs and PTKs (Berton et al. 1999). These involve tyrosine phosphorylation events which are important in the regulation of multiple neutrophil effector functions including oxidant production, migration, chemotaxis, phagocytosis and priming (Gomez-Cambronero et al. 1989, Akimaru et al. 1992).

1.5.1 Regulation of neutrophil signalling

When compared to T lymphocytes, the roles of PTKs and PTPs in neutrophil signalling and activation are much less well characterized. Kinases such as Syk and the Src family kinases Fgr, Lyn and Hck are all expressed by neutrophils and can be activated by engagement of a number of receptors (Gaudry et al. 1992). Using mice lacking these kinases a role in the regulation of integrin signalling has been identified (Mocsai et al. 2002, Pereira et al. 2003). For example it has been observed that lack of Syk significantly impairs neutrophil spreading onto surfaces coated with fibrinogen or serum, through a lack of integrin-mediated signalling (Mocsai et al. 2002). Given the importance of protein phosphorylation by PTKs, appropriate removal of these phosphorylated tyrosine residues to down regulate signalling is of equal importance to prevent unwanted neutrophil activation.

A number of PTPs are expressed by neutrophils including CD45, PTPN22/Lyp, MEG2 and SHP-1 (Hoffmeyer et al. 1995, Brumell et al. 1997, Kruger et al. 2002, Chien et al. 2003). PTPs are known regulators of neutrophil effector functions but the precise role of specific PTPs in receptor initiated signalling pathways is unknown. PTPN22/Lyp expression levels in

neutrophils are comparable if not higher than CD3+ T cells (Chien et al. 2003) implying that this PTP has an important role in neutrophils. Most studies to date have investigated the function of the PTPs in neutrophils by using mice and cell lines lacking the specific PTP of interest, or through the use of blocking antibodies. The CD45 phosphatase is thought to have a role in the regulation of neutrophil phagocytic function (Hoffmeyer et al. 1995) as well as influencing neutrophil motility (Harvath et al. 1991), but mechanisms behind these observations have not yet been identified. A reduction in phosphatase activity induced by oxidation of the active site of CD45 during neutrophil activation has been suggested to be an important regulatory mechanism. Neutrophil activation induces the production of ROS, which have been shown to inhibit CD45 phosphatase activity (Fialkow et al. 1997), suggesting that CD45 oxidation by ROS could be an important means of regulating receptor-mediated signalling in neutrophils. In addition, CD45 and CD148 are involved in the regulation of chemoattractant-mediated migration of neutrophils (Zhu et al. 2011). CD45 is involved in positive regulation whereas CD148 has been shown to have roles in positive and negative regulation, through effects on G-protein coupled receptor signalling. These differential functions imply that CD45 and CD148 dephosphorylate different signalling proteins in neutrophils in order to carry out their regulatory functions. The role of SHP-1 in neutrophil microbicidal function has been investigated using SHP-1 deficient moth-eaten mice and showed that the phosphatase regulates oxidant production as well as adhesion (Kruger et al. 2000). The PTP MEG2 has been found in the cytosol of neutrophils as well as its components being present in secreted granules and vesicles (Kruger et al. 2002), leading to suggestions that MEG2 is involved in the regulation of phagocytosis in neutrophils. Overall, the roles of PTPs in neutrophil signalling and activation are diverse and largely unexplored.

1.6 Interactions between neutrophils and T cells

Given the interplay between different cell types in the normal immune response, it is important to consider potential interactions between cells involved in innate and adaptive

immunity, and how these could by altered in disease. Interactions between neutrophils and T cells are an example of one set of interactions and can potentially occur at a site of tissue damage as well as in the lymph nodes (Figure 1.3). Migration of neutrophils to the lymph nodes is dependent on CCR7 and once they have reached this location they are able to compete with T cells for antigen through their expression of MHC class II (Mantovani et al. 2011). Neutrophils can also interact with DCs to induce their maturation in the lymph nodes or at sites of tissue damage. It has been shown *in vitro* that monocyte-derived DCs matured by interactions with neutrophils could induce Th1 polarisation and proliferation, which *in vivo* could contribute to a more aggressive inflammatory response (van Gisbergen et al. 2005, Megiovanni et al. 2006). T cells can also directly increase activation and survival of neutrophils by release of pro-inflammatory cytokines including granulocyte-macrophage colony stimulating factor (GMCSF), interferon gamma (IFN-γ) and TNF-α (Pelletier et al. 2010). Studies of murine neutrophils have shown that T cells can induce expression of MHC class II on the neutrophil surface which in turn helps to facilitate the differentiation of Th1 and Th17 cells, important for antigen-specific immunity (Abi Abdallah et al. 2011). The number of potential interactions that can occur between neutrophils and T cells illustrates the complexity of cell signalling within the immune system and the importance of considering multiple cell types and their relevance to disease pathogenesis.

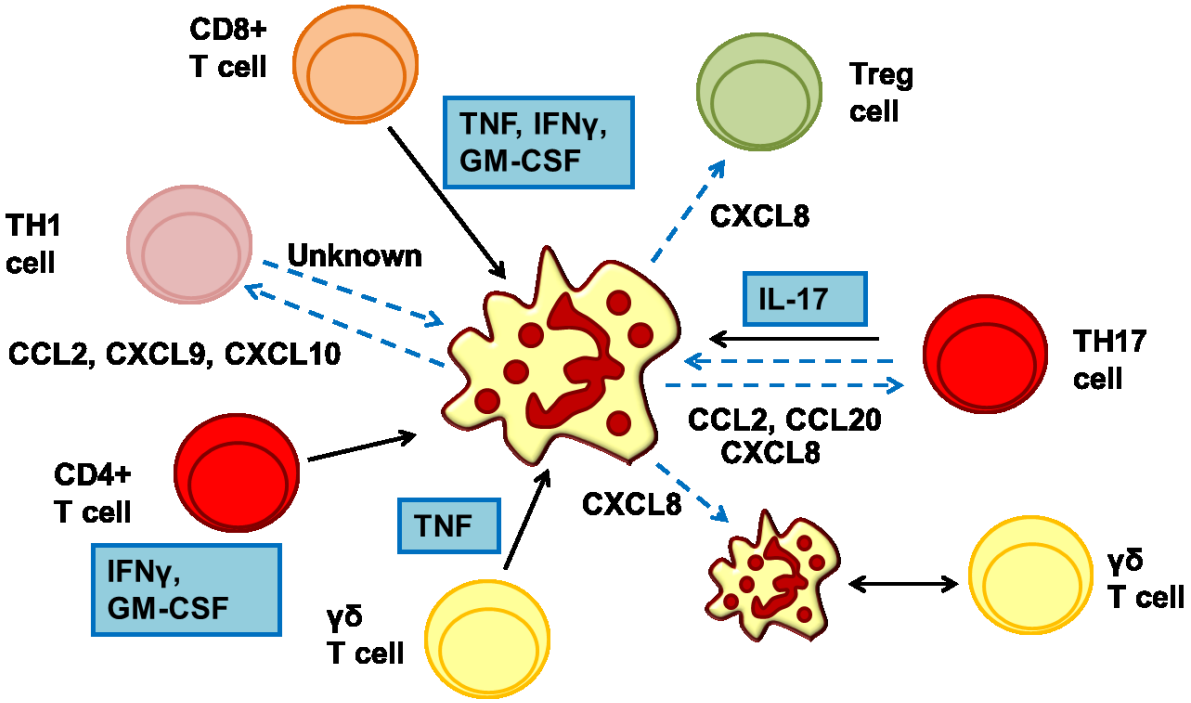


Figure 1.3 Schematic representation of the interactions between neutrophils and T cells. Figure adapted from (Mantovani et al. 2011). Neutrophils can release chemokines to mediate the recruitment of Th1 and Th17 cells to a site of inflammation. T cell subsets including Th1, Th17 and Treg can attract neutrophils through the release of chemokines such as CXCL8. The activation of T cells causes secretion of interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GMCSF), tumour necrosis factor alpha (TNF-α) and interleukin-17 (IL-17). Cytokines such as these increase expression of CD11b on the neutrophil surface and promote cell survival.

1.7 Regulation of signalling by PTPs

An essential requirement for all cells is to efficiently convert signals received from their environment into functional responses. One mechanism by which this is achieved is through reversible phosphorylation events carried out by PTKs and PTPs (Manning et al. 2002). Although the role of PTKs is better characterized, there are more genes within the human genome that code for PTPs responsible for removing phosphotyrosine residues. Specifically, the human genome contains 107 genes encoding PTPs, many of which have a mouse ortholog (105) (Bottini et al. 2004). It is estimated that individual cell types express 30-60% of the total complement of PTKs and PTPs, with hematopoietic and neuronal cells expressing an even larger proportion (Avraham et al. 2000, Bottini et al. 2004). The PTPs CD45 and Lyp are expressed by all leukocytes including T cells and neutrophils. Their specific roles in TCR signalling have been extensively studied however their roles in neutrophil signalling are largely unknown.

1.7.1 CD45

1.7.1.1 CD45 structure and expression

CD45 is a transmembrane PTP consisting of (1) a large extracellular domain (2) a transmembrane domain and (3) a cytoplasmic portion, often described as having a wedge-like structure (Hermiston et al. 2009). It is also referred to as the common leukocyte antigen and is highly expressed by many types of hematopoietic cells (Hermiston et al. 2009). In fact, in some cell types it has been shown to account for 10% of the total cell surface protein (Thomas et al. 1988). The human CD45 protein is 180-220kDa in size and its PTP domains (D1 and D2) are located within the cytoplasmic portion of the protein. Studies have shown that the D1 domain is enzymatically active but for CD45 to function fully as a phosphatase, the D1 and D2 domains must be present (Holmes 2006). The overall organization of the CD45 protein structure is well maintained across species, despite there only being a 35%

homology within the extracellular domain (Hermiston et al. 2009). Eight isoforms of CD45 have been identified at the level of RNA, five of which have been detected as proteins present in humans. Different CD45 isoforms are associated with different stages of T cell activation and differentiation, for example naïve T cells mainly express the RA isoform which then switches to the RO isoform upon activation.

1.7.1.2 CD45 function in T cells

CD45 is thought to be most important in the T cell lineage as profound effects are observed when it is no longer expressed. In CD45 deficient mice, T cell development is partially blocked and signalling through the pre-TCR and TCR is dysfunctional (Hermiston et al. 2009). The kinases Lck and Fyn are hyperphosphorylated on an inhibitory tyrosine residue, suggesting these kinases are the primary substrates for CD45. Further characterization has identified CD45 as the PTP that dephosphorylates tyrosine residue 505 of Lck early on in the TCR signalling pathway (Holmes 2006). This evidence supports the role of CD45 as a positive regulator of signalling through the TCR. There is also some support for CD45 having a role in the negative regulation of TCR signalling and the mechanism by which this occurs is thought to be via dephosphorylation of the activating tyrosine residue 394 of Lck. This has been shown *in vitro*, but only if CD45 is recruited from the plasma membrane into lipid rafts (McNeill et al. 2007). There is debate as to whether this could occur under physiological conditions and if so what the upstream effects on PTPN22/Lyp could be, as it is the conventional PTP acting on tyrosine residue 394 of Lck.

1.7.1.3 CD45 function in other cell types

The absence of CD45 in B cells results in a much less severe consequence, but development of B cells is still impaired at the pro-B cell stage. Upon activation of the B cell receptor (BCR) signalling impairment is observed, as characterized by a decrease in PI3 kinase, nuclear factor kappa B (NFκB) and ERK phosphorylation (Hermiston et al. 2009). The number of natural killer (NK) cells is increased in CD45 deficient mice, implying CD45

has a negative regulatory role in this cell type. Finally, the function of CD45 in cells of the myeloid lineage is less clear. Studies to date have highlighted that regulation of kinases by CD45 is complex in this cell lineage and CD45 is likely to have unique roles in particular myeloid cell types (Hermiston et al. 2009). It should also be taken into consideration the suspected overlap of functions between CD148 and CD45, which could be a potential explanation for functional differences between the lineages.

1.7.1.4 CD45 regulation

The PTP activity of CD45 is likely to be regulated through multiple mechanisms including phosphorylation, oxidation, dimerization, ligand binding and changes in cellular localization (Rider et al. 2003, Hermiston et al. 2009). Phosphorylation of the D2 domain by casein kinase II and oxidation of the catalytic site cysteine residue by ROS have both been observed (Wang et al. 1999, Rider et al. 2003). This significantly reduces the intrinsic activity of the CD45 phosphatase and consequently reduces TCR activation. With regards to dimerization, CD45 dimers have been identified physiologically (Hermiston et al. 2009) and it has been suggested that this alters the availability of different CD45 isoforms. This may determine a cells surface receptor expression at key stages of development and differentiation. Several ligands have been shown to bind non-specifically to CD45, most of which are glycoproteins. The functional significance of this is unclear but a regulatory role is a possibility. Lastly, changes in cellular location are important as this affects the ability of CD45 to access its substrate. For example, during formation of the immunological synapse between a T cell and APC the CD45 phosphatase is initially excluded from the central contact area between the two cells to allow activation of integrins (Johnson et al. 2000). Following this step, CD45 is relocated back to the central area near to the site of TCR engagement. This allows the CD45 phosphatase to activate the Src-family kinase Lck in order to sustain TCR signalling (Johnson et al. 2000). This change in the distribution of CD45

within T cells illustrates the importance of changes in location as a mechanism of regulating CD45 phosphatase function.

1.7.2 PTPN22/Lyp

1.7.2.1 Lyp structure and expression

Lyp is a cytoplasmic PTP encoded by the PTPN22 gene, which can be found on chromosome 1 in region p13 (Cohen et al. 1999). Expression of PTPN22 mRNA is low in most human tissues, but is found at higher levels in primary human blood cells and cell lines (Figure 1.4). CD56+ NK cells and B lymphoblasts have been found to express particularly high levels of PTPN22 mRNA, lower expression can be found in DCs, CD8+ T cells and CD33+ myeloid cells and the lowest levels detected in CD14+ monocytes, CD4+ T cells and CD19+ B cells (Figure 1.4). The Lyp protein is differentially expressed by many types of immune cells, with reports of high levels of expression by neutrophils and monocytes and lower levels in B and T lymphocytes (Cohen et al. 1999, Chien et al. 2003). The human protein Lyp is 105kDa in size and consists of a PTP catalytic domain located at the N-terminal, an interdomain of approximately 300 aa's, and a non-catalytic domain containing four proline-rich motifs (P1-P4) at the C-terminal (Cohen et al. 1999). The proline-rich motifs could be important for the regulation of Lyp by phosphorylation or ligand binding, as proline is critical for many protein-protein interactions (Kay et al. 2000). The aa sequence encoding Lyp is fairly conserved, as it has an overall 70% homology with the murine equivalent protein, PEP. This can be further analysed as 89% homology within the catalytic domain and 61% homology within the non-catalytic regions (Cohen et al. 1999). Consequently, the majority of studies to date looking at the function of Lyp have been carried out using the mouse ortholog.

Three isoforms of Lyp have been identified, Lyp1, Lyp2 and Lyp3 which are produced as a result of alternative splicing. In terms of structure, Lyp1 contains four proline-rich motifs (P1-

P4) whereas Lyp2 only has one (P1). This difference is evident in the protein size as Lyp2 is only 85kDa, which is 20kDa smaller than Lyp1. Lyp3 lacks 28 aa residues which connect the P1 and P2 domains as in the structure of Lyp1 (Wang et al. 2010). This could affect interactions between Lyp and Csk, as this missing aa sequence is found in a structural region thought to be key for this interaction. Lyp1 is the predominant isoform and expression is highest in primary and secondary lymphoid tissues (Wang et al. 2010). At present, *in vitro* testing has revealed no functional differences between Lyp1 and Lyp2, but studies in T cells have suggested Lyp2 may be more important in resting T cells as its expression is reduced following T cell activation (Cohen et al. 1999). An additional isoform, PTPN22.6, has also been identified in CD4+ T cells (Chang et al. 2012) and is produced by alternative splicing of the PTPN22 gene. This particular isoform contains a unique C-terminal sequence and lacks a portion of the Lyp catalytic domain. It has been found that in the context of PTPN22 R620W, expression of the conventional isoform PTPN22.1 leads to attenuation of T cell activation, whereas PTPN22.6 leads to T cell hyperactivity (Chang et al. 2012). Interestingly, expression of PTPN22.6 has also been associated with RA, with levels of PTPN22.6 mRNA correlating with disease activity in a cohort of RA patients (Chang et al. 2012). This suggests that additional splice variants of PTPN22 such as PTPN22.6 could be of importance in RA disease susceptibility and disease severity.

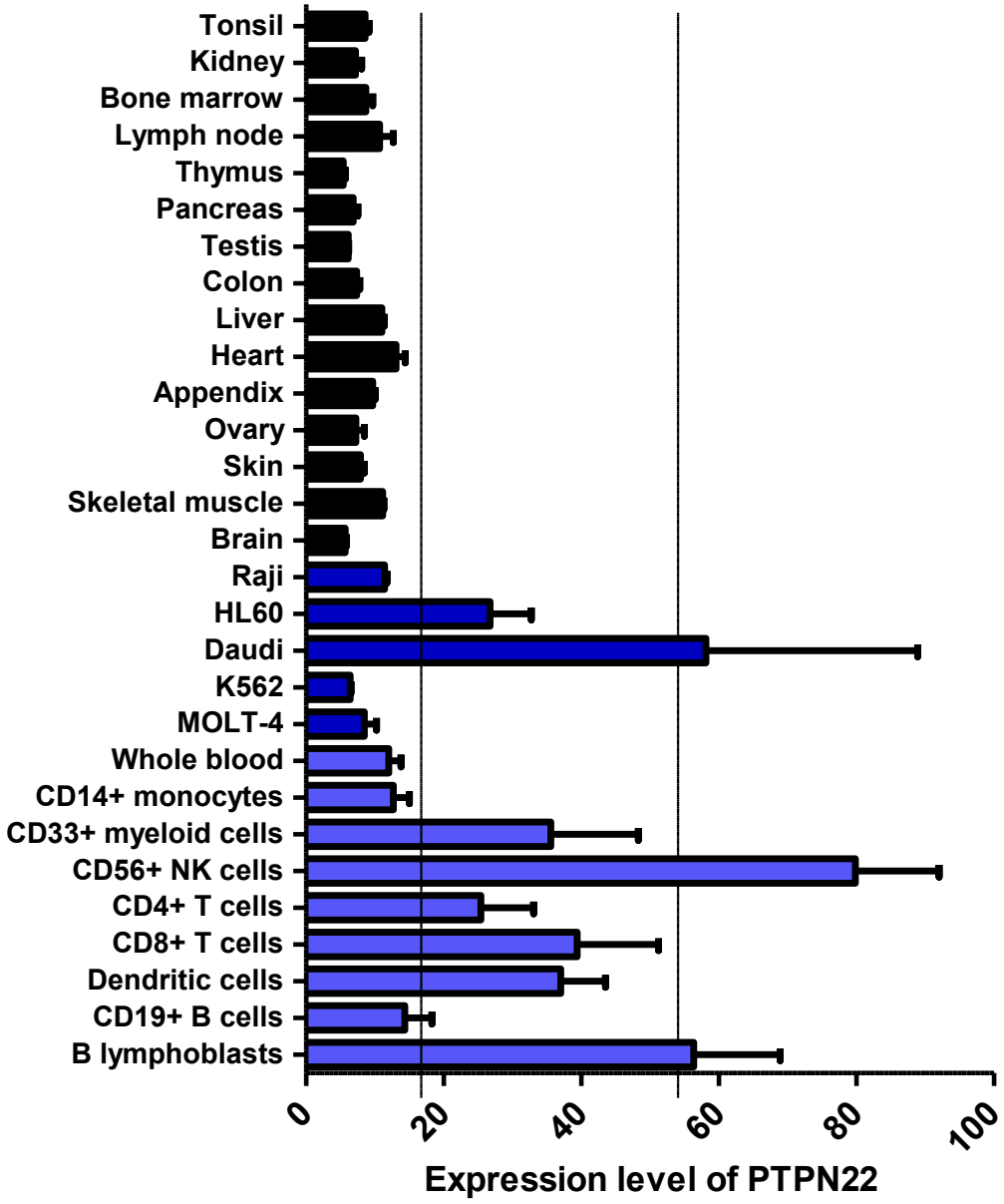


Figure 1.4 Expression of PTPN22 mRNA in different human blood cells, cell lines and tissues. Figure shows data obtained from high-density oligonucleotide arrays used for detecting the expression of PTPN22 mRNA in human blood cells (light blue bars), cell lines (dark blue bars) and tissues (black bars) (http://www.biogps.org/#goto=genereport&id=26191). The first horizontal black line represents the mean expression level (18.98) and the second black horizontal line represents three times the mean expression level (56.94).

1.7.2.2 Lyp function in T cells

In vivo studies using knockout mice and *in vitro* studies using over-expressing cells illustrate that Lyp has an important role in the negative regulation of T cell develpment and function, via its ability to modulate the activation of the Src-family kinase Lck (Gjorloff-Wingren et al. 1999, Hill et al. 2002, Hasegawa et al. 2004). Other targets have been identified using substrate trapping including Zap-70, Vav1, CD3ε and CD3ζ, all of which are downstream of Lck (Wu et al. 2006). Mice which fail to express Lyp/PEP show increased function and expansion of the T effector cell population, elevated serum antibody levels, as well as spontaneous formation of germinal centres (Hasegawa et al. 2004). Most features are likely to be a result of increased signalling and responses mediated through the TCR. Interestingly the numbers and function of naïve T cells is unaltered (Hasegawa et al. 2004). A possible explanation for this is that other PTPs are able to compensate for the loss of function of Lyp in this subset of T cells.

In a resting murine T cell, 25-50% of PEP is associated with its binding partner Csk (Cloutier et al. 1996). This association is solely dependent upon the SH3 domain of Csk and the P1 motif of PEP and is crucial in relocating Lyp near its primary target Lck. In order to modulate signalling through the TCR Lyp and Csk work synergistically, Lyp dephosphorylates tyrosine residue 394 of Lck and Csk rephosphorylates tyrosine residue 505 of Lck, which renders the kinase inactive (Gjorloff-Wingren et al. 1999). In unstimulated T cells, phosphorylated adaptor PAG recruits Csk to the lipid rafts. Upon TCR stimulation, PAG becomes phosphorylated and allows Csk to be released into the cytoplasm where it complexes with PEP. Overall, this sequence of events down regulates signalling through the TCR.

1.7.2.3 Lyp function in other cell types

Although the function of Lyp in T cells is most understood, it is not fully characterized by any means. It is thought Lyp has a similar role in B cells by regulating the signal propagated through stimulation of the BCR. Lyp is likely to be less critical for B cell function as in the

PEP deficient mouse B cell signalling is unaltered, although formation of germinal centres is accelerated (Hasegawa et al. 2004). Regarding Lyp function in other cell types, there are only a handful of studies to date. Lyp has been implicated as a regulator of NK cell proliferation *in vitro*, however the exact signalling pathway involved is yet to be identified (Douroudis et al. 2010). In addition, expression of Lyp has been observed to be induced in monocytes exposed to IFN-γ, with knock down of Lyp resulting in increased production of pro-inflammatory cytokines (Spalinger et al. 2013). These observations suggest a role for Lyp in the regulation of cytokine production and responses by monocytes.

1.7.2.4 Lyp regulation

Determination of the crystal structure of Lyp has provided insights into potential mechanisms regulating Lyp activity and catalysed the development of selective inhibitors (Tsai et al. 2009, Wu et al. 2009). Such studies have revealed Lyp can be serine phosphorylated (Ser-35) within the substrate binding domain, which may act as a negative regulatory mechanism. This was found to reduce the interaction of Lyp with its physiological substrates as well as selective inhibitors (Yu et al. 2007), although further study is required to ascertain the significance of this phosphorylation site.

1.8 PTPN22 R620W single nucleotide polymorphism

1.8.1 PTPN22 R620W and autoimmunity

A variant form of Lyp exists, in which the arginine residue at position 620 is substituted for tryptophan. This single nucleotide polymorphism (SNP) is known as PTPN22 R620W and was originally found to be associated with an increased risk of developing type I diabetes in 2004 (Bottini et al. 2004). Following this publication, the polymorphism has since been associated with a range of autoimmune diseases including Grave's disease, Myasthenia gravis, RA, multiple sclerosis and SLE (Carlton et al. 2005, Burn et al. 2011). PTPN22 R620W is a key genetic risk factor for autoimmunity and it has been estimated that

individuals with this allelic variant are around 1.7 times more at risk of RA when compared to those without (Burn et al. 2011). In addition to R620W other SNPs in the PTPN22 gene have also been associated with RA (Carlton et al. 2005), suggesting that additional variants could be involved in disease susceptibility. The R620W variant is commonly expressed in Caucasian individuals of European ancestry and frequency is highest in countries of northern Europe. The frequency of the allele in the UK is estimated to be 8% (Burn et al. 2011). The allele frequency decreases from northern to southern Europe and the allele is virtually absent in Asian and African populations (Begovich et al. 2004). Based on these observations it has been suggested that the allele may provide some selective advantage, and that it could be under positive selection in certain populations. This idea is supported by the observation that individuals with the allelic variant appear to be more resistant to the development of tuberculosis (TB) infection (Gomez et al. 2005). This could be indicative that this genetic variant could be of benefit by protecting individuals from infection in early life, but increasing risk of autoimmunity in later life.

1.8.2 Mouse models of PTPN22/Lyp function and the effect of R620W

A small number of studies to date have investigated the function of PTPN22/Lyp and the effect of R620W *in vivo* using mouse models. In order to study the function of PTPN22/Lyp in different immune cell types, a knockout mouse has been generated (PTPN22 -/-) (Hasegawa et al. 2004). This mouse model has revealed that Lyp is an important negative regulator of T cell signalling and a number of alterations to T cell numbers and function have been observed. PTPN22 -/-mice exhibit increased expansion of the effector T cell population and enhanced T cell dependent immune responses (Hasegawa et al. 2004). Mice also display increased numbers of single positive CD4 cells, highlighting a role for Lyp in thymic selection. Furthermore, a lack of Lyp/Pep was also associated with an increased expansion of the regulatory T cell population, suggesting an important role for Lyp in controlling the balance of different T cell subsets (Maine et al. 2012). The absence of Lyp/Pep in B cells had much

more modest effects including increased formation of germinal centres and increased levels of selected immunoglobulin subtypes (Hasegawa et al. 2004). In summary, the absence of Lyp/Pep in mice has major effects on lymphocyte function but does not appear to cause disease.

In addition to the PTPN22-/- mouse model, a PTPN22 knock in mouse model has also been generated (Zhang et al. 2011). This model has been used to try and emulate the effects of the R620W polymorphism in humans by engineering the equivalent gene in mice (PEP) to replicate the single aa change which occurs in humans (Pep R619W rather than PTPN22 R620W). Mice with the Pep R619W mutation show alterations in the function of B cells, T cells and myeloid cells. Increased TCR signalling and TCR-mediated proliferation have been reported, as well as the possibility of reduced stability of the Pep R619W encoded protein (Zhang et al. 2011). With regards to B cells, expression of Pep R619W was associated with increased germinal centre area, increased BCR-driven proliferation and a resistance to apoptosis (Zhang et al. 2011, Dai et al. 2013). Lastly when looking at cells of the myeloid lineage, there was found to be an accumulation of CD11c+ cells in the spleen and an increase in the costimulation capacity of DCs (Zhang et al. 2011). Similarly to the Lyp/Pep knock out mouse model, the knock in mouse model also showed that the genetic change in Lyp function alone was not enough to cause disease. Interestingly, when the knock in mouse model was combined with inflammatory arthritis, mice possessing the Pep R619W mutation were unable to suppress the experimentally induced arthritis due to lack of immunoregluation caused by decreased type I interferon production by myeloid cells (Wang et al. 2013). This particular study implicates a role for Pep/Lyp in modifying the immune response to pathogens which may influence disease progression and persistence. Overall, these mouse models of PTPN22/Lyp function and the effect of R620W have provided evidence of Lyp function in multiple cell lineages and highlight that there is still not much known about exactly how alterations in PTPN22 function alter immune responses to promote disease.

1.8.3 Consequences of PTPN22 R620W in T cells

The consequences of PTPN22 R620W and its effects on immune cell function have been widely studied in the context of health and a range of human diseases, but still very little is known about Lyp function in many of the cell types which express the protein. Despite this uncertainty, the effects of the allelic variant have been most well characterized in T cells.

Using purified Lyp protein synthesised in bacteria, it was shown that the R620W polymorphism causes disruption of the binding of Lyp to the kinase Csk. This is due to the aa change being located within one of the SH3 binding sites located in the P1 domain of the Lyp protein, a region critical for Csk binding (Bottini et al. 2004). The effects of this change in binding affinity were not investigated further, but it could prevent Lck from accessing Lyp or vice versa to provide an opportunity for Lyp to act on other substrates. In order for Lyp to down regulate TCR signalling it must be dissociated from Csk to allow recruitment near to the plasma membrane (Vang et al. 2012). This suggests that if the variant Lyp protein shows reduced binding to Csk, Lyp could be more efficiently recruited to the plasma membrane leading to increased down regulation of TCR signalling. In support of this, transfection of the variant Lyp protein in the Jurkat T cell line revealed that the variant Lyp protein has increased intrinsic phosphatase activity, as well as being able to more efficiently inhibit nuclear factor of activated T cells (NFAT)/AP1 (Vang et al. 2005). Both these effects would suggest a gain-of-function Lyp phosphatase which is more efficient at down regulating signalling through the TCR.

In contrast to these data, another study using Jurkat T cells illustrated that expression of the R620W variant induced Erk phosphorylation and enhanced Ca^{2+} release, implying that signalling through the TCR was increased and that the Lyp phosphatase had decreased function under these circumstances (Zikherman et al. 2009). These conflicting results may be explained by the different parameters that were used as a measure of TCR signalling or the

differences in methods used to stimulate the cells. Studies using primary T cells are also conflicting, with reports of the variant resulting in increased and decreased T cell activation. For example, T cells from healthy individuals homozygous for PTPN22 R620W have been shown to exhibit a diminished response to stimulation by antigen, characterized by reduced Ca^{2+} signalling, production of IL-10 and expression of CD25 (Rieck et al. 2007). On the other hand, it has been reported that variant expressing individuals display increased Th1 responses and decreased Th17 responses (Vang et al. 2013) suggesting that perhaps the variant Lyp in these cells has lost its ability to down regulate TCR signalling. The consequences of a change in signalling threshold could have a number of implications within the T cell lineage. There is a possibility of impaired regulation of the effector T cell population, as well as a reduction in negative selection of autoreactive T cells which could result in autoimmunity. In addition, variant Lyp has been shown to reduce integrin mediated adhesion and migration of T cells (Svensson et al. 2011), highlighting a role for Lyp in the regulation of T cell adhesive and migratory responses.

1.8.4 Consequences of PTPN22 R620W in other cell types

Effects in B cells are important, as they are the direct source of the autoantibodies characteristic of autoimmune conditions. B cells from individuals with the R620W variant display a decrease in signalling through the BCR, which is characterized by decreased Ca^{2+} signalling, reduced proliferation and a reduction in tyrosine phosphorylation of key signalling proteins (Rieck et al. 2007, Arechiga et al. 2009). PTPN22 R620W is highly associated with autoimmune diseases in which autoantibodies are a predominant feature (Carlton et al. 2005, Burn et al. 2011) and furthermore a higher frequency of autoreactive B cell clones is observed even in healthy individuals expressing R620W (Menard et al. 2011). As with T cells, a reduction in signalling through the BCR could give rise to an increased number of autoreactive B cells, which could contribute to the development of autoimmunity.

With regards to other types of leukocytes, virtually nothing is known about the role of Lyp or the effect of the R620W variant. As mentioned, Lyp is widely expressed by most immune cells and high levels can be found in neutrophils, NK cells and monocytes (Cohen et al. 1999, Chien et al. 2003). A single study has highlighted a role for Lyp in the regulation of NK cell proliferation *in vitro,* but the *in vivo* relevance of this is uncertain (Douroudis et al. 2010). It was also shown that expression of PTPN22 R620W in healthy individuals was associated with a reduced fraction of NK cells in culture (Douroudis et al. 2010), suggesting the variant may alter the balance of NK and T cells. Also, using neutrophils obtained from anti-neutrophil cytoplasmic antibody (ANCA) vasculitis patients, it was shown that the phosphatase activity of Lyp was increased in patients with the R620W variant compared to those without (Cao et al. 2012). The role in Lyp in the signalling mediated through neutrophil surface receptors is not known. The functional consequences of PTPN22 R620W in neutrophils are important as this cell type makes a significant contribution to the processes resulting in tissue damage in RA and other inflammatory diseases (Wright et al. 2010).

1.9 Oxidation of PTPs

Regulation of PTP function is important for fine tuning signalling responses and can occur via genetic alterations in PTP coding genes or environmental changes which induce post-translational modifications to the PTP active site. One environmental mechanism by which PTP function can be altered is through oxidation. The catalytic site of classical PTPs contains a cysteine residue, the oxidation state of which directly impacts on the ability of the PTP to dephosphorylate its target substrate. A cysteine in this location has been estimated to have a much lower pKa than is usual for a cysteine, making it vulnerable to the effects of positively charged nucleophiles such as ROS. Using calculations based on experimental data, it has been shown that the pKa of the PTP catalytic cysteine is 4-6 (Peters et al. 1998), much lower than the 8-9 exhibited by other cysteines (Winterbourn et al. 2008). Reversible oxidation of this cysteine functional group thiol from R-PH to R-SOH (sulfenic acid) through its reaction

with ROS is a common occurrence and an important regulatory mechanism used by PTPs (Figure 1.5). Oxidation to R-SOH is easily reversed by reducing agents such as glutathione (GSH), dithiothreitol (DTT) and tris (2-carboxyethyl) phosphine (TCEP) (Rider et al. 2003). However, further oxidation to $-SO_2H$ (sulfinic acid) or further still to $–SO_3H$ (sulfonic acid) is considered irreversible and renders the PTP permanently inactive (Takakura et al. 1999).

PTPs show differential susceptibility to oxidation, which may be explained by the position of their active site cysteine or cellular location (Groen et al. 2005). For example, CD45 is a cell surface receptor PTP and its active site cysteine residue is fairly exposed, which may be indicative of a high susceptibility to oxidation by intra and extra cellular ROS (Holmes 2006). A high level of oxidation could be important in regulating CD45 phosphatase activity in order to alter the strength of TCR signalling. Experiments in mice have shown that only 3% of the normal level of CD45 surface protein expression is required for sufficient TCR signalling (McNeill et al. 2007) and a decrease in CD45 activity through oxidation could be one of the physiological mechanisms employed to down regulate TCR activation. In contrast to CD45, the Lyp phosphatase is intracellular and its cysteine residue is in vicinity of two other cysteine residues. These residues can readily form a disulfide bond without a considerable change in conformation, and thus possibly protect Lyp from oxidation (Tsai et al. 2009). Oxidation as a regulatory mechanism may not be as important for the Lyp phosphatase as the Lyp protein is expressed at much lower levels when compared to the CD45 protein in T cells (Hermiston et al. 2009).

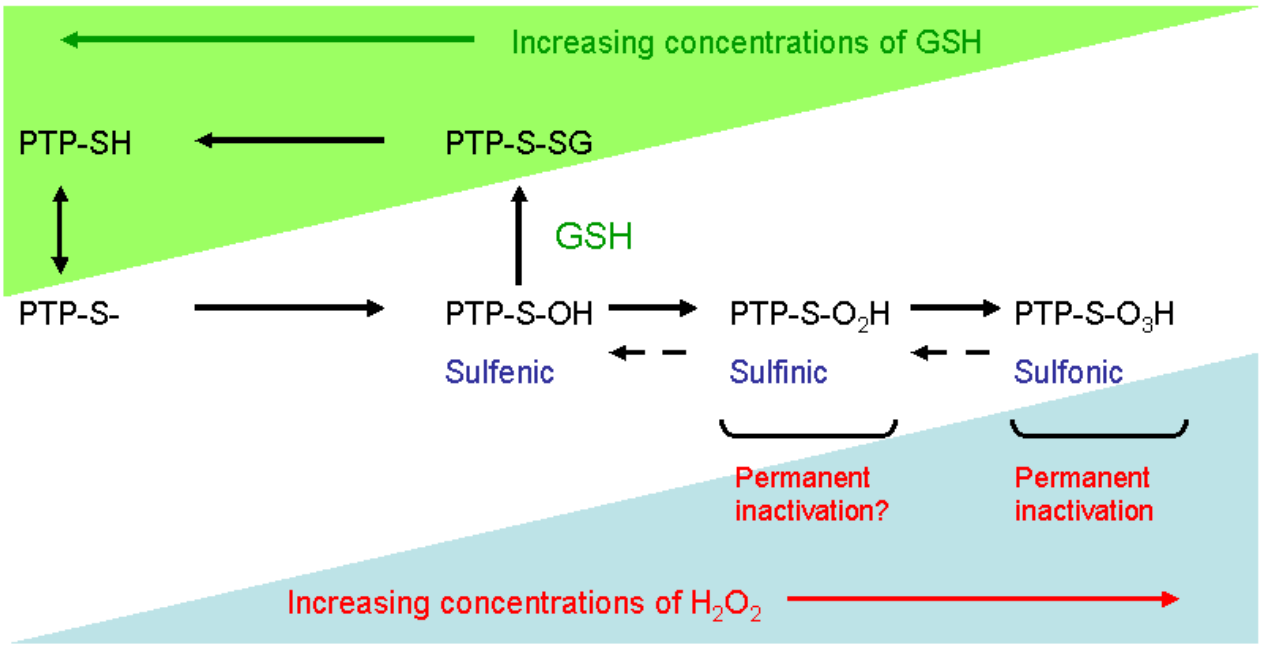


Figure 1.5 A diagram to show reversible and irreversible oxidation of PTPs. PTPs can be oxidised by H_2O_2, which can be reversed by GSH. However, if oxidation goes too far the PTP can no longer by reduced by GSH and is permanently inactivated. PTP, protein tyrosine phosphatase; GSH, glutathione; H_2O_2, hydrogen peroxide.

1.10 Reactive oxygen species

ROS are derivatives of molecular oxygen and include a number of molecules and free radicals. They are formed as part of normal cellular metabolic processes, with the predominant source being aerobic respiration carried out by the mitochondria (Turrens 2003). Abundant ROS include hydrogen peroxide (H_2O_2), nitric oxide (NO-), superoxide ($O_{2^-}^{\bullet}$) and hydroxyl radical ($OH^{\bullet}$) which can be produced enzymatically or non- enzymatically. $O_{2^-}^{\bullet}$ is a fairly weak oxidant, but often participates in the prorogation of chain reactions and is a pre-cursor for most other ROS (Turrens 2003). ROS are important in immune cell signalling and maintaining a balance between production and removal of ROS is critical. If production is greater than removal, this can cause 'oxidative stress' defined generally as the deleterious processes caused by overproduction of ROS and under activity of the antioxidant system. This could cause irreversible oxidation of various cellular proteins, including the PTPs. However, some ROS are important to maintain effective signalling through redox regulation. For example, it has been observed *in vitro* that the induction of regulatory T cells by macrophages is dependent on ROS production (Kraaij et al. 2010), highlighting that a low level of ROS is required for the proper regulation of immune responses. There are a variety of endogenous sources of ROS, such as the oxidative burst during activation of macrophages and neutrophils. Over-activation of these cell types may cause a large increase in the overall amount of ROS in the joint and initiate inflammatory process associated with RA. A number of specific components of the joint have shown to be oxidatively damaged in RA, as well as non-specific damage to immunoglobulins, lipids and DNA being observed (Hitchon et al. 2004). The antioxidant defence system is essential to provide cells with protection from ROS, whether endogenously produced by normal cellular reactions or introduced from the environment.

1.11 The antioxidant defence systems

GSH is a tripeptide thiol (L-cysteine, L-glutamic acid, and glycine) with powerful reducing properties and a key component of the antioxidant defence system. It has two major functions of importance in the context of inflammatory diseases and these are regulation of NFκB and scavenging of ROS. NFκB is a transcription factor which can be activated by over 150 different stimuli indicative of cellular stress such as ultraviolet light, cytokines and ROS (Pahl 1999). GSH is a potent inhibitor of NFκB whereas ROS activate NFκB and increase transcription of a number of immunologically relevant genes, many of which have pro-inflammatory effects (Pahl 1999). It is essential that the suppressive action of GSH on NFκB is balanced correctly, as in the case of an infection the occurrence of inflammation via NFκB activation is beneficial. On the other hand, excessive activation of NFκB may result in higher basal levels of inflammation and promote inflammatory conditions such as RA. Even small changes in GSH have profound effects on immune function, which is relevant to the joint as synovial perfusion alters greatly. Frequent ischemic-reperfusion injury cycles affecting the joint have been shown to activate NFκB (Han et al. 2003), which could be due to fluctuations in GSH.

In the case of RA, depletion of GSH is thought to be a key mechanism perpetuating the emergence of a pro-inflammatory environment in which many cell types experience oxidative stress. This is supported by observations of increased markers of oxidative stress and impaired antioxidant status in RA patients (Jaswal et al. 2003). Furthermore, antioxidant supplementation has been shown to increase circulating antioxidants and reduce markers of oxidative stress in patients (Jaswal et al. 2003). A decrease in GSH could play an important role in the pathogenesis of RA, which may interact with pre-determined genetic factors to promote disease development.

1.12 Gene-environment interactions in rheumatoid arthritis

Evidently, alterations in PTPN22/Lyp function alone are not enough to directly cause autoimmune disease, as shown in studies using PEP (the mouse equivalent of PTPN22) knockout mice in which autoimmune disease is absent (Hasegawa et al. 2004). However in the presence of a genetically susceptible background, T cells lacking PEP promote production of autoantibodies by hyper responsive B cells expressing a mutated form of CD45 (CD45E613R), which manifests as a lupus-like disease (Hermiston et al. 2009). This emphasises the importance of gene-gene and gene-environment interactions in determining disease susceptibility.

1.12.1 Interactions between PTPN22 R620W and cigarette smoking

As discussed there are genetics factors (for example HLA-DRB1 and PTPN22 R620W) and environmental factors (smoking, ageing and infection) which have been associated with an increased risk of developing RA. RA is complex and the observed phenotype is likely to be a result of gene-gene and gene-environment interactions. This principle is best illustrated by a study which assessed the interaction between HLADRB1, PTPN22 R620W and smoking (Kallberg et al. 2007). It was observed that the odds ratio (OR) of developing anti-CCP positive RA with one risk factor alone was as follows, 7.5 (HLADRB1), 2.0 (PTPN22 R620W) and 1.5 (smoking) (Figure 1.6). If HLADRB1 and PTPN22 R620W were present the OR rose to 13.2, indicative of more than an additive effect (Kallberg et al. 2007). Additionally if both genetic factors were present as well as a history of smoking, the OR increased further to 23.4. This study provides sound evidence that gene-gene and gene-environment interactions occur and risk of RA greatly increases with the presence of more than one additional risk factor. Studies such as this illustrate the importance of interactions between genetic and environmental risk factors, however the mechanisms by which these interactions occur have not yet been elucidated. This study also found that these interactions were not associated

with the development of anti-CCP negative RA. This suggests PTPN22 R620W and smoking could be making a direct contribution to the generation of citrullinated peptides and production of anti-CCP antibodies, given the absence of their associations with anti-CCP negative RA.

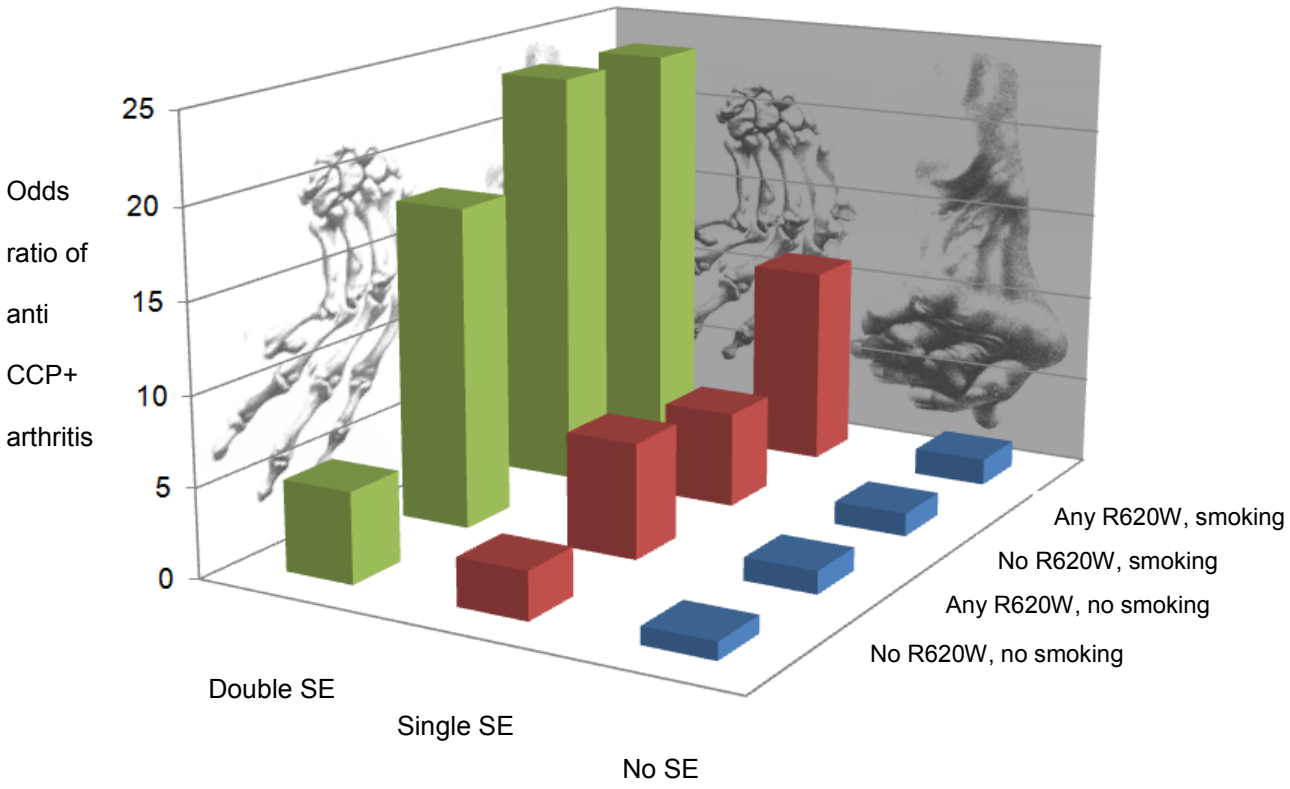


Figure 1.6 Interactions between genetic polymorphisms (HLA-DRB1, PTPN22 R620W) and environmental factors (smoking) drive RA. Figure adapted from (Kallberg et al 2007) SE, shared epitope, R620W, PTPN22 R620W.

1.12.2 Mechanisms of interactions between PTPN22 R620W and cigarette smoking

Changes in PTPN22/Lyp function through expression of PTPN22 R620W and cigarette smoking have been investigated separately and both were found to have significant effects on immune cell signalling. However, many of these studies have raised further questions as the findings are often contradictory. For example, it has been observed that T cells expressing the PTPN22 R620W variant exhibit reduced Ca^{2+} flux in response to stimulation of the TCR, which would suggest a decrease in T cell responses and function (Rieck et al. 2007). More recently, investigations have shown that expression of the same variant favours Th1 responses, implicating a stronger signal through the TCR which increases T cell activation and function (Vang et al. 2013). Such studies could be confounded by certain aspects of experimental approach taken, but illustrate that there is still much more to investigate with regards to the effects of PTPN22 R620W on immune cell responses. There are no data currently available on PTPN22 R620W in certain leukocyte subtypes, for example the function of Lyp and its variants in neutrophils is unknown. This is an important line of investigation given the role of neutrophils in innate immunity to infection and their proposed involvement in the pathogenesis of RA.

Smoking and smoke extract have also been found to have direct effects on immune cell activation. The effects of smoking are widespread and effect cells that participate in innate and adaptive immune responses (Goncalves et al. 2011). Studies to date investigating the effects of smoking on T cell function have produced mixed results. T cell proliferation has been shown to be decreased (Glader et al. 2006, Mortaz et al. 2009) or unaffected (Zavitz et al. 2008) when exposed to smoke, and cytokine production has been shown to be altered (Ouyang et al. 2000, Lambert et al. 2005, Vassallo et al. 2005, Robbins et al. 2008) or equivalent (Zavitz et al. 2008). Additionally, cigarette smoke has been found to upregulate the activity of the enzyme PADI, which could promote production of CCPs (van der Helm-van Mil et al. 2007). These CCPs could then be recognised by anti-CCP antibodies commonly

found in RA (Yamada et al. 2005). The components of cigarette smoke extract (CSE) and chronic smoking in humans have also been found to have profound effects on the activation status of neutrophils. Smokers have increased numbers of neutrophils in the blood (Kuschner et al. 1996) and they appear to be more active and inflammatory even in the absence of infection. For example, acute exposure to CSE has been shown to increase neutrophil elastase activity (Drost et al. 1993), a serine proteinase released by neutrophils to aid destruction of bacteria and host tissue.

Apart from the direct effects on immune cell signalling, cigarette smoking also creates a pro-oxidant environment. Acute exposure to CSE in human subjects increases the content of H_2O_2 in exhaled breath, suggesting an increased level of oxidative stress (Guatura et al. 2000). Peripheral blood levels of cysteine are also depressed following even just one cigarette (Tsuchiya et al. 2002). Availability of cysteine is the rate limiting factor for synthesis of the antioxidant GSH, which suggests smoking not only introduces more oxidants but also impairs antioxidant defence. This lack of antioxidant defence leaves proteins vulnerable to oxidation and damage. In particular this is true for the PTPs due to their active site cysteine being highly susceptible to regulation by oxidation.

1.13 Summary and aims

Alterations in the function of Lyp including the PTPN22 R620W variant are known to play a crucial role in the development of RA (Carlton et al. 2005). To date, the role of R620W expression in RA pathogenesis is thought mainly to be via impaired regulation of T cell signalling and function (Gregersen et al. 2005, Vang et al. 2005, Rieck et al. 2007) but changes in the function of B cells are also present (Rieck et al. 2007, Arechiga et al. 2009, Menard et al. 2011). In support of this, characteristic features of RA include hyporesponsive T cells and the presence of autoantibodies produced by B cells (Firestein 2003, Rider et al. 2003). However, a genetically susceptible background alone does not automatically mean an individual will develop RA. Studies have shown that disease concordance is 15% in monozygotic twins and 4% in dizygotic twins (Cook et al. 1993) and that some patients with autoimmune disease do not possess any of the susceptibility alleles (Kallberg et al. 2007). These findings illustrate the importance of environmental factors and their contribution to disease development. In genetically susceptible individuals, smoking and insufficient intake of antioxidants have been shown to increase risk of RA (Hitchon et al. 2004, Kallberg et al. 2007, Costenbader et al. 2008). Both these factors deplete the antioxidant GSH and RA patients have been identified as deficient in GSH and show increased levels of oxidative stress (Jaswal et al. 2003). This suggests depletion of GSH and oxidative stress could be important contributors to immune dysfunction seen in RA.

Based upon the role of Lyp in autoimmunity and indications that diseased individuals are likely to be under oxidative stress, it was hypothesised that these two factors would synergise to promote the development of RA through alterations in leukocyte signalling. The role of PTPN22 R620W and smoking were investigated in the context of innate and adaptive immune responses, through studying effects on neutrophils and T lymphocytes.

The aim of the work described in this thesis was to investigate how genetic and environmental factors alter the function of PTPN22/Lyp, and how these factors may synergise to induce aberrant activation of innate immune cells (neutrophils) and adaptive immune cells (lymphocytes), which could promote inflammation and RA. Studies focussed on the effects of the genetic variant PTPN22 R620W and the environmental factor of cigarette smoking.

The four main aims of this study were:

- To develop a suitable assay to measure Lyp phosphatase activity
- To determine the effects of PTPN22 R620W on T lymphocyte and neutrophil function in health and rheumatoid arthritis
- To determine the effects of cigarette smoke extract on T cell receptor signalling
- To determine possible mechanisms of synergy between PTPN22 R620W and cigarette smoking which would operate to promote the development of rheumatoid arthritis

CHAPTER TWO: MATERIALS AND METHODS

This chapter lists the antibodies and other reagents used throughout they study and also includes detailed descriptions of the experimental methods used. References are included for any non-standard methods which were used and developed throughout the project.

For the first part of the study, B lymphocytes were isolated from the PB of vasculitis patients with a known PTPN22 R620W genotype in order to generate preliminary data. For the majority of the work a large cohort of healthy controls, disease controls and RA patients were recruited and genotyped for the PTPN22 R620W variant. Following identification of variant expressing individuals, CD4+ T cells and neutrophils were isolated from PB for signalling and functional analyses.

To investigate the effects of PTPN22 R620W on T cell function, CD4+ T cells were isolated from healthy individuals and RA patients expressing the R620W variant and their Lyp phosphatase activity and Lyp substrate phosphorylation status were assessed. Lyp activity was measured using an immunocapture assay developed throughout the study and Lyp substrate phosphorylation was assessed by flow cytometry and immunoblotting. T cell phenotype was also assessed by measuring cell proliferation using CFSE and cytokine production using intracellular cytokine staining and ELISA following stimulation with anti-CD3 and anti-CD28.

To determine the effects of PTPN22 R620W on neutrophil function, neutrophils were isolated from the PB of healthy individuals and RA patients expressing the R620W variant and their effector functions were assessed. These included assessment of Ca^{2+} flux by fluorimetry, production of ROS by flow cytometry and ability to adhere and migrate across inflamed endothelium using adhesion and migration assays.

Finally, methods were employed to characterise the effects of exposure to CSE on T cell signalling in the Jurkat T cell line. CSE was generated, Jurkat T cells were treated and then TCR signalling parameters were measured. These included measurement of PTP activity by

immunocapture, Ca^{2+} flux by fluorimetry and Lck phosphorylation status by flow cytometry. Lastly, investigations were conducted into potential mechanisms by which CSE could alter T cell receptor signalling. These experiments particularly focused on the potential of CSE to oxidise TCR signalling proteins and involved measurement of cellular GSH content, removal of particulate matter from the extract by filtration and assessing levels of protein oxidation using a novel cysteinyl labelling method.

2.1 Materials

All reagents were purchased from Sigma Aldrich (United Kingdom), unless otherwise stated.

Table 2.1 Antibodies

Name	Purchased from	Clone	Stock concentration	Dilution
Anti-human CD19 PE	Immunotools, Germany	LT19	not known	1/20
Anti-human CD28	Immunotools, Germany	15-E8	1mg/ml	1/200
Anti-human CD3	Immunotools, Germany	MEM-57	1mg/ml	1/333
Anti-human CD4 FITC	Immunotools, Germany	MEM-241	not known	1/20
Anti-human CD45	AbD Serotec, UK	F-10-89-4	1mg/ml	1/100
Anti-human IFN-γ Pacific blue	Biolegend, UK	4S.B3	0.5mg/ml	1/60
Anti-human IL-10 PE/Cy7	Biolegend, UK	JES3-9D7	0.2mg/ml	1/5
Anti-human IL-17 PE	Biolegend, UK	BL168	0.2mg/ml	1/10
Anti-human IL-4 FITC	Biolegend, UK	MPF-25D2	0.5mg/ml	1/10
Anti-human Lck	Santa Cruz Biotechnology, USA	3A5	0.2mg/ml	1/1000
Anti-human Lyp	R and D Systems, UK	340113	0.5mg/ml	1/50
Anti-human pLck (Tyr 394)	Santa Cruz Biotechnology, USA	polyclonal	0.1mg/ml	1/1000 IB 1/00 FCM
Anti-human pLck (Tyr 505)	Santa Cruz Biotechnology, USA	pY505.4	0.1mg.ml	1/1000
Anti-human pLck (Tyr 505) PE	BD Biosciences, USA	MOL 171	not known	1/10
Anti-human Zap70 (Tyr 493)	Santa Cruz Biotechnology, USA	polyclonal	0.2mg/ml	1/1000
Anti-phosphotyrosine	Santa Cruz Biotechnology, USA	PY20	0.2mg/ml	1/1000
Donkey anti-rabbit IgG-HRP	GE Healthcare, UK	polyclonal	1mg/ml	1/10,000
Goat anti-rabbit IgG FITC	Southern Biotech, USA	polyclonal	1mg/ml	1/200
Mouse IgG1 kappa pacific blue isotype control	Biolegend, UK	MOPC-21	0.5mg/ml	1/24
Mouse IgG1 kappa PE isotype control	Biolegend, UK	MOPC-21	0.2mg/ml	1/10
Rat IgG1 kappa FITC isotype control	Biolegend, UK	RTK2071	0.5mg/ml	1/500
Rat IgG1 kappa PE/Cy7 isotype control	Biolegend, UK	RTK2071	0.2mg/ml	1/200
Sheep anti-mouse IgG-HRP	GE Healthcare, UK	polyclonal	1mg/ml	1/10,000
Streptavidin-HRP	GE Healthcare, UK	polyclonal	1mg/ml	1/10,000

FCM, flow cytometry; IB, immunoblotting.

Table 2.2 Other chemicals and reagents

Name	Purchased from
6,8-Difluoro-4-Methylumbelliferyl Phosphate (DiFMUP)	Invitrogen, USA
96 well qPCR plates	Roche, Switzerland
Amersham ECL Prime Western blotting detection reagent	GE Healthcare, UK
Amphotericin B	Gibco Invitrogen Compounds, UK
Annexin V APC	BD Biosciences, USA
BD Phosflow Fix buffer I	BD Biosciences, USA
BD Phosflow Perm-Wash buffer	BD Biosciences, USA
CD4+ T cell isolation kit II	Miltenyi Biotec, Germany
CFSE	Invitrogen, USA
Complete protease inhibitor cocktail tablets	Roche, Switzerland
Cytokine ELISA Kits (IL-4, IL-10, IL-17, IFN-γ and TNF-α)	eBioscience, USA
Epidermal growth factor	Gibco Invitrogen Compounds, UK
FIX AND PERM® Reagents	Invitrogen, USA
Fluorescein Diphosphate, Tetraammonium Salt (FDP)	Invitrogen, USA
Gentamycin	Gibco Invitrogen Compounds, UK
Hanks buffered salt solution	Invitrogen, USA
Heat inactivated foetal calf serum	Labtech, UK
Hydrocortisone	Gibco Invitrogen Compounds, UK
Indo-1 AM	Invitrogen, USA
Iodoacetamide (IAA)	Fisher Scientific, UK
Iodoacetylpolyethylene oxide (IAP) probes	Fisher Scientific, UK
Lightcycler 480 Genotyping Master mix	Roche, Switzerland
Lightning-Link® Horseradish peroxidase	Innova Biosciences, USA
M199 medium	Invitrogen, USA
Nanosep 3K Omega filters	Pall Corporation, USA
Nunc® MaxiSorp™96 well plates	Nunc, Denmark
Percoll	GE Healthcare, UK
Phosphate buffered saline	Oxoid, UK
Pre-stained protein standard	Invitrogen, USA
Propidium iodide	Invitrogen, USA
Protein A/G PLUS Agarose	Santa Cruz Biotechnology, USA
Protogel	Geneflow, UK
PTPN22 anchor and sensor probes	TIB MOLBIOL, Germany
PTPN22 forward and reverse primers	TIB MOLBIOL, Germany
PTPN22 recombinant protein	Abnova, Taiwan
QuickGene DNA Whole blood kit	Fujifilm, Japan
Recombinant tumour necrosis factor alpha	R and D Systems, UK
Streptavidin-sepharose beads	GE Healthcare, UK
Tris-glycine Electro-blotting buffer	Geneflow, UK
Tris-glycine-SDS PAGE buffer	Geneflow, UK

2.2 Methods

2.2.1 Generation of EBV cell lines

$1.0x10^7$ peripheral blood mononuclear cells (PBMCs) were resuspended in 0.5ml of RPMI-1640 supplemented with 10% heat-inactivated foetal calf serum (HIFCS) and 1% glutamine penicillin streptomycin (GPS) (complete culture medium). 4ml of supernatant from the B95.8 marmoset cell line culture was then added. Cells were incubated for 1 hour at 37°C and 1µg/ml of Cyclosporin A (CsA) added to prevent activation and growth of T cells. 50µl/well of cells was transferred to a 96-well round-bottomed tissue culture plate. Cells were incubated overnight at 37°C and fed by dilution via the addition of 50µl of complete culture medium containing 1µg/ml of CsA. After 1 week, cells were fed by removing half of the supernatant after centrifugation at $300xg_{av}$ for 6 minutes using a Mistral 3000i centrifuge and replacing this with fresh complete culture medium containing CsA (feeding by retention). This feeding process was continued for 6 weeks, alternating between dilution and retention. Upon the appearance of large clumps of cells, cultures were transferred to progressively larger plates to allow for maximum growth of cells. To check the purity of the generated lines, $1.0x10^6$ EBV-transformed cells were washed in phosphate buffered saline (PBS) by centrifugation at $300xg_{av}$ for 6 minutes. Cells were resuspended in 200µl of FACS buffer (2% bovine serum albumin (BSA)/PBS) and transferred to FACS tubes. Anti-human CD4 FITC and anti-human CD19 PE were added and incubated for 20 minutes at room temperature (RT) in the dark. Cells were washed in FACS buffer and resuspended in 0.5ml of PBS for FACS analysis using a CyAn ADP (Dako).

2.2.2 Maintenance of cell lines

Jurkat T cells, which were first isolated in 1980 and identified as a stable tumour cell line capable of producing IL-2 (Gillis et al. 1980), were grown in complete culture medium in an environment containing 5% CO_2. Cells were passaged at least twice a week by dilution in

additional medium and cultures were maintained at a density of $1.0x10^6$ cells/ml – $2.0x10^6$ cells/ml. EBV transformed B cell lines were grown in complete culture medium in an environment containing 5% CO_2. Cells were passaged three times a week by removing a third of the cell suspension and replacing it with fresh medium to maintain a maximum density of $2.0x10^6$ cells/ml.

2.2.3 Isolation of PBMC, CD4+ T cells and neutrophils

PB was obtained in EDTA-containing 50ml falcon tubes. 2% Dextran was added (1ml/6ml of blood) and the blood left to settle for 30-60 minutes to separate red blood cells from the buffy coat. 90% Percoll was made using 45ml of Percoll (neat from stock) and 5ml of 10x NaCl (4.5g NaCl in 50ml sterile H_2O). 80% Percoll (40ml 90% Percoll and 10ml 1x NaCl) and 56% Percoll (28ml 90% Percoll and 22ml 1x NaCl) were made for use in Percoll gradients. For each gradient 5ml of 56% Percoll was pipetted into a 15ml falcon tube. A Pasteur pipette was then used to add 2.5ml of 80% Percoll underneath. The buffy coat was added to Percoll gradients containing 80% and 56% Percoll and centrifuged at $190xg_{av}$ for 25 minutes with no brake using a Mistral 3000i centrifuge. The neutrophil layer was removed from the gradient using a Pasteur pipette and transferred to a 15ml falcon tube containing pre-warmed complete culture medium. Cells were washed twice in pre-warmed complete culture medium, counted using a haemocytometer and then used in experiments.

The PBMC layer was also removed from the gradient using a Pasteur pipette and subjected to further isolation to obtain purified CD4+ T cells. This was done using a kit containing biotinylated antibodies and anti-biotin microbeads to remove irrelevant cells. PBMC were washed twice in pre-warmed complete culture medium via centrifugation at $300xg_{av}$ for 6 minutes. Cells were counted using a haemocytometer, centrifuged as before and resuspended in 40µl of MACS buffer (0.5% BSA/2mM EDTA/PBS) per $1.0x10^7$ cells. 10µl of biotin-antibody cocktail (anti-CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and

Glycophorin A) was added per $1.0x10^7$ cells. Cells were mixed well and incubated for 10 minutes at 4°C. 30µl of MACS buffer was added per $1.0x10^7$ cells, followed by 20µl of anti-biotin microbeads and cells were incubated for 10 minutes at 4°C. Cells were washed by addition of 10 times the volume of MACS buffer via centrifugation at $300xg_{av}$ for 6 minutes. The supernatant was removed and the cell pellet resuspended in 500µl of MACS buffer. A MACS LS column was washed with 3ml of MACS buffer prior to magnetic separation of cells. The cell suspension was added to the column and collected in a universal. The column was washed three times using 3ml of MACS buffer and the flow through collected in the same universal as used previously. The flow through containing purified CD4+ T cells was centrifuged at $300xg_{av}$, the supernatant removed and cells resuspended in pre-warmed complete culture medium. Cells were washed a further two times in complete culture medium, counted using a haemocytometer and then used in experiments. Cells used in experiments were >95% pure, based on flow cytometric analysis using antibodies to CD3 and CD4.

2.2.4 Stimulation of CD4+ T cells

A 96-well round-bottomed tissue culture plate was coated with a solution of sterile PBS containing 3µg/ml anti-CD3 and 5µg/ml anti-CD28 (50µl/well) overnight at 4°C. The plate was washed with 50µl of sterile PBS. CD4+ T cells were isolated as described in Section 2.2.3. Cells were resuspended at $1.0x10^6$/ml in complete culture medium and 100µl added to each well of the plate coated with anti-CD3 and anti-CD28. Cells were maintained at 37°C for between 48-120 hours depending on the experimental design.

2.2.5 Extraction of DNA for PCR

PB was obtained in EDTA-containing vacutainers and stored at 4°C. DNA was extracted from whole blood samples using the QuickGene-810 system (Fujifilm, Japan). Briefly, 30µl of EDB buffer was added to a 1.5ml microcentrifuge tube. 200µl of whole blood and 250µl of

LDB buffer was added and samples vortexed for 15 seconds. Samples were incubated at 56°C for 2 minutes and then 250µl of >99% ethanol was added. Samples were vortexed as before and loaded into a QuickGene-810 system set on DNA whole blood mode. The QuickGene-810 system is a nucleic acid extraction technique which employs the use of a porous polymer membrane which selectively traps nucleic acids (http://www.autogen.com/product-quickgene-810.htm). When ethanol is added to lysates of whole blood samples the polarity is reduced which promotes the adsorption of nucleic acids into the membrane. The membrane is washed with a low polarity solution (wash buffer) under low pressure to remove any contaminating components without desorbing the nucleic acids. Finally, the nucleic acids are eluted using a high polarity solution (elution buffer) under low pressure and a pure solution of nucleic acids is obtained. This method of DNA extraction was chosen because of its simplicity, accuracy and speed which were required to efficiently process the large number of samples collected for this study. Following extraction using the QuickGene-810 system, DNA samples were stored at 4°C for later processing.

2.2.6 PTPN22 R620W genotyping

The Roche LightCycler 480 System (Roche Diagnostics Ltd. UK) was used to identify the single nucleotide polymorphism of PTPN22 (rs2476601) by real-time PCR and melting curve analysis. This method has been described previously in detail (Toms et al. 2011). Primers and probes included; primers: 5–GCCTCAATGAACTCCTCAAAC–3 (forward) and 5 CTGATAATGTTGCTTCAACGGA–3 (reverse), probes: the sensor (A) 5–CAGGTGTCCATACAGGAAGTG–3–FLU and the anchor 5–LCRED640–GGGGATTTCATCATCTATCCTTGGAGCAGTTG–PH. Primers and probes were used at concentrations of 10µM and 3µM respectively. The components for each PCR reaction (5.5µl PCR grade water, 0.5µl forward primer, 0.5µl reverse primer, 0.3µl anchor probe, 0.2µl sensor probe and 2µl genotyping master mix) were added to a 1.5ml microcentrifuge tube

and thoroughly vortexed. 9µl of the reagent mix was added to each well of a 96-well white qPCR plate. 1µl of DNA extracted as described in Section 2.2.5 was added to each well.

2.2.7 Treatment of cells with hydrogen peroxide

Jurkat T cells and CD4+ T cells were exposed to concentrations of hydrogen peroxide (H_2O_2) ranging from 50µM to 5mM depending on the experimental design. Cells were isolated and resuspended at $1.0x10^6$/ml in complete culture medium in 15ml falcon tubes. Tubes were wrapped in foil to avoid light exposure before direct addition of the appropriate amount of H_2O_2. Cells were exposed for 15 minutes at RT and then washed once by centrifugation at $300xg_{av}$ in complete culture medium using a Mistral 3000i centrifuge.

2.2.8 Treatment of cells with cigarette smoke extract

Cigarette smoke extract (CSE) was made by smoking one Marlboro Red cigarette in a fume hood for 10 minutes. The cigarette was lit, the smoke passed through a filter and bubbled into 30ml of sterile PBS at RT using a vacuum pump (Figure 2.1). The CSE preparation was filtered using a filter with a 0.2µM pore size and 1ml aliquots were frozen at -80°C and thawed out for each experiment. Cells were resuspended at $1.0x10^6$/ml in complete culture medium and transferred to a 6, 24 or 96-well tissue culture plate depending on experimental design. The CSE solution was added at concentrations of 3%, 15% and 30% and cells were treated at 37°C for 24 hours. Some cells were treated with an equal volume of sterile PBS to act as a 0% CSE control. To remove particulate matter (PM) from the CSE, Nanosep 3K Omega filters were washed twice with 0.5ml of PBS by centrifugation at $13,000xg_{av}$ for 10 minutes using an Eppendorf centrifuge 5415 R. 0.5ml of CSE was added to the filters and centrifuged as before. Flow through containing filtered CSE with molecules of <3000 molecular weight was collected and frozen in 1ml aliquots at -80°C. Aliquots were thawed out for each experiment.

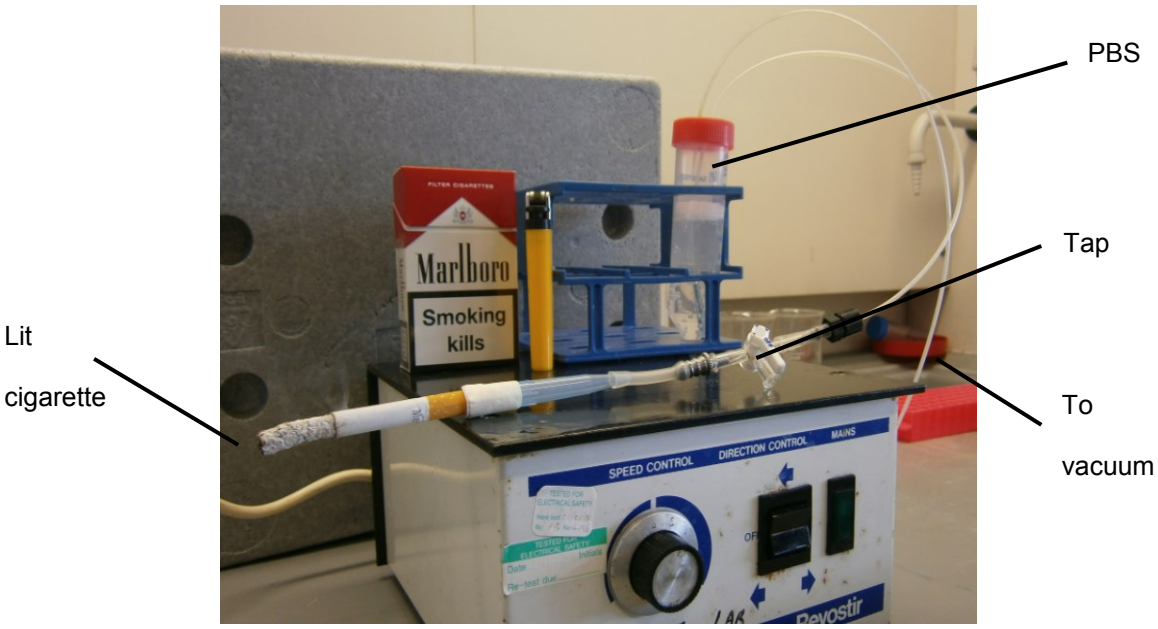


Figure 2.1 Generation of cigarette smoke extract. One Marlboro Red cigarette was lit in a fume hood for 10 minutes. The smoke was passed through a filter and collected by bubbling into 30ml of PBS at room temperature using a vacuum pump.

2.2.9 Treatment of cells with catalase

Jurkat T cells were resuspended at $1.0x10^6$/ml in complete culture medium and transferred to a 6, 24 or 96-well tissue culture plate depending on experimental design. Catalase from bovine liver or catalase polyethylene glycol were dissolved in sterile PBS and added to cells at a final concentration of 1μM. Cells were treated with the two types of catalase at the same time as CSE treatment, which was carried out for 24 hours at 37°C.

2.2.10 Measurement of Lyp phosphatase activity

Protein A (10μg/ml) in carbonate-bicarbonate buffer (50mM, pH 9.6) was added to wells of a 96-well Maxisorp™ plate overnight at 4°C. Wells were washed 3 times using 0.05% Tween 20/PBS and 5μg/ml anti-human Lyp monoclonal antibody in 1% BSA/0.05% Tween 20/PBS was added for 2 hours at 37°C to allow antibody binding to protein A. Wells were washed as before and blocked with 2% BSA/0.05% Tween 20/PBS for 1 hour at 37°C. Jurkat T cells, CD4 + T cells or EBV-transformed B cells were removed from culture and counted using a haemocytometer. The required number of cells were transferred to a 15ml falcon tube and washed twice in Ca^{2+} containing Hanks Buffered Salt Solution (HBSS) via centrifugation at $300xg_{av}$ for 6 minutes using a Mistral 3000i centrifuge. Cells were washed once in ice-cold 0.05% Tween 20/PBS, transferred to a 1.5ml microcentrifuge tube and lysed in TNE buffer (20mM Tris, 150mM NaCl, 1mM EDTA, pH 7.4) supplemented with protease inhibitors. Lysis was carried out by incubating tubes on ice for 30 minutes with regular agitation. Cell debris was removed by centrifugation at $13,000xg_{av}$ for 10 minutes at 4°C using an Eppendorf centrifuge 5415 R. After aspiration of the blocking solution, cell lysate was added to the wells and incubated for 3 hours at 37°C. Wells were washed 3 times and the activity of Lyp was detected by the addition of 0.2mM 6, 8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) in phosphate reaction mixture (0.1M sodium acetate, 1mM EDTA, 0.2% Triton X-100, pH 6.0) with 50mM DTT. Accumulation of fluorescent product was measured at 37°C over a 2-hour

time period using a Fluoroskan Ascent plate reader (Labsystems, UK) with the 355/460nm filter pair. Parallel wells bound with Lyp protein from identical lysates were probed with 3µg/ml chicken anti-human Lyp-HRP antibody in 1% BSA/0.05% Tween 20/PBS for 1 hour at 37°C. The standard ELISA substrate tetramethylbenzidine (TMB) was added for 15 minutes at RT and the reaction stopped by addition of 2M H_2SO_4. A single reading of absorbance at 405nm was measured using an Anthos HTIII spectrophotometric plate reader and Stingray software. The amount of Lyp was determined by comparison to a standard curve using purified Lyp protein. An overall summary of the assay procedure is shown in Figure 2.2 and the structure of the two substrates (FDP and DiFMUP) used in the assay are shown in Figure 2.3.

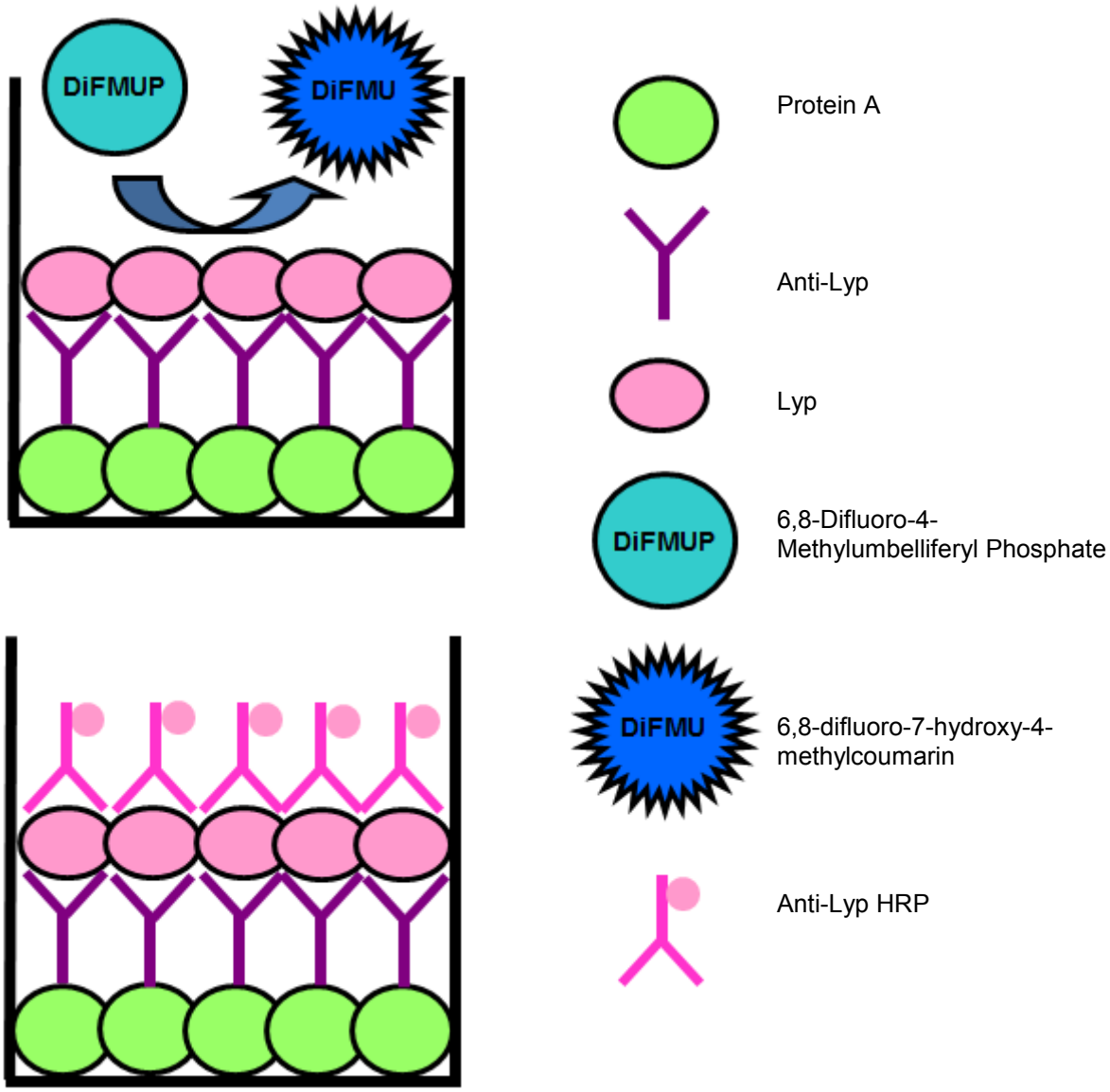
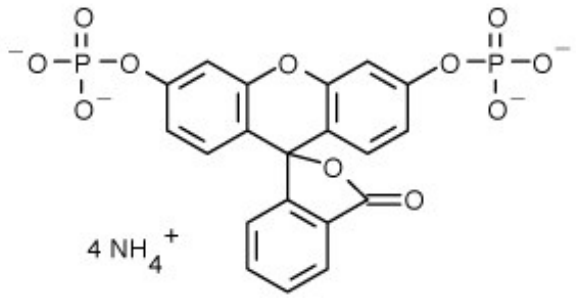
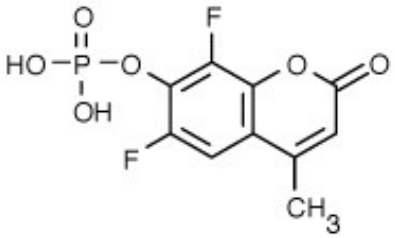


Figure 2.2 Principle of the Lyp phosphatase assay procedure. Lyp is captured from cell lysates using a protein A and anti-Lyp monoclonal antibody coated 96-well plate, and activity measured by dephosphorylation of the fluorescent substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). The amount of protein is measured using an anti-Lyp HRP conjugate, with reference to a standard curve generated with purified Lyp. These two measurements are then used to calculate the specific phosphatase activity.



Fluorescein Diphosphate (FDP)



6,8-Difluoro-4-Methylumbelliferyl Phosphate (DiFMUP)

Figure 2.3 The chemical structure of Fluorescein Diphosphate (FDP) and 6,8-Difluoro-4-Methylumbelliferyl Phosphate (DiFMUP), the two substrates used in the Lyp phosphatase assay method. Figures taken from www.lifetechnologies.com/order/catalog/product/F2999 and http://www.lifetechnologies.com/order/catalog/product/D6567.

2.2.11 Measurement of other protein tyrosine phosphatases

For global PTP activity, $1.0x10^6$ cells/well were lysed as for the Lyp phosphatase assay. 50µl of cell lysate was added to each well of a flat-bottomed 96-well plate and the global PTP activity measured by addition of 50µl of 30µM fluorescein diphosphate (FDP) in phosphate reaction mixture with 50mM DTT. The accumulation of fluorescent product was measured at 37°C over a 1 hour time period using a Fluoroskan Ascent plate reader (Labsystems, UK) with the 485/530nm filter pair. To measure CD45 phosphatase activity, the same procedure for measuring Lyp phosphatase activity was used as described in section 2.2.10. All conditions were identical except an anti-CD45 capture antibody was used.

2.2.12 Measurement of maximum fluorescence intensity

Serial dilutions of alkaline phosphatase (AP) were mixed with various concentrations of DiFMUP and readings taken every 30 seconds at 355/460nm using a Fluoroskan Ascent plate reader. The increase in fluorescence intensity (FI) following cleavage of 1nmol of substrate was calculated by dividing the maximum FI obtained for each concentration of AP by the number of moles of DiFMUP present in the wells.

2.2.13 Measurement of cellular reduced glutathione content

Cells were lysed as in the Lyp phosphatase assay (Section 2.2.10), supernatants were removed and 30µl of 30% perchloric acid (PCA)/0.5ml of lysate was added to allow precipitation of protein from the sample. Samples were vortexed, centrifuged at $13,000xg_{av}$ for 5 minutes using an Eppendorf centrifuge 5415 R, the supernatant removed and stored at -20°C for later processing. After sample thawing, 25µl of each sample was added in triplicate to a flat-bottomed 96-well plate with 100µl of working buffer (68.4mM $Na_2HPO_4.2H_2O$, 31.6mM $NaH_2PO_4.2H_2O$, 10mM EDTA, 0.02% BSA, pH 7.2) and 125µl of 20mM of dithio-bis 2-nitrobenzoic acid (DTNB). A single reading of absorbance at 405nm was measured using

an Anthos HTIII spectrophotometric plate reader and Stingray software. The reduced glutathione (GSH) concentration in the cell lysate was determined using the absorbance value with reference to a standard curve and represents the GSH content when $0.5x10^6$ cells were lysed in 0.5ml of lysis buffer.

2.2.14 Immunoblotting

Sample preparation

For immunoprecipitations (IPs) cells were lysed as described in the Lyp phosphatase assay method (Section 2.2.10). The lysates were pre-cleared by addition of 30µl of protein A/G plus agarose and incubated for 30 minutes at 4°C while rotating. The beads were pelleted by centrifugation at $300xg_{av}$ for 4 minutes using an Eppendorf centrifuge 5415 R, the supernatant removed and the beads discarded. 5µg/ml of anti-phosphotyrosine monoclonal antibody was added to the supernatant and incubated for 1 hour at 4°C while rotating, to allow IP of proteins phosphorylated on a tyrosine residue. 50µl of protein A/G plus agarose beads were added and the lysates incubated overnight at 4°C while rotating. Beads were pelleted as before and washed twice in PBS. The beads were reconstituted by suspension in 100µl of SDS loading buffer (see Appendix 1 for recipe). Samples were heated at 100°C for 5 minutes using a heating block and stored at -20°C until ready to load onto the gel. When IP was not used, cells were removed from culture, counted using a haemocytometer and the required numbers of cells transferred to 1.5ml microcentrifuge tubes. Cells were pelleted by centrifugation at $300xg_{av}$ for 5 minutes at 4°C and the supernatant removed. Samples were resuspended in hot SDS loading buffer (40µl/million cells) and heated at 100°C for 5 minutes using a heating block. Samples were stored at -20°C until ready to load onto the gel.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Samples equivalent to $1.0x10^6$ cells were loaded onto a 10% SDS-PAGE gel layered with a 5% stacking gel (see Appendix 1 for recipe). A broad range of pre-stained protein markers were loaded onto the gel to allow for determination of molecular weight. The gel was run at 100V for 1 hour and 30 minutes, or until optimal protein separation had occurred. Using a wet-blotting system, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane at 450mA for 1 hour and 30 minutes. The membrane was soaked in blocking buffer (5% BSA/0.05% Tween 20/PBS) for 1 hour on a shaker at RT to block non-specific binding. The membrane was probed with primary antibody (mouse anti-human beta actin, mouse anti-human Lck, rabbit anti-human pLck Y394 or rabbit anti-human pZap70 493) diluted 1:1000 in 0.05% Tween 20/PBS and left overnight on a shaker at 4°C to allow antibody binding. The membrane was washed 3 times for 10 minutes in 0.05% Tween 20/PBS. A secondary horseradish peroxidise linked antibody (sheep anti-mouse or donkey anti-rabbit) was added to the membrane diluted 1:10,000 in 0.05% Tween 20/PBS for 1 hour at RT. The membrane was washed 3 times as previously, before addition of enhanced chemoiluminescence (ECL) reagents (1:1) for 5 minutes. Visualisation of proteins was carried out by exposure to X-ray autoradiography film or using the ChemiDoc™ XRS+ gel imaging system (Bio-rad).

Stripping membranes

Membranes were stripped of antibodies to enable re-probing of the membrane with a different primary antibody. To do this, membranes were washed twice in stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween 20/ dH_2O, pH 2.2) for 5 minutes at RT on a shaker. Membranes were washed twice in PBS and twice in 0.05% Tween 20/PBS for 5 minutes at RT on a shaker. The immunoblotting method was then followed (as described previously), starting by blocking the membrane with 5% BSA/0.05% Tween 20/PBS for 1 hour at RT while on a shaker.

2.2.15 Cysteinyl labelling assay

An assay which labels reversibly oxidised SH groups was employed to detect possible oxidation of the cysteine residue found within the active site of PTPs. This method has been described previously in detail (Boivin et al. 2008) and employs a number of steps to label and detect reversibly oxidised SH groups (Figure 2.4). Briefly, cells were treated with oxidising agents and then lysed in a degassed buffer at pH 5.5 containing iodoacetamide (IAA), which alkylates any SH groups which are in a reduced state. Any SH groups which have been oxidised by the treatment are protected from alkylation by IAA. Lysates were subject to buffer exchange using a size exclusion chromatography column to remove IAA and through the use of a reducing buffer to allow any oxidised SH groups to be reduced back to their original state. These reduced SH groups then react with the biotinylated compound iodoacetylpolyethylene oxide (IAP) to enable purification by streptavidin pull-down and finally visualisation of proteins using a streptavidin-horseradish peroxidise linked antibody.

Cysteinyl labelling

Jurkat T cells were counted using a haemocytometer, transferred to a 15ml falcon tube and centrifuged at 300xg_{av} for 6 minutes using a Mistral 3000i centrifuge. Cells were washed in ice-cold PBS, transferred to a 1.5ml microcentrifuge tube and 600µl of ice-cold freshly prepared lysis buffer (25mM sodium acetate, 1% Triton X-100, 150mM NaCl, 10% vol/vol glycerol, protease inhibitors) supplemented with 10mM IAA was added. Prior to use, lysis buffer was degassed on a vacuum line for 10 minutes and placed on ice. Cells were agitated regularly on ice for 30 minutes before centrifugation at 13,000xg_{av} for 5 minutes using an Eppendorf centrifuge 5415 R. Lysates were placed on a rotating wheel for 1 hour at RT to allow complete alkylation of any free thiols. Cell debris was cleared by centrifugation at 13,000xg_{av} for 3 minutes and the cell lysate was slowly applied to a desalting column, which had been equilibrated with IAA-free lysis buffer. 1.5µM DTT was added to the lysate and

placed on a rotating wheel for 30 minutes at RT. 1mM IAP probes were added to the lysate and incubated for 1 hour while rotating at RT. Enrichment of biotinylated proteins was carried out by addition of 20µl of streptavidin-sepharose beads and incubation for 16 hours at 4°C while rotating. Beads were washed in PBS by centrifugation at 13,000xg_{av} for 2 minutes and reconstituted by resuspension in 40µl of hot SDS loading buffer. Samples were heated at 100°C for 5 minutes using a heating block, and stored at -20°C until ready to load onto the SDS gel.

SDS-PAGE and immunoblotting

Samples were prepared as described in the cysteinyl labelling method and heated at 100°C for 5 minutes using a heating block before use. To detect overall oxidation of SH groups immunoblotting was carried out as described in Section 2.2.14, however no primary antibody was required. Membranes were probed with an alternative secondary antibody which was streptavidin-horseradish peroxidase linked and diluted 1:10,000 in 0.05% Tween 20/PBS.

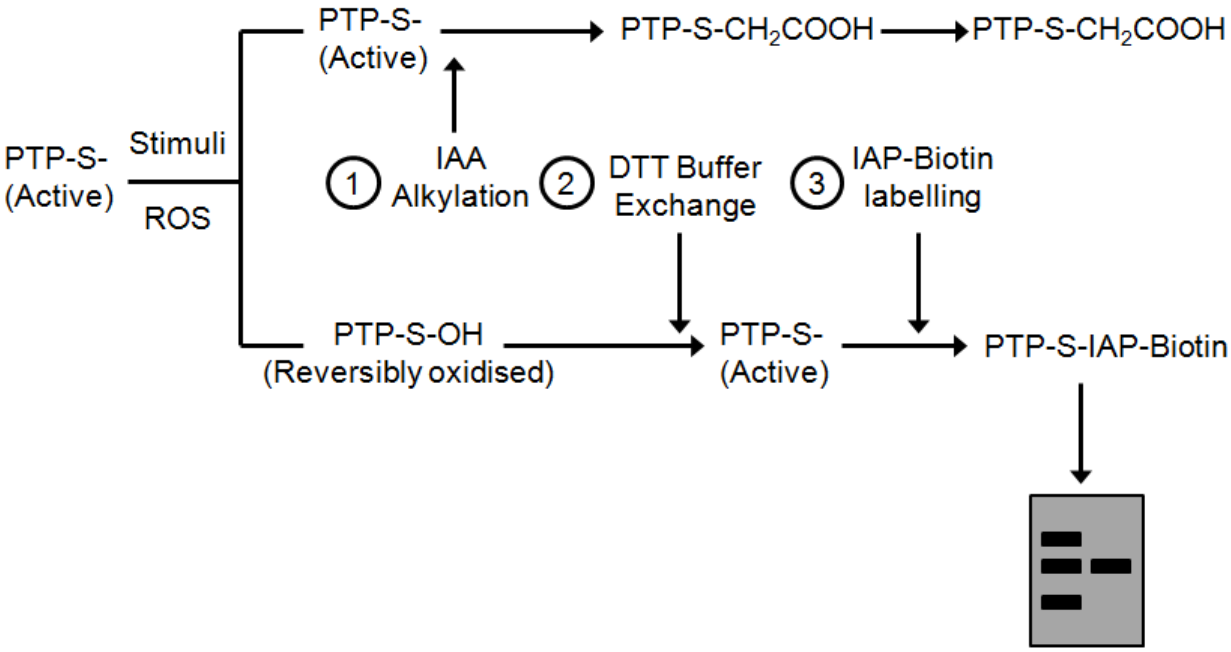


Figure 2.4 Principle of the cysteinyl-labelling assay. (**1**) After a physiological stimulus, the cells were lysed in a degassed buffer at pH 5.5 containing IAA. The low-pK_a cysteine residue at the active site of those PTPs that remained in a reduced state are alkylated, terminally inactivating this pool of PTPs. Conversely, the cysteine residues that were oxidised by second-messenger ROS molecules were protected from irreversible alkylation. (**2**) IAA was then removed from the lysate by buffer exchange using size-exclusion spin columns, and the oxidised cysteine residues were reduced back to the thiolate ion with DTT. (**3**) The oxidised PTPs were maintained in pH 5.5 buffers and incubated with a sulfhydryl-reactive IAP probe. Purification by streptavidin pull-down and immunoblotting permits identification of ROS-targeted PTPs. Figure adapted from (Boivin et al. 2008). DTT, Dithiothreitol; IAA, Iodoacetamide; IAP, Iodoacetylpolyethylene oxide; PTP, Protein tyrosine phosphatase; ROS, Reactive oxygen species.

2.2.16 Determination of cell viability

Jurkat T cells were resuspended at $1.0x10^6$/ml in complete culture medium and transferred to a 24-well tissue culture plate. CSE solution was added at concentrations of 3%, 15% and 30% or an equal volume of sterile PBS as a control, and cells were treated at 37°C for 24 hours. Cells were removed from culture, transferred to FACS tubes and washed in PBS by centrifugation at $300xg_{av}$ for 6 minutes using a Mistral 3000i centrifuge. Cells were resuspended at $1.0x10^6$/ml in Annexin V binding buffer (10μM Hepes/NaOH pH 7.4, 140μM NaCl and 2.5μM $CaCl_2$) and 5μl of Annexin V APC added for 15 minutes at RT in the dark. 2μl of propidium iodide was added for 10 minutes at RT and cells were analysed using flow cytometry (Cyan ADP, Dako, UK).

2.2.17 Measuring Lck phosphorylation status

This method employs the use of intracellular staining using fluorescent-conjugated antibodies specific to phosphorylated tyrosine residues of the kinase Lck (Y394 and Y505). Jurkat T cells or CD4+ T cells were resuspended at $1.0x10^6$/ml and 500μl transferred to each well of a 24-well tissue culture plate. The plate was incubated on ice for 15 minutes and 3μg/ml anti-CD3 added to the wells. The plate was moved to RT and after 5 minutes 4μg of goat anti-mouse was added to the wells containing anti-CD3 to cross link the receptors. Cells were stimulated using this method for 1 minute before the addition of 500μl pre-warmed Phosflow fix buffer and incubation at 37°C for 15 minutes to maintain cells phosphorylation status. Cells were transferred to FACS tubes and washed twice in PBS via centrifugation at $300xg_{av}$ for 6 minutes using a Mistral 3000i centrifuge. The supernatant was removed and 500μl of Phosflow Perm/Wash buffer was added for 10 minutes at RT. Cells were washed in Perm/Wash buffer via centrifugation at $300xg_{av}$ for 6 minutes. 100μl of Perm/Wash buffer containing primary antibodies (anti-pLck Y394 and anti-pLck Y505 PE) was added to each sample and incubated for 20 minutes at RT in the dark. Samples were washed twice in

Perm/Wash buffer as before. 50µl of Perm/Wash buffer containing secondary antibody (goat anti-rabbit IgG FITC) was added to each sample and incubated for 15 minutes at RT in the dark. Cells were washed twice in Perm/Wash buffer as before and resuspended in 500µl of PBS for analysis by flow cytometry (Cyan ADP, Dako, UK).

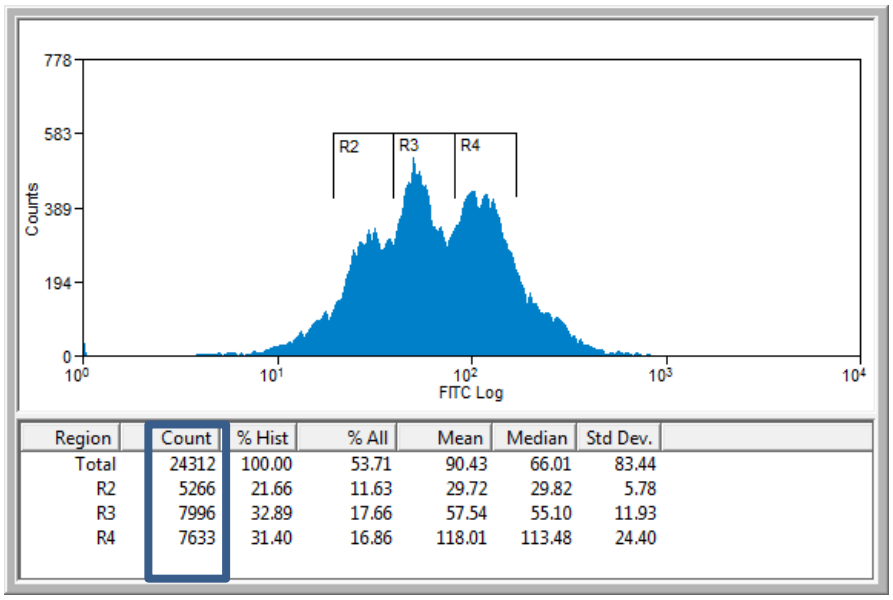
2.2.18 Analysis of reactive oxygen species production

Neutrophils were isolated as described in Section 2.2.3 and resuspended at $1.0x10^6$/ml in complete culture medium in a 15ml falcon tube. 200,000 cells were transferred to each FACS tube required for the experiment. Some neutrophils were pre-incubated with 0.5µg TNF-α for 15 minutes at 37°C in order to prime the cells for activation. 10µM dihydrorhodamine (DHR) in DMSO was added for 5 minutes at 37°C, and cells were then stimulated with 1µM fMLP. Excess DHR was removed by washing cells with ice-cold PBS via centrifugation at $300xg_{av}$ for 6 minutes using a Mistral 3000i centrifuge. Cells were fixed using 2% paraformaldehyde overnight at 4°C, washed as before and resuspended in 0.5ml of PBS for analysis. The level of DHR fluorescence was assessed using flow cytometry (Cyan ADP, Dako, UK).

2.2.19 Measurement of cell proliferation

CD4 + T cells were isolated as described in Section 2.2.3. Cells were resuspended at $1.0x10^6$/ml in pre-warmed sterile PBS in a 15ml falcon tube. CFSE was added at a final concentration of 1µM and cells incubated for 10 minutes at 37°C in the dark. The tube was wrapped in foil to avoid light exposure. CFSE staining was quenched by adding 5 times the volume of cold complete culture medium for 5 minutes on ice. Cells were washed 3 times in complete culture medium via centrifugation at $300xg_{av}$ for 6 minutes using a Mistral 3000i centrifuge and cultured as described in Section 2.2.4. Cells were removed from culture at Day 2 and 5, and their proliferation analysed by flow cytometry (Cyan ADP, Dako, UK). Proliferation was measured by calculation of the proliferation index, an example of which is shown on the next page.

Example calculation of the proliferation index



Region	Count	% Hist	% All	Mean	Median	Std Dev.
Total	24312	100.00	53.71	90.43	66.01	83.44
R2	5266	21.66	11.63	29.72	29.82	5.78
R3	7996	32.89	17.66	57.54	55.10	11.93
R4	7633	31.40	16.86	118.01	113.48	24.40

3 division gates in total (R2, R3 and R4)

$$\frac{\text{Total number of cells in each division gate}}{\text{Number of cell divisions}}$$

$$R4 = \frac{7633}{1} + R3 = \frac{7966}{2} + R2 = \frac{5266}{3} = 13,371$$

13, 371 = the total number of starting cells

$$\frac{\text{Total number of cells}}{\text{Total number of starting cells}} = \frac{24,312}{13,371} = 1.8$$

= 1.8 = proliferation index

2.2.20 Cytokine detection by flow cytometry

CD4+ T cells were isolated as described in Section 2.2.3 and stimulated as described in Section 2.2.4 for 48 hours at 37°C. Following stimulation CD4+ T cells were treated with 100µg/ml brefeldin A for 3 hours at 37°C in order to retain cytokines within the cells. Cells were transferred to a 96-well flexi plate and washed in 100µl of FACS buffer via centrifugation at $300xg_{av}$ for 6 minutes at 4°C using a Mistral 3000i centrifuge. The supernatant was removed and 50µl of FIX® reagent added for 15 minutes at RT. Cells were washed twice in FACS buffer as before, the supernatant was removed and 50µl of PERM® reagent containing fluorescent conjugated cytokine antibodies (IL-4, IL-10, IL-17 and IFN-γ) or appropriate isotype controls were added for 30 minutes on ice and in the dark. Cells were washed twice as before and resuspended in 100µl FACS buffer. Samples were transferred to FACS tubes containing 400µl of PBS and analysed using flow cytometry (Cyan ADP, Dako, UK).

2.2.21 Cytokine detection by ELISA

CD4+ T cells were isolated as described in Section 2.2.3 and stimulated as described in Section 2.2.4 for 48 hours at 37°C. Following stimulation 100µl of supernatant was aspirated from each well of the tissue culture plate, transferred to a 1.5ml microcentrifuge tube and stored at -80°C. The supernatant was then used to asses the production of IL-4, IL-10, IL-17, IFN-γ and TNF-α using ELISA kits, which were used according to the manufacturer's instructions.

2.2.22 Measurement of intracellular calcium

This method monitors Ca^{2+} release using the Ca^{2+} indicator Indo-1 AM by measuring changes in fluorescence which occur in response to the addition of a stimulus (Carruthers et al. 2000). Cells were placed in 15ml falcon tubes, washed by centrifugation at $300xg_{av}$ for 6

minutes in Ca^{2+} containing HBSS using a Mistral 3000i centrifuge and resuspended at $2.0x10^6$/ml in complete culture medium. Indo-1 AM ester was reconstituted in DMSO just before use and added to the cells at a final concentration of 1μM. Cells were incubated for 40 minutes at 37°C in the dark to allow incorporation of the dye. After 2 washes in HBSS, cells were resuspended at $1.0x10^6$/1.5ml and transferred to acrylic cuvettes. Samples were placed in a water bath at 37°C for 5 minutes prior to any measurements being taken. Samples were placed in a Perkin Elmer LS50B spectrofluorimeter and maintained at 37°C when taking measurements. To maintain cells in suspension, a magnetic stirrer was placed in the cuvette. T cells were stimulated with 5μg anti-CD3 and neutrophils were stimulated with 50μM fMLP. 2μM ionomycin was also added as a positive control. Data was collected using FL Winlab Version 2.1 software using a dual-emission experimental protocol for Indo-1 AM loaded cells.

2.2.23 Adhesion and migration assays

This method and its principals have been described previously in detail (McGettrick et al. 2007, McGettrick et al. 2009). Human umbilical endothelial cells (HUVEC) were isolated from umbilical cords and cultured in Medium 199 (M199) supplemented with 20% FCS, 10ng/ml epidermal growth factor, 35ng/ml gentamycin, 1ng/ml hydrocortisone and 2.5ng/ml amphotericin B. Primary HUVEC were dissociated using trypsin/EDTA and seeded on 12-well tissue culture plates at a density to yield confluent monolayers within 24 hours. TNF-α (0.05 and 5ng/ml) was added to confluent monolayers for 4 hours before the adhesion assay. HUVEC were washed with M199 supplemented with 0.15% BSA (M199BSA) to remove residual cytokines and purified neutrophils ($1x10^6$ cells/ml in M199BSA) were added for 6 minutes. Non-adherent neutrophils were removed by gentle washing with M199BSA. Digitised images of the endothelial surface were made using phase contrast microscopy at 37°C at 2 and 9 minutes from the end of the settling period and analysed offline using Image-Pro Plus software (DataCell Ltd, Finchampstead, UK). Adhesion was determined as a percentage of neutrophils that adhered by counting all adherent cells per image, then

calculating the number of cells per well and dividing this number by the total number of neutrophils added. Transmigration was calculated as a percentage of the adherent cells that appeared phase dark (i.e. underneath the endothelial monolayer). Migration velocities (μm/min) of phase-dark lymphocytes underneath the HUVEC were determined the average distance moved by the centre of the cell per minute.

2.2.24 Statistical analysis

Results were analysed using GraphPad Prism Software Version 5.1. Non-parametric data were tested for normality using the D'Agostino-Pearson test. The statistical tests used were determined according to if the data were normally distributed and paired or unpaired. The specific statistical tests used were as follows: (1) normally distributed paired data = paired t test, (2) not normally distributed paired data = Wilcoxon matched-pairs signed rank test, (3) normally distributed unpaired data = unpaired t test, and (4) not normally distributed unpaired data = Mann-Whitney test. For data comparing more than three groups, analysis was carried out using ANOVA followed by Bonferonni post-test. $P<0.05$ was considered to be of statistical significance.

CHAPTER THREE: LYP PHOSPHATASE ASSAY DEVELOPMENT

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3.1 Introduction

The PTPN22 gene encodes the protein Lyp, a protein tyrosine phosphatase (PTP) expressed by all leukocytes. Lyp is an important regulator of signalling mediated through engagement of immune cell receptors and small changes in its phosphatase activity could have a big effect on such signalling cascades. Changes in Lyp activity could be induced by genetic or environmental factors and it is of importance to be able to accurately assess these. The genetic variant PTPN22 R620W is associated with an increased risk of autoimmunity and is known to cause changes in the phosphatase activity of the Lyp protein (Burn et al. 2011). A number of environmental factors are also known to induce changes to phosphatases such as Lyp. For example, the activity of Lyp and other PTPs with an active site cysteine have been shown to be decreased by oxidising agents like H_2O_2 (Meng et al. 2002). These oxidising agents can also be found in cigarette smoke (CS) (Borgerding et al. 2005) which is an environmental factor associated with an increased risk of RA and also known to interact with PTPN22 R620W via an unknown mechanism (Kallberg et al. 2007).

Given the association of PTPN22 R620W and cigarette smoking with an increased risk of autoimmune conditions such as RA, it is of importance to be able to measure the effects of these factors on the activity of the Lyp protein directly using a reliable and reproducible method. Such a method was not in existence at this time, and so it was decided to develop a new method. Thus this chapter focuses on development of a Lyp phosphatase assay to allow rapid and reproducible capture of Lyp protein from a variety of immune cells.

3.1.1 Current methods for measuring phosphatase activity

To assess the effects of alterations in PTP activity on immune cell receptor signalling, an assay procedure which directly measures PTP activity is required. At present there are a number of phosphatase assays used to detect the activity of PTPs *in vitro*, as reviewed in (Montalibet et al. 2005). All begin with an IP or biochemical purification of the PTP of interest, then the varied preparation steps that follow function to provide suitable conditions under which the PTP can dephosphorylate a target substrate. It is important that the IP is done using a highly specific antibody to minimise capture of other PTPs and also the chosen antibody does not bind and interfere with the active site of the PTP.

Following the initial purification procedure there are three main methods of detecting PTP activity, (1) colorimetric (2) radioactive and (3) fluorimetric (Lorenz 2011). The most extensively used method is colorimetric, which commonly employs use of the substrate p-Nitrophenyl Phosphate (pNPP). When dephosphorylated under alkaline conditions, pNPP generates the yellow substance p-nitrophenol which is detectable using a spectrophotometer (Lorenz 2011). Another example of colorimetric detection is the use of malachite green, a dye which complexes with inorganic phosphate and molybdate under acidic conditions to produce a visible colour change from yellow to green (Van Veldhoven et al. 1987). Radioactive methods employ the use of a substrate containing a radioactive isotype of phosphorus within its moeity of phosphotyrosine residues, which upon dephosphorylation is released and emits measureable levels of radioactivity (Frank et al. 1999). More recently, the use of fluorescent substrates such as fluorescein diphosphate (FDP) and 6, 8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) has occurred. These phosphorylated substrates emit fluorescence upon dephosphorylation by a PTP, and are around 50 times more sensitive in comparison to pNPP (Huang et al. 1992).

The choice of detection method should be based on consideration of the advantages and disadvantages of each procedure, and is usually specific to the PTP of interest. For example colorimetric methods are often cheap and simple to set up, however the sensitivity is low when compared to radioactive and fluorimetric methods. Using radioactivity provides increased sensitivity, but reagents must be made up more frequently due to radioactive decay over time. Fluorescent substrates provide the increased sensitivity that is also obtained by radioactivity, and in addition are stable when stored at low temperatures. In summary there are advantages and disadvantages to all three methods, as shown in Table 3.1.

As mentioned, the ideal method of measuring PTP activity is highly dependent on the PTP of interest and a number of approaches to date have been used to measure the activity of the Lyp phosphatase. pNPP, DiFMUP and a novel fluorescent substrate have all been used previously to detect the activity of Lyp immunoprecipitated from a variety of cell lines transfected with recombinant Lyp protein (Cohen et al. 1999, Hill et al. 2002, Orru et al. 2009, Fiorillo et al. 2010). However, none of these methods were extended to detect the activity of native Lyp in primary cells. More recently, pNPP was used again as a Lyp substrate to show that Lyp phosphatase activity is higher in leukocytes obtained from vasculitis patients with the PTPN22 R620W SNP, widely believed to be a gain-of-function mutant (Cao et al. 2012). Despite this finding, Lyp phosphatase activity was only detected in leukocytes with this SNP, and not in patients without the polymorphism. In fact, activity in cells without the polymorphism was equivalent to that of the negative control, even though the same amount of Lyp protein was present as in the leukocytes isolated from individuals with the SNP. This implies that using pNPP is not sensitive enough to detect subtle differences in Lyp phosphatase activity and a more sensitive method is required.

Table 3.1 Comparison of the advantages and disadvantages of different methods of detecting the activity of protein tyrosine phosphatases *in vitro*.

Phosphatase activity detection method					
Colorimetric		Radioactive		Fluorimetric	
+	-	+	-	+	-
Cheap and widely available reagents	Low sensitivity	High sensitivity	Reagents are unstable	High sensitivity	Requires a fluorescent plate reader
Reagents can be stored for an extended time period	Often uses non-specific substrates	Allows exact quantification of PTP activity	Expensive and time consuming	Choice of substrates for use at acid and alkaline pH	Can require a large number of primary cells
	Can require a large number of primary cells		Can require a large number of primary cells	Reagents can be stored for an extended time period	

PTP, protein tyrosine phosphatase.

3.2 Results

3.2.1 Determination of a suitable test cell type

Aside from choosing a suitable substrate to measure Lyp activity, it was also necessary to consider which type of immune cells would be most useful in development of the assay. Previous studies have shown that a variety of primary immune cell types express Lyp (Cohen et al. 1999, Chien et al. 2003). However, to optimise and develop a successful assay large cell numbers were likely to be required and thus it was decided to use readily available cell lines. Two widely used cell lines are Jurkat and U937, however conflicting results have been published as to their expression levels of Lyp (Cohen et al. 1999, Chien et al. 2003). Given that Jurkat cells are a T cell line and U937 cells are a monocytic cell line, it could be that expression levels of Lyp are different in these two cell types. To confirm expression of the Lyp protein in Jurkat and U937 cells, immunoblotting was carried out and revealed both cell lines expressed Lyp (Figure 3.1). As no loading control was carried out, a comparison between the two cell lines in terms of Lyp expression could not be done. However, as Jurkat and U937 cells were shown to express Lyp both were used in the development of the assay.

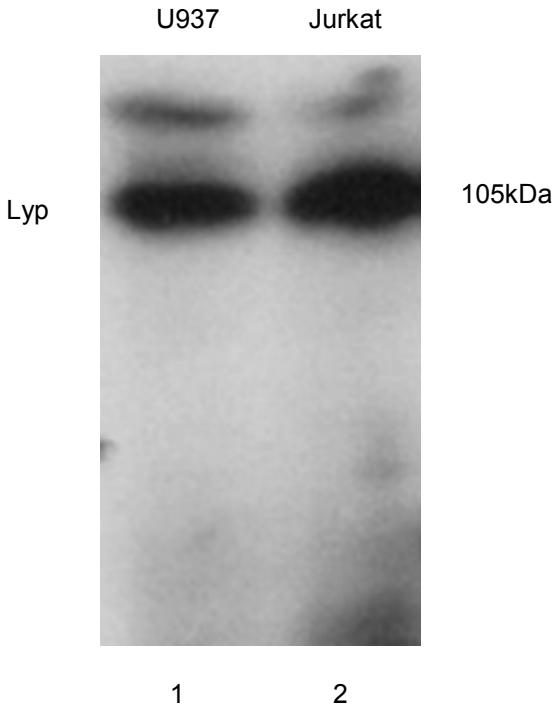


Figure 3.1 U937 and Jurkat cells express the Lyp protein. U937 (Lane 1) and Jurkat (Lane 2) cell lysates were run on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with a primary antibody to Lyp followed by a HRP-linked secondary antibody. This image is taken from one single experiment.

3.2.2 Use of fluorescent substrate for measuring Lyp phosphatase activity

Given the advantages of using a fluorimetric method of assessing phosphatase activity, this was chosen as a basis for the development of a new and more sensitive Lyp phosphatase assay. The assay needed to be suitable for a wide range of cell types including cell lines and primary cells, which can be isolated from the blood and other fluids. The assay also needed to be fairly rapid and require only a small number of cells. Such a method would be useful to compare the activity of Lyp in healthy and diseased individuals, and to identify differences between different human disease states. The initial approach to developing a successful assay was based upon a method previously used for measuring the activity of the CD45 phosphatase (Rider et al. 2003). This method monitors phosphatase activity by measuring the increase in fluorescence generated by dephosphorylation of the substrate FDP. However, using FDP appeared problematic and the phosphatase activity of Lyp could not be detected in a number of cell lines (Figure 3.2) even with a large excess of $8.0x10^6$ cells (results not shown).

As FDP was unsuccessful, the next step was to try an alternative fluorescent substrate. DiFMUP was chosen based on limited evidence of its use to detect Lyp activity from purified Lyp protein (Orru et al. 2009). This was more suitable and Lyp activity was detected in fairly small numbers ($1.0x10^6$) of HL60, U937 and Jurkat cells (Figure 3.2). After a 2 hour time period the largest increase in fluorescence intensity (FI) was observed in HL60 cells (473±97), and the least in U937 cells (153±17). The fact that the Lyp protein can effectively dephosphorylate DiFMUP and not FDP may be due to differences in the chemical structure of the two fluorescent substrates (Figure 2.3).

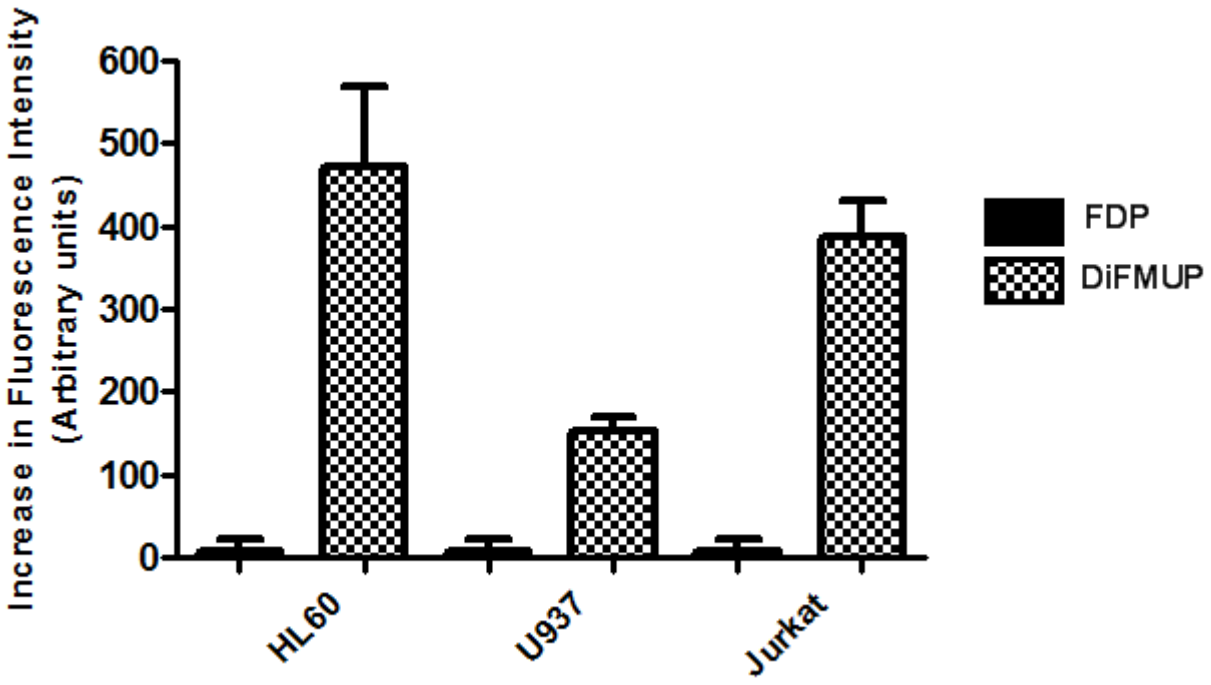


Figure 3.2 Comparison of use of the fluorescent substrates FDP and DiFMUP. $1.0x10^6$ Jurkat T cells, HL60 cells and U937 cells were lysed and added to triplicate wells of a 96-well plate coated with anti-Lyp. Lyp activity was then measured as described in Materials and Methods, using FDP (black bars) or DiFMUP (checked bars) as the substrate in the assay. Readings were taken at time 0 and 2 hours and the increase in fluorescence intensity (FI) calculated by subtraction of the reading at time 0 from the reading after 2 hours at 37°C. Data represents the mean (±SEM) of three separate experiments.

3.2.3 Measuring the activity of Lyp

Following confirmation that Lyp phosphatase activity could be detected using the fluorescent substrate DiFMUP, it was necessary to investigate the optimal number of cells required to carry out an assay using this method. To do this Jurkat T cells were used, as they have been found to express the Lyp protein at reasonable levels (Cohen et al. 1999) and availability of cells would not be an issue. It was found that Lyp phosphatase activity could be detected in as little as $0.2x10^6$ Jurkat T cells (Figure 3.3 A), with an average increase in FI of 103±8. When larger numbers of cells were used there was little difference in the increase in FI, ($0.8x10^6$ cells = 279±16, $1.0x10^6$ cells = 308±21, $2.0x10^6$ cells = 297±53) and so in future assays using Jurkat T cells $1.0x10^6$ cells per well was used. This number of cells is a considerable improvement when compared to previous phosphatase assays, which suggest the number of cells required to be between $5x10^6$ and $5x10^7$ (Lorenz 2011).

An important consideration for the Lyp phosphatase assay was its suitability for use with primary cells. Thus the next issue to address was if the number of primary cells required to detect Lyp activity was comparable to the number of Jurkat T cells needed. Due to the transformed nature of the Jurkat T cell line, they are likely to have a much higher protein turnover when compared to untransformed primary T cells, so it was possible that more primary cells would be needed. To investigate this, CD4+ T cells freshly isolated from PB were used in the Lyp phosphatase assay. Indeed it was observed that more CD4+ T cells were required to produce a similar increase in FI to smaller numbers of Jurkat T cells (Figure 3.3 B). $1.0x10^6$ Jurkat T cells produced an increase in FI of 308±21 whereas this was around 40% less when using the same number of primary CD4+ T cells (189±21). This difference was minimised when larger numbers of CD4+ T cells were used ($2.0x10^6$ Jurkat = 297±53, $2.0x10^6$ CD4+ = 247±19) and thus $2.0x10^6$ CD4+ T cells per well were used in future assays.

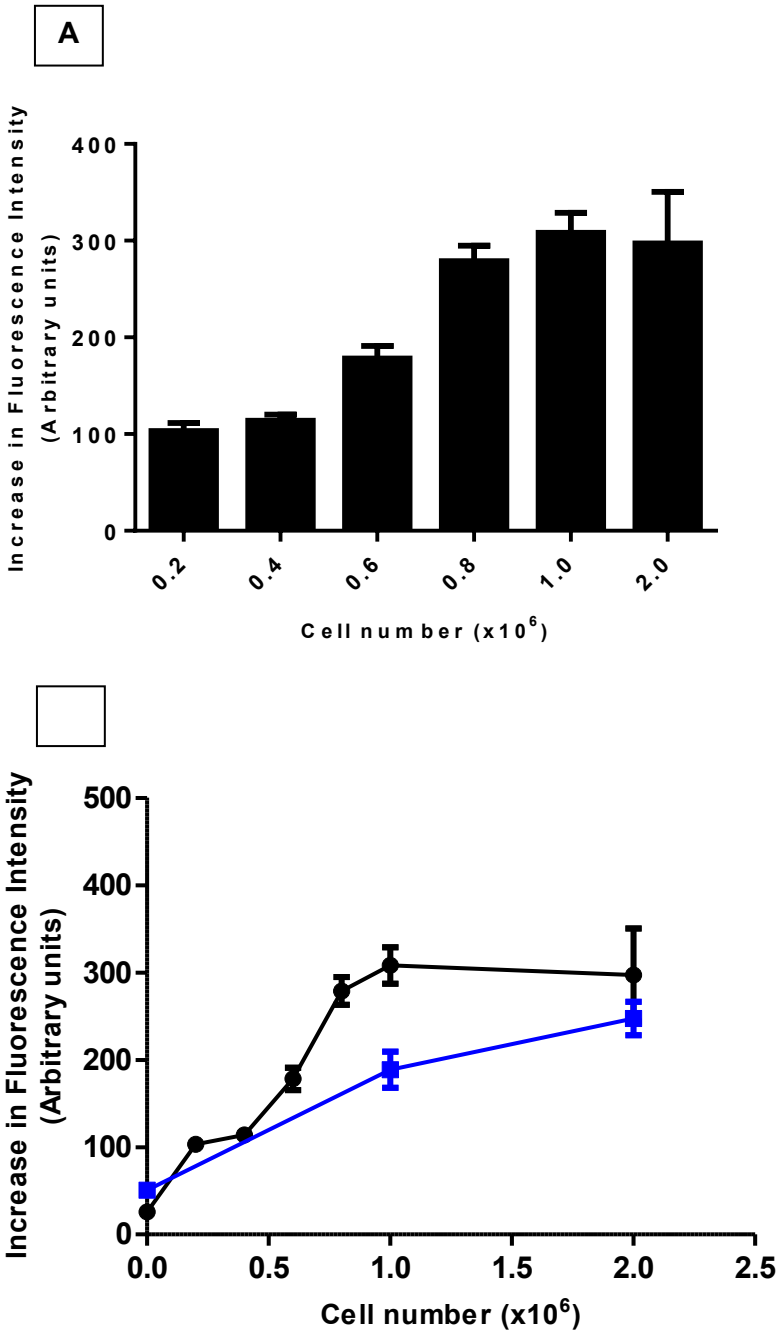
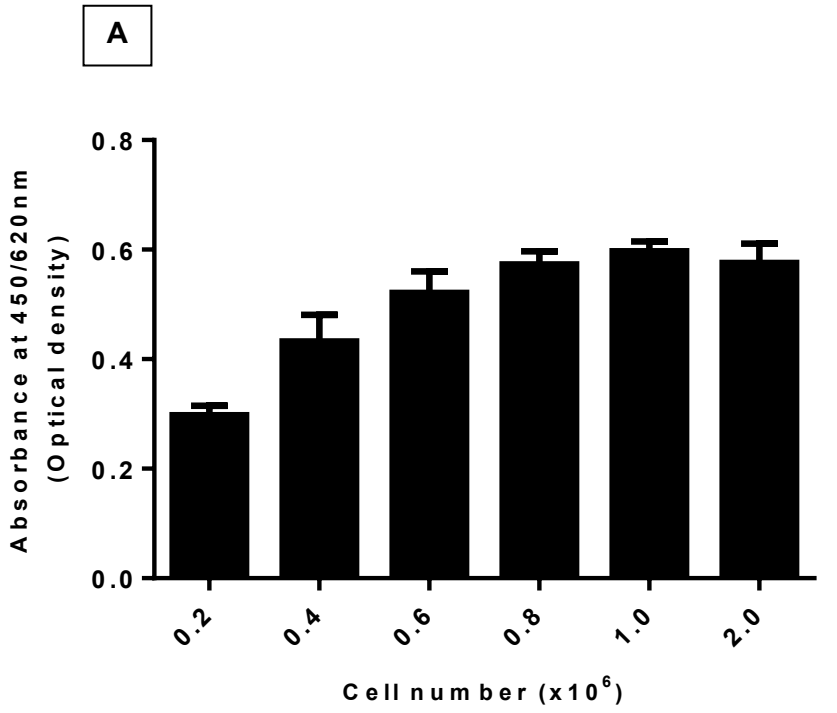


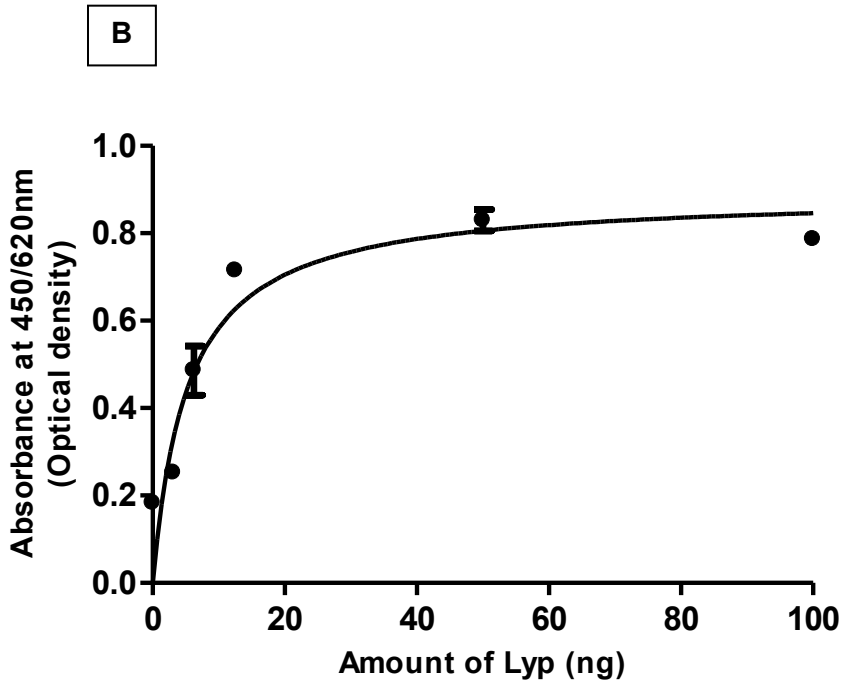
Figure 3.3 Lyp phosphatase activity in Jurkat T cells and primary CD4+ T cells. Different numbers of Jurkat T cells or CD4+ T cells were lysed in identical amounts of lysis buffer and 50µl of lysate added to triplicate wells of a 96-well plate coated with protein A and anti-Lyp antibody. The plate was incubated at 37°C for 3 hours, and wells were washed 3 times using 0.05% Tween 20/PBS. The activity of Lyp was detected by addition of 0.2mM DiFMUP and 50mM DTT. Activity was measured over a 2 hour time period with readings taken in triplicate. Results show the mean (±SEM) of three separate experiments. **(A)** The activity of the Lyp phosphatase in different numbers of Jurkat T cells. **(B)** Comparrison of the Lyp phosphatase activity in Jurkat T cells (black line) and primary CD4+ T cells (blue line).

3.2.4 Measuring the amount of Lyp

The next step in development of the assay was to be able to correlate the amount of Lyp protein bound during the assay with Lyp phosphatase activity. This is more useful as it accounts for any Lyp protein bound but in an inactive state. To date, only a small number of phosphatase assays have been able to measure activity and amount (Rider et al. 2003, Montalibet et al. 2005), with only one assay specific to the Lyp protein (Montalibet et al. 2005). This was achieved by probing wells with an anti-Lyp-HRP antibody and showed that Lyp protein could be detected even when phosphatase activity could not. To enable measurement of protein amount, the activity assay was modified by using a polyclonal anti-Lyp HRP antibody after Lyp protein capture. This modification to the method was tested using Jurkat T cells and the absorbance of HRP increased with increasing numbers of cells (Figure 3.4 A). Similarly to the activity assay, saturation appeared to occur at around $1.0x10^6$ cells, with similar absorbance values obtained for cell numbers between $0.8x10^6$ and $2.0x10^6$ ($0.8x10^6$ cells = 0.573±0.02, $1.0x10^6$ cells = 0.597±0.02, and $2.0x10^6$ cells = 0.576±0.03).

Although using anti-Lyp HRP allowed estimation of the amount of Lyp bound to the plate, it provided no actual quantification of the absolute amount of Lyp protein. To overcome this, a standard curve using purified Lyp protein was produced using the assay (Figure 3.4 B), which could then be used as a reference to calculate the actual quantity of Lyp protein. Using the absorbance of HRP (Figure 3.4 A) and the standard curve (Figure 3.4 B), the actual amount of Lyp protein bound from different numbers of Jurkat T cells was determined (Figure 3.5 C). The amount of Lyp protein captured during the assay increased with increasing numbers of cells (Figure 3.4 C). This varied from 1.65±0.29ng captured from $0.2x10^6$ cells, to 11.99±3.35ng captured from $2.0x10^6$ cells. This increase in protein amount was closely proportional to the number of cells used in the assay procedure. For example, the average amount of Lyp bound from $0.4x10^6$ cells was 5.38±1.72ng, which proportionally increased to 10.54±1.31ng when twice as many cells ($0.8x10^6$) were used.


A
Absorbance at 450/620nm
(Optical density)
0.0
0.2
0.4
0.6
0.8
0.2
0.4
0.6
0.8
1.0
2.0
Cell number (x10^6)


B
Absorbance at 450/620nm
(Optical density)
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
Amount of Lyp (ng)

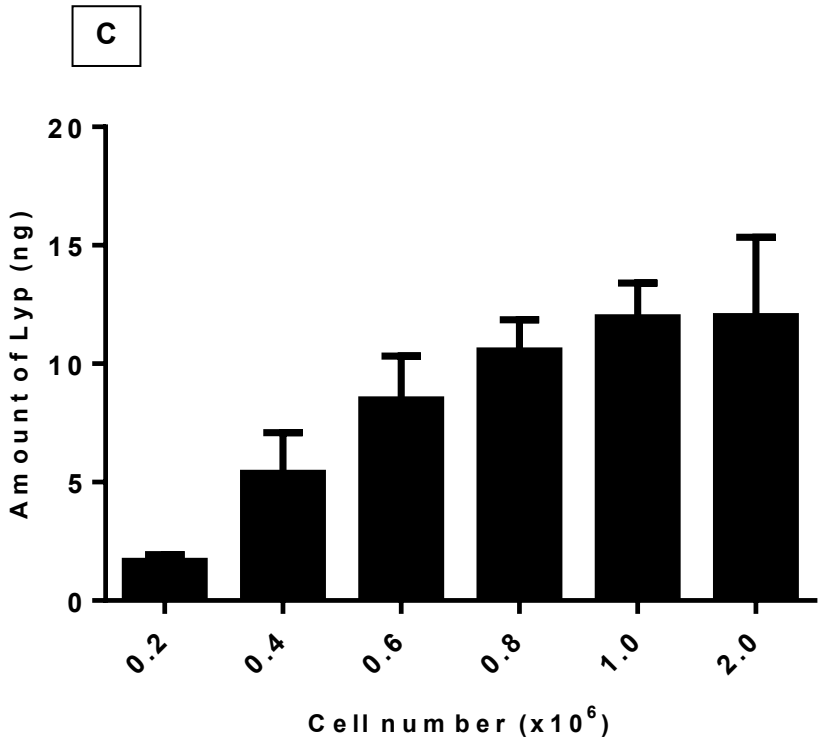


Figure 3.4 Calculation of the amount of Lyp protein captured from Jurkat T cells using a standard curve. Different numbers of Jurkat T cells were lysed in identical amounts of lysis buffer. Purified Lyp protein was serially diluted to form a standard curve. 50µl of lysate or purified Lyp protein added to duplicate wells of a 96-well plate coated with protein A and anti-Lyp antibody. The plate was incubated at 37°C for 3 hours, and wells were washed 3 times using 0.05% Tween 20/PBS. The amount of Lyp was detected by adding 30µl of a 3µg/ml solution of anti-Lyp HRP polyclonal antibody to each well and incubating for 1 hour at 37°C. Wells were washed 3 times as before and the activity of HRP was measured using the substrate tetramethylbenzidine (TMB). Measurements were taken in duplicate and results show the mean (±SEM) of three separate experiments. **(A)** The absorbance of HRP from different numbers of Jurkat T cells **(B)** Standard curve for the amount of purified Lyp protein. Results show a typical standard curve obtained during the assay procedure. **(C)** Actual amount of Lyp protein captured from different numbers of Jurkat T cells as determined using absorbance of HRP with reference to a standard curve using purified Lyp protein.

3.2.5 Specificity of the assay

In the development of any new assay the use of a suitable negative control to assess the specificity of the procedure must be considered. Previous phosphatase assays have used a number of negative controls, most commonly irrelevant antibodies during the phosphatase purification process (Lorenz 2011). If available, the best negative control for any cell based experiment is to use cells which lack the protein of interest. In this case, as Lyp is a protein specifically expressed by cells of the immune system, synovial fibroblasts were used to determine the specificity of the Lyp phosphatase assay. In addition to using cells which lack the Lyp protein, an irrelevant capture antibody was also used to test the specificity of the assay. It was found that the assay was specific to measuring the activity and amount of Lyp, as there was a minimal increase in FI and absorbance in wells containing the negative controls (Figure 3.5). The absorbance for both negative controls was comparable (fibroblasts = 0.183±0.004 and irrelevant antibody = 0.136±0.027), and there was more variability in the FI (fibroblasts = 37±6 and irrelevant antibody = 4±5). Based on these data, it was concluded that both types of negative control could be used to illustrate the specificity of the Lyp phosphatase assay.

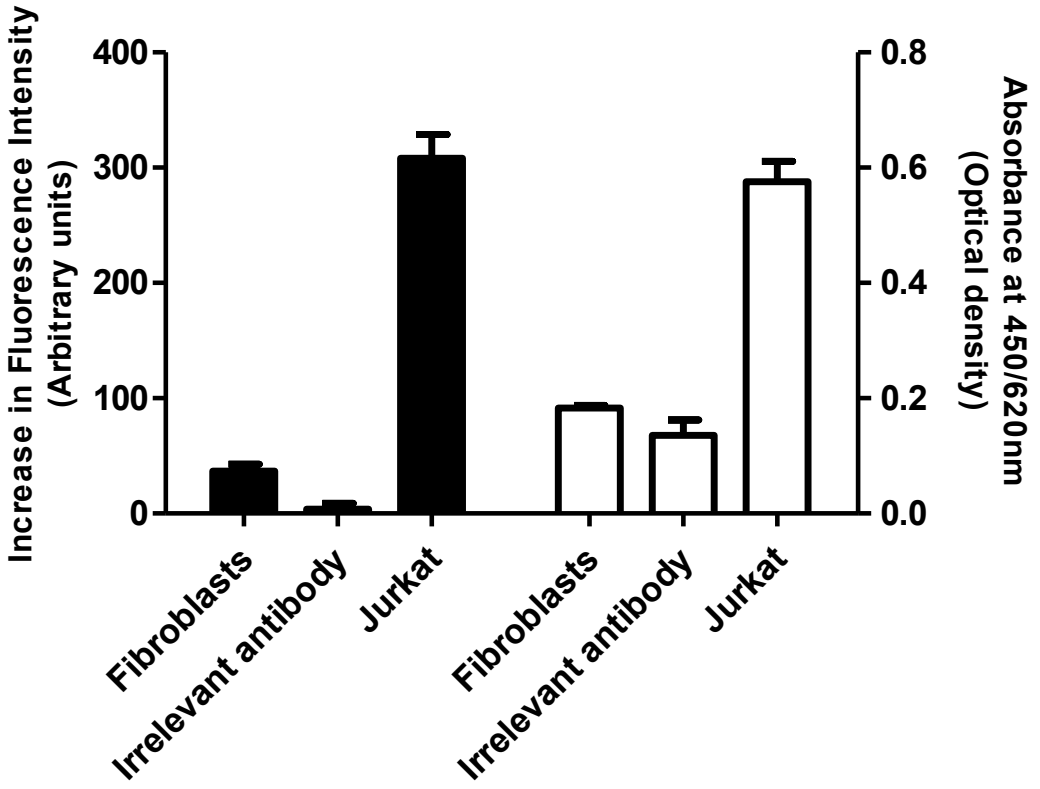


Figure 3.5 Amount and activity of the Lyp phosphatase in cells which do not express Lyp. $1.0x10^6$ Jurkat T cells and synovial fibroblasts were lysed and added to triplicate wells of a 96-well plate coated with anti-Lyp or irrelevant antibody. The activity of Lyp was assayed as described in Figure 3.4 A. The amount of Lyp was measured as described in Figure 3.4 C. Results show the phosphatase activity of Lyp (black bars) and the amount of Lyp protein (white bars). Data show the mean (±SEM) of three separate experiments.

3.2.6 Variability of the assay

As small changes in the activity of PTPs can have big implications on overall cell signalling, it was essential to examine the reliability and reproducibility of the Lyp phosphatase assay. If the method was not accurate enough, subtle changes in Lyp activity may remain undetected. To investigate this, the intersample and intrasample variability were determined by doing a number of experiments using Jurkat T cells (Table 3.2). To determine the variability between the same cell extract sample placed in different wells, the usual triplicate measurements were increased to ten wells and the intrasample variation calculated to be 9.57% (FI = 247.5±7.49). To investigate the variability between assays, the Lyp phosphatase activity was measured in eight separate experiments over a four month time period using Jurkat T cells from the same tissue culture flask. This allowed calculation of the intersample variability which was 11.8% (FI = 223.9±5.4). These relatively small percentages illustrate that the Lyp phosphatase assay method is accurate and reproducible.

Table 3.2 Intersample and intrasample variability of the Lyp phosphatase activity assay. Lyp phosphatase activity in Jurkat T cells was measured in 8 separate experiments over a period of 4 months to determine the intersample variability. To measure the intrasample variability, a single sample was split into 10 separate wells, and Lyp phosphatase activity was measured in each separate well.

	Inter	**Intra**
Number of values	24	10
Minimum	187.5	212.1
25% Percentile	201.8	232.3
Median	225.1	243.8
75% Percentile	236.2	260.1
Maximum	287.2	289.5
Mean	223.9	247.5
Standard Deviation	26.43	23.67
Standard Error	5.4	7.49
Lower 95% CI	212.8	230.6
Upper 95% CI	235.1	264.4
Coefficient of variation	**11.8%**	**9.57%**
Geometric mean	222.5	246.5

3.2.7 Measuring the specific activity of Lyp

One of the advantages of using a method which measures the activity and amount of Lyp is that this allows specific phosphatase activity to be calculated. This is much more accurate when compared to measuring activity as an increase in fluorescence alone, as this accounts not just for changes in detectable phosphatase activity but also the effects of alterations in the amount of protein present. Specific activity is calculated using a formula which incorporates the amount and activity of Lyp, and the amount of fluorescence generated by cleavage of one unit of substrate. In order to determine the latter, various concentrations of alkaline phosphatase (AP) were incubated with the substrate DiFMUP, and the maximum FI for each concentration recorded (Table 3.3). Purified AP is very efficient at dephosphorylating DiFMUP, and can therefore completely dephosphorylate all of the substrate in the well during the assay. The increase in FI can then be divided by the number of nanomoles of DiFMUP to calculate how much fluorescence is generated upon dephosphorylation of 1nmol of DiFMUP. When the average of three concentrations of AP is taken and this calculation is carried out, the increase in FI equates to 2981±209.5. This value is specific to the fluorimeter used here, but can also be calculated to calibrate other similar machines.

Table 3.3 Maximum fluorescence intensity obtained using alkaline phosphatase and the substrate DiFMUP for use in calculation of specific phosphatase activity. 20µl of various concentrations of alkaline phosphatase (AP) were added to triplicate wells of a 96-well plate. 50µl of DiFMUP at various concentrations was then added to the same wells. Measurement of fluorescence was taken every 30 seconds until the maximum fluorescence intensity was reached. Results show the mean (±SEM) of three separate experiments.

Amount of AP (µg)	**Amount of DiFMUP (nmol)**		
	0.157	0.313	
40	514(±46.11)	921.7(±87.18)	
60	495.9(±39.81)	854.9(±73.91)	
80	492.9(±54.02)	826(±95.3)	
MEAN	500.9(±6.59)	867.5(±28.34)	**MEAN**
FI/DiFMUP	3191(±166.5)	2772(±154.3)	**2981(±209.5)**

Specific activity calculation

To determine the specific activity of the Lyp phosphatase, the activity and amount of Lyp are taken into account using the formula below. A similar formula was first employed in calculation of the specific activity of the CD45 phosphatase (Rider et al. 2003).

Specific activity (nmol/ng/hour) =

$$\left[\frac{\textit{Increase in fluorescence intensity}}{\textit{Amount of substrate cleaved per fluorescence unit}}\right] \; X \; \left[\frac{1}{\textit{Nanograms (ng) of Lyp protein}}\right]$$

For example, 1.0×10^6 Jurkat T cells produced an increase in fluorescence of 308 and the amount of Lyp bound to the plate was 12ng:

$$\left[\frac{308}{2981}\right] \; X \; \left[\frac{1}{12}\right]$$

= 0.0086 / 2 (as activity is measured over a 2 hour time period)

= 0.0043 nmol/ng/hour

When the specific activity of Lyp is calculated from different numbers of Jurkat T cells, it is the same regardless of the number of cells present in the wells (Figure 3.6). For example, the specific activity of Lyp captured from $0.8x10^6$ Jurkat T cells was 0.00465±0.0003nmol/ng/hour, and the value of 0.00459±0.0004nmol/ng/hour for $1.0x10^6$ cells was very similar. These comparable values further illustrate the reliability of the assay, showing a direct correlation between the amount of Lyp protein and the phosphatase activity measured.

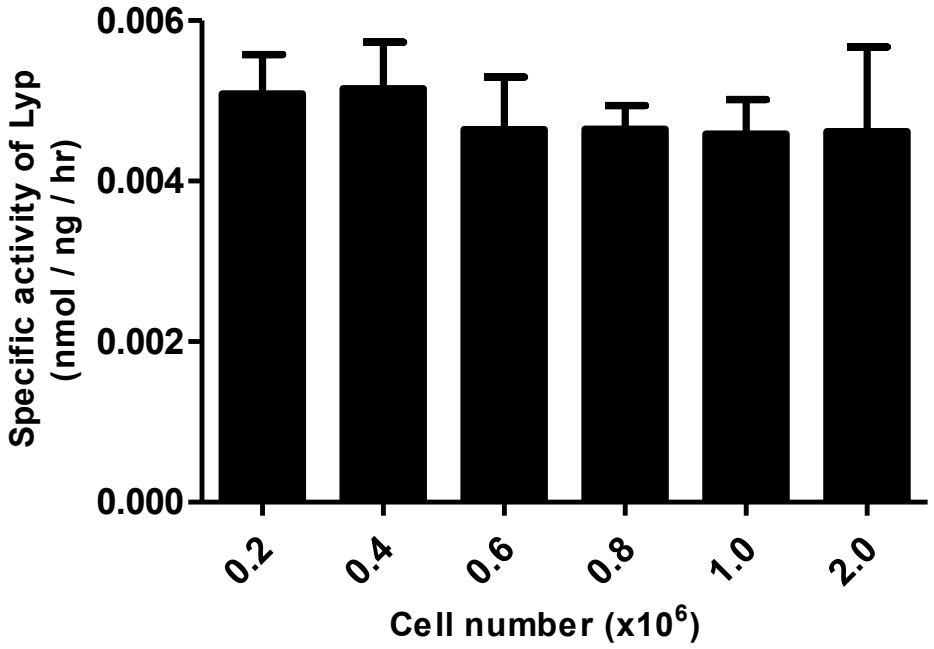


Figure 3.6 Specific activity of the Lyp phosphatase in different numbers of Jurkat T cells. Jurkat T cells were lysed in identical amounts of lysis buffer and 50µl of lysate added to triplicate wells of a 96-well plate coated with protein A and anti-Lyp antibody. The amount and activity of Lyp were determined as described in Figures 3.4 A and 3.4 C. Specific activity was then calculated as described on the previous page. Results show the mean (±SEM) of three separate experiments.

3.2.8 Measurement of other protein tyrosine phosphatases

The approach to development of the Lyp phosphatase assay was to modify a method previously used for measuring activity of the CD45 phosphatase (Rider et al. 2003). This was necessary as the substrate used in this assay (FDP) was not compatible for use with the Lyp phosphatase. This approach was originally taken with a view to comparing the activity of CD45 and Lyp using the same PTP assay. This would be useful as CD45 and Lyp are two of the three main PTPs controlling signalling through the TCR, and being able to monitor both their activities in the same cells would be advantageous. In line with this rationale, the new assay using DiFMUP as the substrate was tested in order to detect the activity of CD45. This was successful and CD45 activity was detected in small numbers of Jurkat T cells ($0.5x10^6$) and primary CD4+ T cells ($2.0x10^6$) (Figure 3.7). In Jurkat T cells, $0.5x10^6$ cells produced an increase in FI of 74±13 when measuring Lyp, and the equivalent of 963±129 for CD45 in the same number of cells. This difference in FI is most likely due to greater expression of CD45 when compared to Lyp, as CD45 has been reported to contribute up to 10% of a cells total surface protein (Thomas 1989).

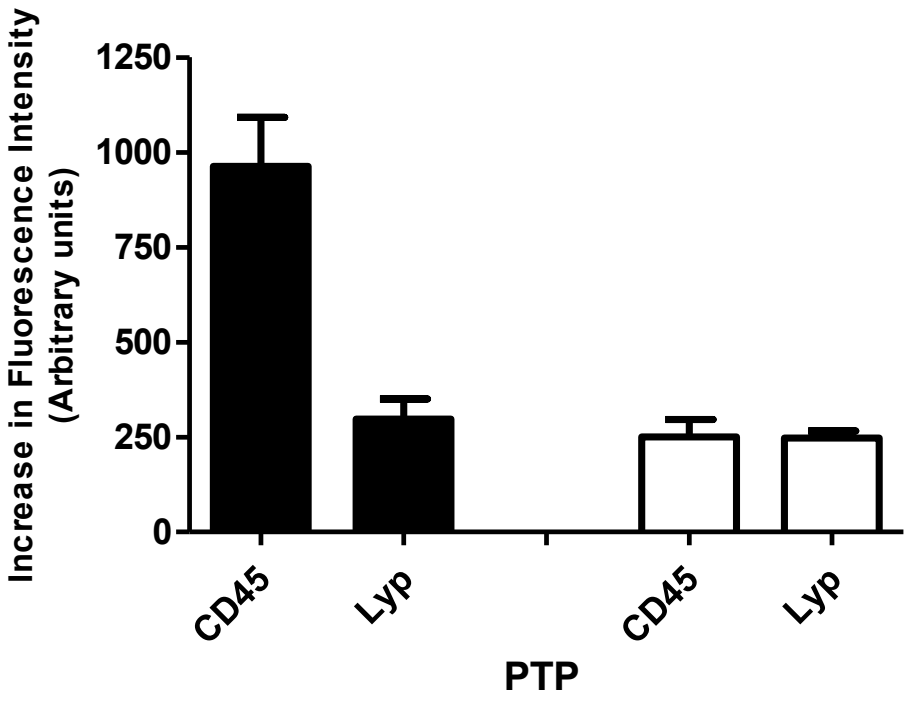


Figure 3.7 Comparison of CD45 and Lyp protein tyrosine phosphatase activity in Jurkat T cells and primary CD4+ T cells. Jurkat T cells (black bars) and CD4+ T cells (white bars) were lysed and 50µl of lysate added to triplicate wells of a 96-well plate coated with protein A and anti-CD45 antibody or anti-Lyp antibody. The plate was incubated at 37°C for 3 hours, and wells were washed 3 times using 0.05% Tween 20/PBS. The activity of CD45 and Lyp were detected by addition of 0.2mM DiFMUP and 50mM DTT. Activity was measured over a 2 hour time period with readings taken in triplicate. $0.5x10^6$ cells per well were used for measuring CD45 activity and $2.0x10^6$ cells were used for measuring Lyp activity. Results show the mean (±SEM) of three separate experiments.

3.3 Discussion

As mentioned in the introduction to this chapter, there are a number of methods which are currently used to measure the activity of PTPs. However, these methods all have certain limitations or were not specifically designed to measure the activity of the Lyp phosphatase. The method developed and described in this chapter fulfils the necessary criteria lacking in previous methods and allows rapid and reproducible measurement of Lyp activity. This is achieved through combining measures of Lyp activity and protein amount to calculate a specific phosphatase activity, measured in activity per nanomole of substrate per nanogram of Lyp per hour. Using this assay Lyp activity could be detected from a variety of cell lines, as well as primary CD4+ T cells freshly isolated from PB. The assay is a considerable improvement on current methods and requires reasonably small numbers of cells ($1.0x10^6$ Jurkats and $2.0x10^6$ CD4+ T cells). The assay was proven to be reliable, as illustrated by findings of a fairly low intrasample and intersample variability of 9.57% and 11.8% respectively. The immunocapture method and use of the substrate DiFMUP were extended to measure the activity of CD45, showing the assay could potentially be used to assess the activity of a number of other PTPs. In addition, the immunocapture of Lyp was highly specific as when cells lacking Lyp were used, the activity and amount detected was minimal. This minimal activity and amount were also present when an irrelevant antibody was used in the assay, indicating that either negative control can be used.

The method described here has a number of advantages when compared to previously used methods. To our knowledge, this is the only method which allows efficient and rapid capture of Lyp protein to allow measurement of the amount and activity to be accurately correlated. This is particularly important for accurate analysis of results from Lyp inhibitor studies, to assess the extent to which the specific activity of Lyp is inhibited in human cells. This method could also be used to further characterise the effect of the autoimmune-associated SNP PTPN22 R620W. It is believed that the SNP results in a gain-of-function Lyp phosphatase,

but one study has shown that the SNP increases proteasomal degradation of the Lyp protein (Zhang et al. 2011). This discrepancy between an increased activity and decreased amount of Lyp protein could be further investigated using this method. Overall, this method for measuring the specific activity of the Lyp phosphatase is a very useful technique and can be employed to detect the presence of Lyp and its phosphatase activity in a range of immune cells.

CHAPTER FOUR: EFFECTS OF PTPN22 R620W ON LEUKOCYTE SIGNALLING

4.1 Introduction

Genes make a significant contribution to an individual's susceptibility to autoimmune conditions including RA. Variants of the MHC class II genes found on chromosome 6 and a variant of the PTPN22 gene found on chromosome 1 are known to contribute to the majority of genetic risk for RA and a range of autoimmune diseases (Burn et al. 2011). The PTPN22 variant R620W is highly expressed among individuals of European ancestry, despite its association with increased disease risk (Burn et al. 2011). The PTPN22 gene encodes the protein Lyp which is widely expressed by all leukocytes (Chien et al. 2003). However, the function of Lyp in many cell types is at present unknown and thus the effect of the R620W variant is also not determined. Highest expression of Lyp is found in polymorphonuclear leukocytes (PMNs) and mononuclear leukocytes (MNCs), and lower levels in CD3+ T cells (Chien et al. 2003). Although expression is lowest in CD3+ T cells the function of Lyp is most well characterized in this cell type, where the Lyp protein functions as a negative regulator of T cell activation through its ability to down regulate signalling through the TCR (Hermiston et al. 2009). Lyp has been shown to have a similar role in B cells, where it modulates signalling through the BCR (Hermiston et al. 2009). In contrast to lymphocytes, nothing is known about the function of Lyp in neutrophils even though expression of the protein is very high.

As mentioned previously, the aetiology of RA is complex and thought to involve changes in the function of a number of leukocytes including B cells, T cells and neutrophils. Given the association of R620W with an increased risk of RA, the wide expression profile of the Lyp protein and the involvement of multiple cell types in RA pathogenesis, this chapter of work investigated the role of the Lyp protein in different types of leukocytes. These included cells involved in both innate immunity (neutrophils) and adaptive immunity (B and T cells). Study particularly focussed on the effect of R620W on Lyp protein activity, to try and identify potential mechanisms by which functional alterations in leukocytes could occur. Identification of functional changes induced by R620W would give insight into how this specific genetic risk

factor could be involved in the pathogenesis of RA. In order to fully investigate how altered Lyp function affects leukocyte signalling, a number of methods were employed. There is debate regarding if R620W confers a loss or gain of function Lyp phosphatase (Vang et al. 2005, Zikherman et al. 2009) and thus a direct approach was taken by measuring the specific activity of Lyp in cell lines expressing R620W. These cell lines also proved useful in determining if the R620W variant changed the expression of Lyp at the protein level, which has been reported previously (Zhang et al. 2011). Although cell lines were advantageous to initially assess Lyp activity and expression, the transformed nature of these lines did not allow measurement of more functional analyses such as cytokine production and Ca^{2+} signalling (Miller et al. 1993). Thus the majority of the work in this chapter is focussed on primary T cells and neutrophils freshly isolated from individuals expressing R620W.

4.2 Results

4.2.1 Effects of PTPN22 R620W on lymphocyte signalling and function

4.2.1.1 B lymphocytes

Following development of the Lyp phosphatase assay (Chapter 3), the next step was to determine the effects of the R620W variant on Lyp phosphatase activity. This is an important question as current literature regarding the activity of Lyp in cells expressing R620W is conflicting. Some studies have suggested a gain-of-function phosphatase (Vang et al. 2005, Aarnisalo et al. 2008) and others provide evidence for a loss-of-function phosphatase (Lefvert et al. 2008, Zikherman et al. 2009). Because of the conflicting results described in the literature, it was chosen to measure the specific activity of Lyp without the need for transfection or overexpression. Initially, peripheral blood mononuclear cells (PBMC) were obtained from vasculitis patients that had been genotyped for R620W. B cell lines were generated using EBV transformation to obtain a large number of cells expressing R620W GG (control) and AA (homozygous), which were then used in the Lyp phosphatase assay and other experimental procedures. The use of transformed B cell lines was an essential step to determining the effects of R620W expression on lymphocyte signalling, before investigating effects in primary lymphocytes.

4.2.1.2 Generation of B lymphocyte cell lines expressing the PTPN22 R620W variant

An important element to investigating the effects of PTPN22 R620W on lymphocyte signalling was to find a way of obtaining a large number of cells at regular time points. To achieve this, B cell lines were made using EBV transformation. PB was obtained from vasculitis patients that had been genotyped for the PTPN22 R620W SNP and PBMC were isolated. From this cell population, B cells were used along with the B95.8 marmoset cell line to create stable B EBV transformed B cell lines. This was carried out as described in Chapter 2 (Section 2.2.1).

After creating these cell lines, it was necessary to check the purity of the lines for potential T cell contamination. This was done by staining the cell lines with an antibody to CD19, a cell surface marker used to identify B cells. A very high percentage of the cells were CD19+ and this was similar when comparing the control (GG = 98.5% CD19+) and homozygous (AA = 97% CD19+) B cell lines that had been generated (Figure 4.1). Based upon this high percentage purity, transformation of the B cells expressing the PTPN22 R620W variant was considered successful and the B cell lines were deemed suitable for use in further experiments.

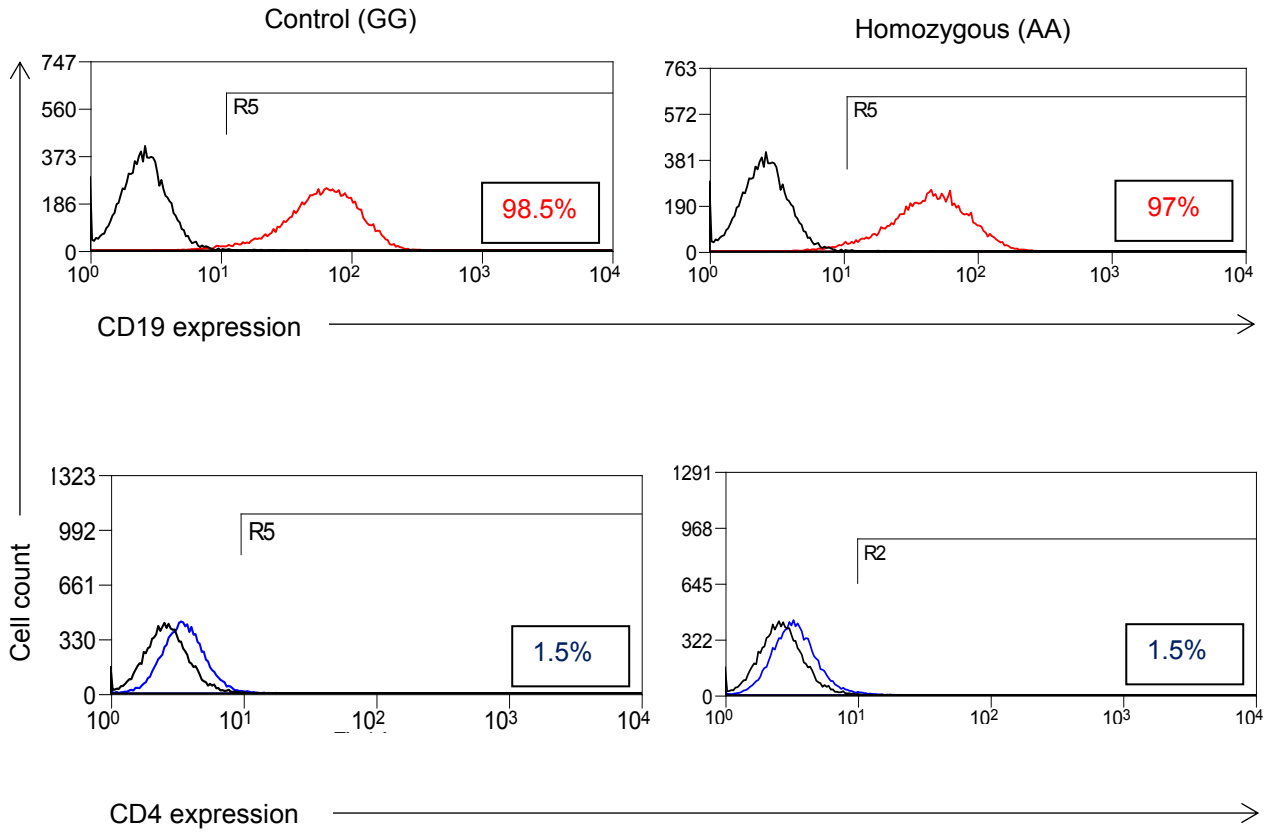


Figure 4.1 Percentage of CD19+ and CD4+ cells in EBV cell lines control (GG) and homozygous (AA) for the PTPN22 R620W variant. B cell lines were generated using PBMC isolated from vasculitis patients who had been genotyped for the PTPN22 R620W variant and the B95.8 marmoset cell line. Cells were stained using fluorescent conjugated antibodies to CD19 and CD4 and analysed by flow cytometry. Plots show CD19+ cells (red), CD4+ cells (blue) and isotype controls (black). Results shown are taken from one single experiment.

4.2.1.3 Decreased protein amount and increased specific activity of Lyp in B lymphocytes expressing PTPN22 R620W

Following successful generation of EBV B cell lines, cells were then prepared for a Lyp phosphatase assay to determine the effect of R620W expression on Lyp phosphatase activity and Lyp protein amount. It was observed in B cell lines homozygous (AA) for R620W that the amount of Lyp protein present was statistically significantly decreased when compared to control cells expressing GG (Figure 4.2 A). The average amount of Lyp bound during the assay from GG and AA cells was 28ng±13 and 9ng±4 respectively. Despite this difference in amount, the activity measured from the two cell types was similar (GG=702.95±192 and AA=687.83±107), as can be seen in Figure 4.2 B. With regards to specific activity, this was found to be much higher in cells expressing AA when compared to control GG cells, however this was not statistically significant (Figure 4.2 C). The average specific activity of the Lyp phosphatase in AA cells (16.9nmol/µg/hour) was more than 3 times that of the control GG cells (4.69nmol/µg/hour). No studies to date have attempted to define the specific activity of Lyp in this manner, however by simply measuring the activity of immunoprecipitated Lyp it has been estimated that PTPN22 R620W AA is 50% more active than PTPN22 R620W GG (Vang et al. 2005). The results found here measuring specific activity may explain the conflicting findings of previous studies, as they show the intrinsic phosphatase activity of R620W to be enhanced (Vang et al. 2005), however the total amount of Lyp present in the cell is decreased (Zhang et al. 2011).

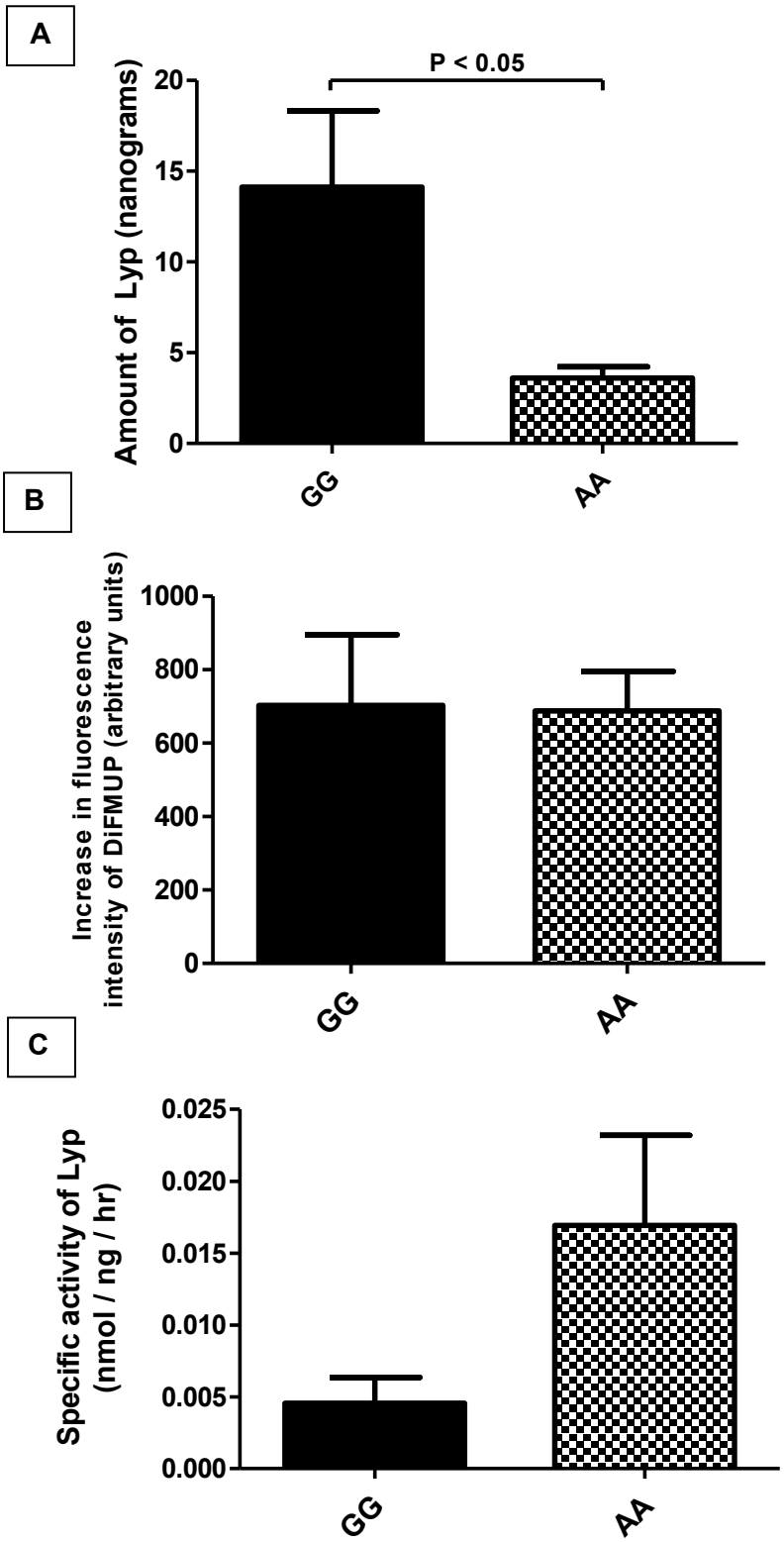


Figure 4.2 Decreased protein amount and increased specific activity of Lyp in B lymphocytes expressing PTPN22 R620W. EBV-transformed B cells ($1.0x10^6$) expressing the different forms of Lyp (GG and AA) were lysed and their Lyp phosphatase activity and amount of Lyp protein captured were measured. **(A)** The amount of Lyp protein captured in nanograms **(B)** The activity of the Lyp phosphatase measured as an increase in fluorescence intensity (FI) **(C)** The specific activity of the Lyp phosphatase. Results show the mean (±SEM) of three separate experiments. Measures of activity were taken in triplicate and measures of amount were taken in duplicate. $P<0.05$, Unpaired t-test compared with control (GG) cells.

4.2.1.4 The Lyp protein is tyrosine phosphorylated in B lymphocytes

Protein phosphorylation and dephosphorylation is an important and reversible mechanism by which signalling proteins can be activated when required (Tonks 2006). Although Lyp is a PTP involved in removing tyrosine residues from target proteins, Lyp itself can also be regulated by phosphorylation. Previous work has shown that the Lyp protein encoded by R620W is less phosphorylated on an inhibitory tyrosine residue (Fiorillo et al. 2010), suggesting this may a regulatory mechanism which is altered by expression of the variant. Based on this evidence, we wanted to investigate if tyrosine phosphorylation of Lyp in B lymphocytes expressing R620W was altered.

To study this possibility, total phosphorylated protein was immunoprecipitated from control (GG) and homozygous (AA) B cells using an antibody against tyrosine phosphorylated proteins. This was then complexed with protein A/G beads and the bound proteins run on an SDS gel which was probed for Lyp protein expression. This method revealed no significant differences in tyrosine phosphorylation between GG and AA B cells (Figure 4.3) however tyrosine phosphorylation was present in both samples. This suggests that tyrosine phosphorylation is not a likely regulatory mechanism contributing to altered function of the variant Lyp protein in B cells and other mechanisms should be investigated. It is possible due to the nature of cells transformed using EBV, that a large number of proteins are phosphorylated non-specifically which would not normally be the case in primary, untransformed cells. In addition, no loading control was carried out for this experiment which would be needed to confirm this result. Based on this observation and its limitations, it is necessary to study how Lyp function could be altered in primary lymphocytes.

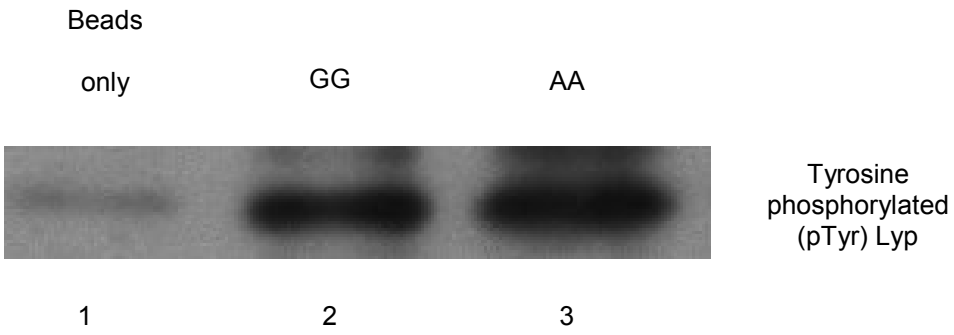


Figure 4.3 Lyp is tyrosine phosphorylated in B lymphocytes transformed using EBV. GG (Lane 2) and AA (Lane 3) B cells were lysed and proteins phosphorylated on a tyrosine residue were immunoprecipitated using a pTyr antibody. Immunoprecipitates were complexed with protein a/g beads which were then run on a 10% SDS-PAGE gel. Proteins were transferred to a PVDF membrane, which was probed with a primary antibody against Lyp, then a HRP-linked secondary antibody. This image is representative of two experiments.

4.2.1.5 T lymphocytes

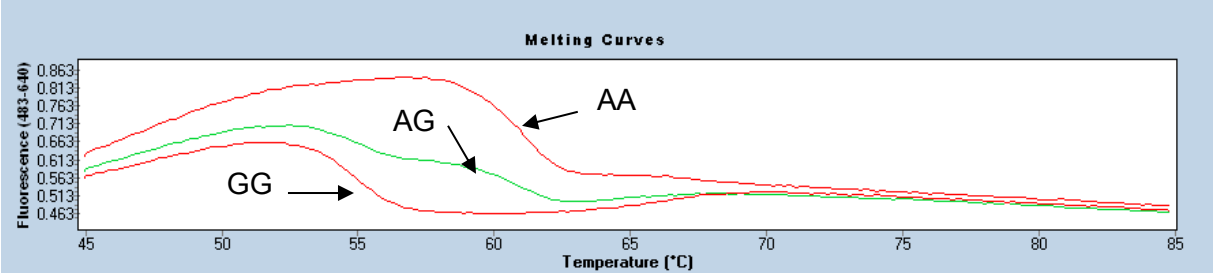
Preliminary work studying the R620W variant was carried out in B lymphocytes, however the main line of investigation was to study the effect of the variant on T lymphocyte signalling and function. There are a number of known substrates of Lyp in T cells including Lck, Zap-70, Vav1, CD3ε and CD3ζ (Wu et al. 2006) indicating there could be many ways in which the PTPN22 variant could alter T cell activation. Studies to date have highlighted alterations in phosphatase activity, decreased association with its binding partner Csk, changes in transcription factor expression and reduced Ca^{2+} signalling (Bottini et al. 2004, Vang et al. 2005, Rieck et al. 2007). Only a handful of these observations have been made in primary T cells and thus one of the main aims of this project was to further characterize the effects of R620W in primary T cells. To achieve this, a large cohort of individuals with a known PTPN22 R620W genotype was required.

4.2.1.6 Cohort recruitment and PTPN22 R620W genotyping

To fully study the effects of R620W and the mechanisms by which this variant confers an increased risk of autoimmunity, a cohort of healthy individuals and patients with autoimmune diseases was needed. To do this, ethical approval was obtained and a number of healthy controls (n=107) and patients (n=508) were recruited from two sites. These sites were the Queen Elizabeth Hospital in Birmingham and Russell's Hall Hospital in Dudley. The patient group was predominantly patients diagnosed with RA, but also included disease controls with other types of inflammatory arthritis, for example psoriatic arthritis. Whole blood was obtained from all subjects and DNA was extracted from these samples. It was chosen to extract the DNA directly from whole blood samples because this method was simple, accurate and quick and would enable efficient processing of the large number of samples collected for this study. PTPN22 R620W genotype was determined using real time polymerase chain reaction (RT-PCR) and melting curve analysis (Figure 4.4) (Toms et al. 2011). This genotyping allowed identification of individuals control (GG), heterozygous (AG), and homozygous (AA) for the PTPN22 variant (Table 4.1).

There was high expression of the heterozygous (AG) form of R620W in healthy individuals, disease controls and RA patients (healthy=20%, disease controls=21%, RA patients=23%) and this was also similar to the published frequency in healthy controls (21.6%, www.HapMag.org). There was increased expression of the homozygous (AA) form in RA patients and disease controls when compared with healthy individuals (healthy=1%, disease controls=2%, RA patients= 2%). A reasonable number of variant expressing individuals were identified within the cohort recruited, and the next step was to isolate T cells from these individuals to analyse T cell signalling and function.

A



B

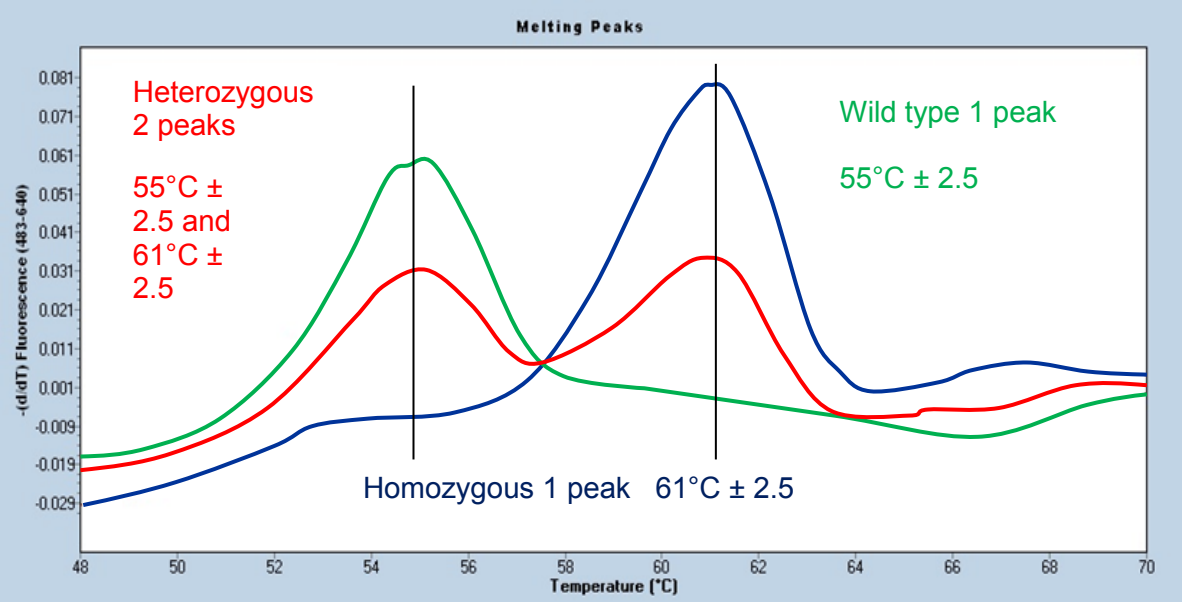


Figure 4.4 Typical results from real time polymerase chain reaction (RT-PCR) of the PTPN22 R620W variant. DNA was extracted from whole blood. RT-PCR was carried out and resulting melting curves were analysed to determine PTPN22 R620W genotype. **(A)** Typical melting curve obtained for each genotype. **(B)** Typical melting peaks obtained for each genotype. DNA melting temperatures for each genotype are shown. Genotypes: control (GG), heterozygous (AG) and homozygous (AA).

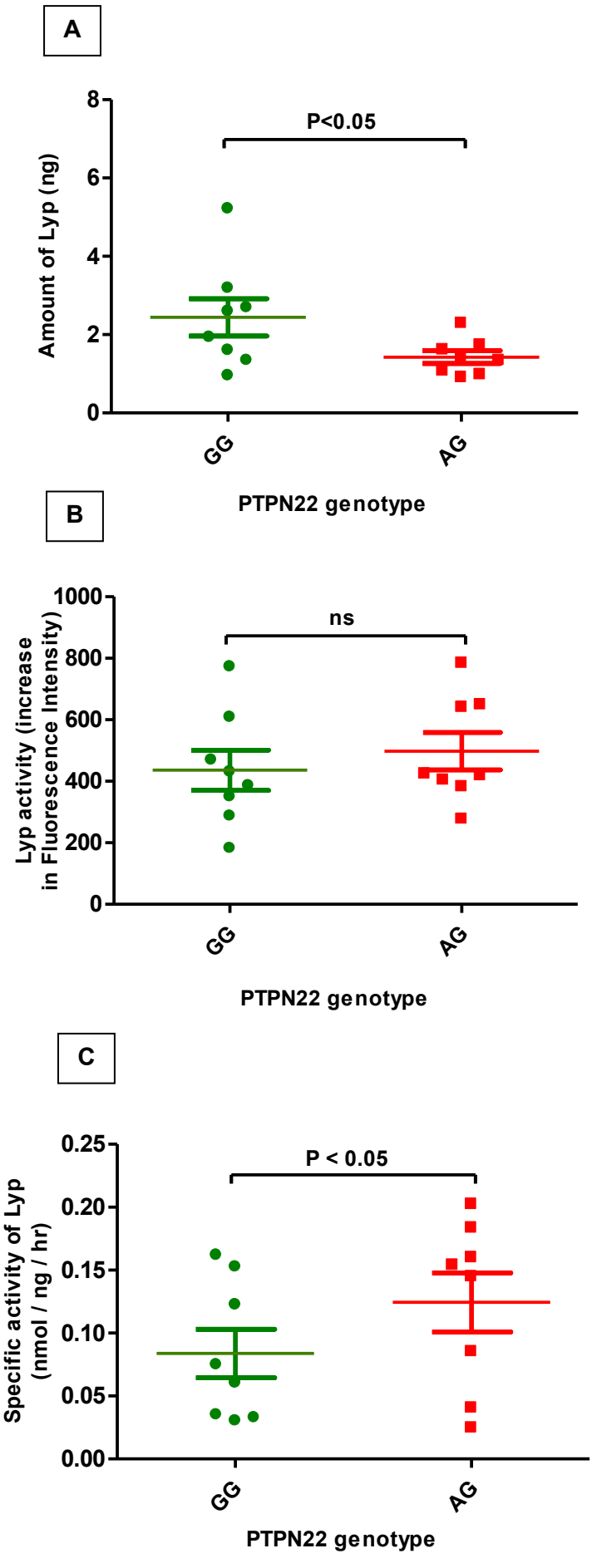
Table 4.1 Frequency of the PTPN22 R620W variant in healthy individuals, individuals with rheumatoid arthritis, and individuals with other types of inflammatory arthritis.

PTPN22 R620W genotype	Healthy controls (n=107)	Disease controls (n=128)	Rheumatoid arthritis (n=380)
Control (GG)	79% (n=85)	77% (n=98)	75% (n=284)
Heterozygote (AG)	20% (n=21)	21% (n=27)	23% (n=87)
Homozygote (AA)	1% (n=1)	2% (n=3)	2% (n=9)

4.2.1.7 Decreased protein amount and increased specific activity of Lyp in T lymphocytes expressing PTPN22 R620W

Further blood samples were obtained from the genotyped cohort to isolate T cells from individuals control (GG), heterozygous (AG) and homozygous (AA) for R620W. The first functional analysis carried out was to measure the specific activity of Lyp in these T cells. To determine if Lyp phosphatase activity was altered by the R620W variant, T cells were isolated from age and sex matched healthy individuals, with and without the variant. T cells were prepared for the Lyp phosphatase assay and their Lyp activity and amount of Lyp protein assayed (Figure 4.5).

It was found that CD4+ T cells heterozygous (AG) for the R620W variant had statistically significantly less Lyp protein (Figure 4.5 A) when compared to control (GG) T cells (GG=2.4ng±0.48, AG=1.4ng±0.16). Although the amount of Lyp protein was less in AG T cells the activity assay (Figure 4.5 B) showed that Lyp phosphatase activity was equivalent in cells with either genotype (GG=FI 436±66, AG=FI 498±61). Considering AG T cells had less Lyp protein with similar activity to GG T cells, when specific Lyp phosphatase activity was calculated (Figure 4.5 C) this was shown to be significantly higher in AG T cells (GG=0.08nmol/ng/hr±0.02, AG=0.12nmol/ng/hr±0.02). When samples were paired according to age and sex, this increase in specific activity in AG T cells can be seen more clearly (Figure 4.5 D). The global PTP activity from GG and AG T cells was also measured to ensure the overall amount of lysate added to each well was equivalent. This was done by carrying out the assay in the absence of the Lyp capture antibody, to measure the ability of all the cellular PTPs to dephosphorylate the substrate used in the assay. It was observed that the overall PTP activity of GG and AG T cells was similar (GG=FI 3711±329, AG= FI 3792±317), suggesting that the increase in specific Lyp phosphatase activity was not due to more AG lysate being added to the wells.


A
P<0.05
Amount of Lyp (ng)
8
6
4
2
0
GG
AG
PTPN22 genotype
B
ns
Lyp activity (increase in Fluorescence Intensity)
1000
800
600
400
200
0
GG
AG
PTPN22 genotype
C
P < 0.05
Specific activity of Lyp (nmol / ng / hr)
0.25
0.20
0.15
0.10
0.05
0.00
GG
AG
PTPN22 genotype

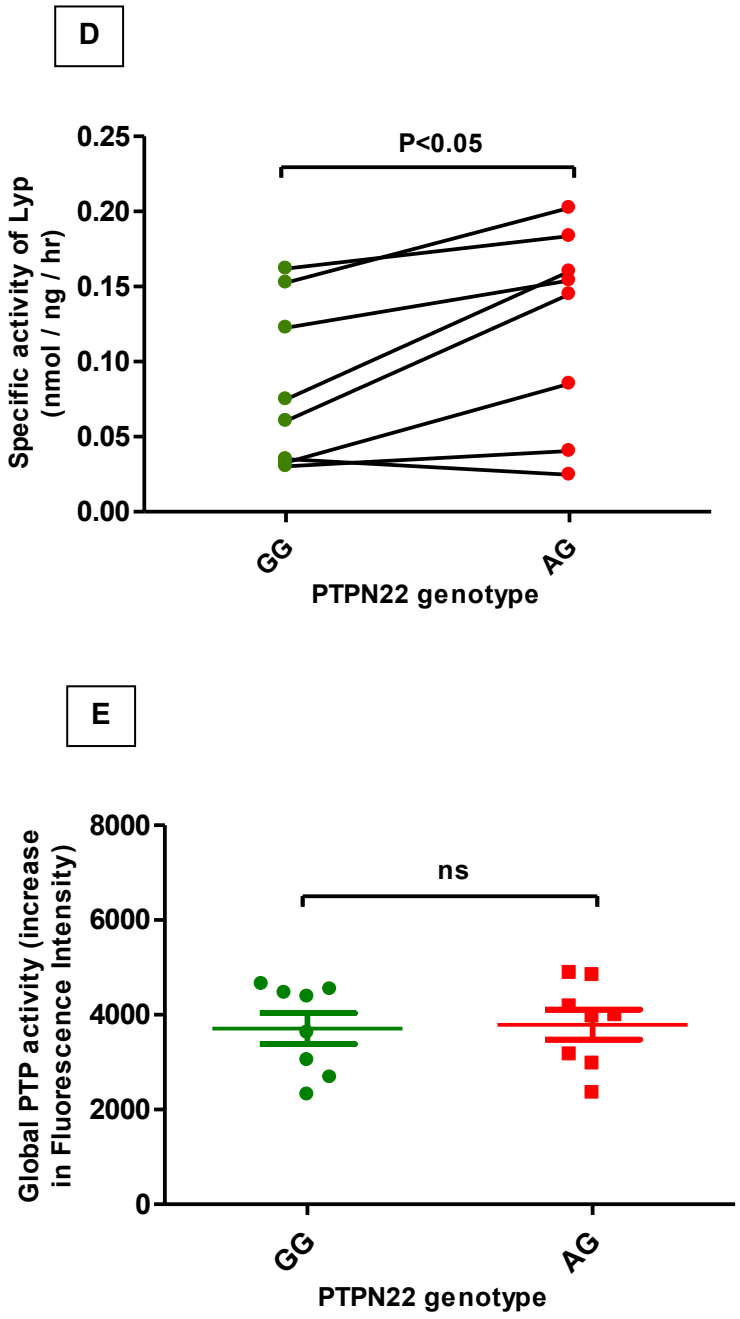


Figure 4.5 Specific activity of the Lyp phosphatase in CD4+ T cells isolated from healthy individual's control (GG) and heterozygous (AG) for the PTPN22 R620W variant. CD4+ T cells ($2.0x10^6$) expressing the different forms of Lyp (GG and AG) were lysed and their Lyp phosphatase activity and amount of Lyp protein captured were measured. **(A)** The amount of Lyp protein captured in nanograms **(B)** The activity of the Lyp phosphatase measured as an increase in fluorescence intensity (FI) **(C)** The specific activity of the Lyp phosphatase **(D)** The specific activity of the Lyp phosphatase from age and sex matched samples **(E)** The global protein tyrosine phosphatase activity. Results show the mean (±SEM) of eight separate experiments. Measures of activity were taken in triplicate and measures of amount were taken in duplicate. P<0.05, Paired t-test compared with control (GG) cells.

4.2.1.8 Phosphorylation of Lyp substrates in T lymphocytes expressing PTPN22 R620W

Lyp has a number of known substrates in T cells which have been identified using binding studies and substrate trapping (Wu et al. 2006). However, the effect of R620W on substrate availability and binding in primary T cells has not been determined. Studies in Jurkat T cells transfected with the R620W variant gene have shown that variant Lyp has reduced association with its binding partner Csk (Bottini et al. 2004) and other work has highlighted that for Lyp to down regulate TCR signalling it must be dissociated from Csk (Vang et al. 2012). These two observations suggest that the R620W variant could be a more potent negative regulator of TCR activation. This lack of binding to Csk could increase the availability of Lyp to known substrates such as Lck and Zap-70, however this has not been investigated in primary T cells expressing R620W. Thus we wanted to examine the activation state of Lyp substrates by measuring their phosphorylation status. Many kinases and phosphatases can be activated by phosphorylation of their activating residues (Yamaguchi et al. 1996) and so we measured the phosphorylation status of two known Lyp substrates (Lck and Zap-70) to determine if the R620W variant caused changes in their activation. To do this, CD4+ T cells were obtained from healthy individual's control (GG), heterozygous (AG) and homozygous (AA) for the R620W variant. Lck and Zap-70 phosphorylation were assessed by immunoblotting, and additional measurement of Lck phosphorylation was carried out by flow cytometry.

The Lck kinase is important in early TCR triggering and is activated when the CD45 phosphatase dephosphorylates the inhibitory tyrosine residue (Y505), which triggers autophosphorylation of the activating tyrosine residue (Y394) (Mustelin et al. 1990, Zikherman et al. 2009). Lyp is involved in Lck inactivation through its action of dephosphorylating the activating Y394 tyrosine residue (Zikherman et al. 2009), and so it is possible that the R620W variant alters this function of Lyp.

Flow cytometric analysis revealed there to be no difference in the phosphorylation status of the activating tyrosine residue (Y394) or the inhibitory tyrosine residue (Y505) of Lck when comparing GG, AG and AA CD4+ T cells (Figure 4.6 A+B). This was determined by measuring mean fluorescence intensity (MFI) which was similar in unstimulated (Y394: GG=33±2, AG=30±4, AA=34±0, Y505: GG=29±4, AG=28±3, AA=21±0) and stimulated (Y394: GG=36±5, AG=32±4, AA=45±0, Y505: GG=103±18, AG=104±16, AA=52±0) T cells of all three genotypes. The rise in Lck Y505 phosphorylation following T cell stimulation appeared to be decreased in AA CD4+ T cells (Figure 4.6 B), however as it was only possible to carry out experiments on one healthy individual, the significance of this could not be determined.

The phosphorylation status of Lck and Zap-70 were also assessed by immunoblotting to determine if expression of the activated tyrosine residues of Lck (Y394) and Zap-70 (Y493) were different in R620W CD4+ T cells. Using this method it was found that phosphorylation of the Lck kinase on residue Y394 was significantly decreased in heterozygous (AG) and homozygous (AA) CD4+ T cells when compared to control (GG) cells (Figure 4.6 C+D). When comparing the relative quantity of p-Lck 394 in R620W and non-R620W T cells, it was found that phosphorylation on this residue in AG and AA T cells was around 50% less than that of GG T cells. When the relative quantity of p-Lck 394 in GG T cells was fixed at 1 for analysis purposes, AG T cells measured only 0.48±0.13, and AA T cells even less at 0.39. The phosphorylation of Zap-70 493 was also shown to be reduced in CD4+ T cells expressing R620W (Figure 4.6 E+F). This difference in Zap-70 493 phosphorylation was not statistically significant (GG=1, AG=0.72±0.22, and AA=0.36), suggesting that Lck phosphorylation is particularly affected by the variant Lyp.

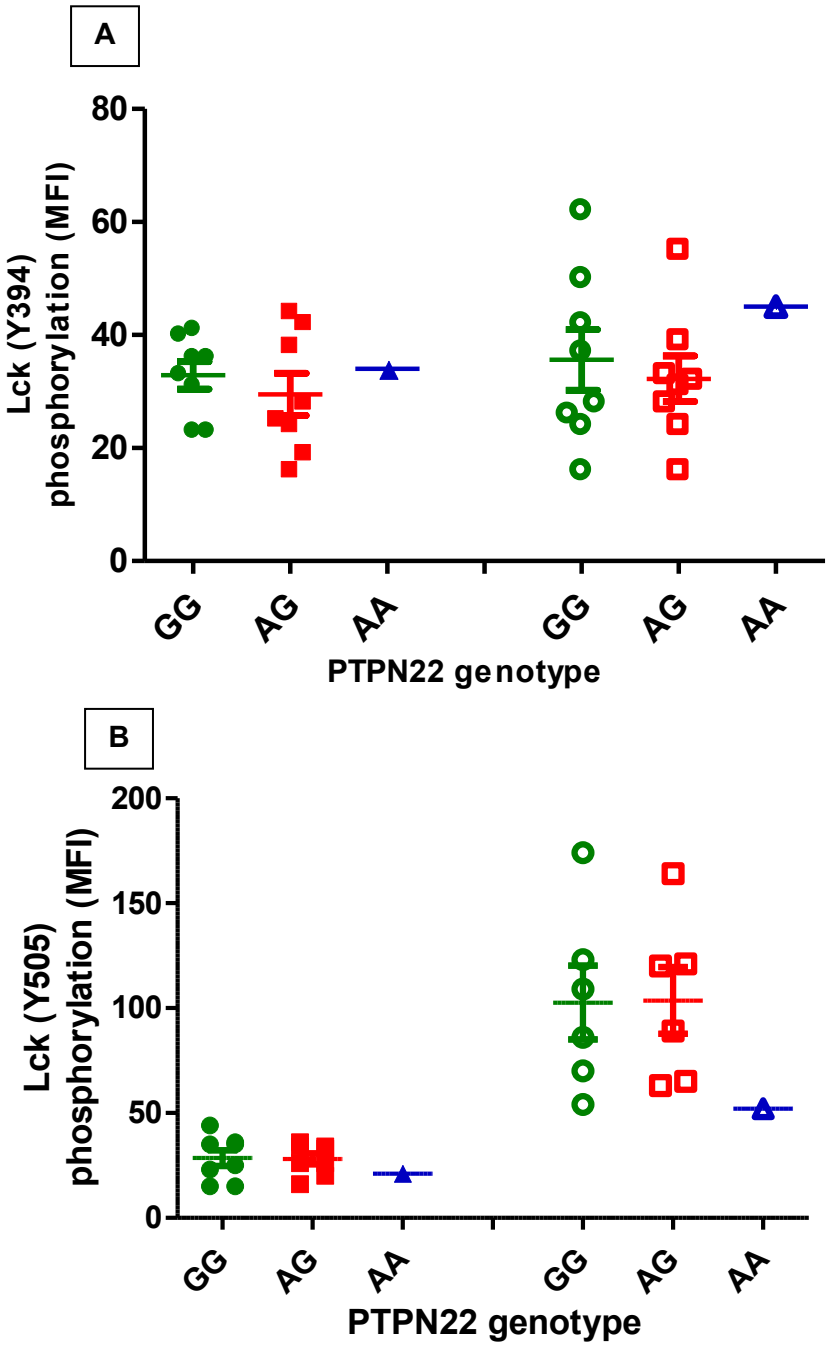


Figure 4.6 A+B Phosphorylation of the Lck kinase in CD4+ T cells isolated from healthy individual's control (GG), heterozygous (AG), and homozygous (AA) for the PTPN22 R620W variant assessed by flow cytometry. CD4+ T cells expressing the different forms of Lyp (GG, AG and AA) were isolated and stimulated for 1 minute with 3µg/ml anti-CD3 and 4µg goat anti-mouse to cross link receptors. Cells were fixed and permeabalised to allow for intracellular staining. Cells were stained with antibodies to the phosphorylated residues of Lck; Lck (Y505) PE and Lck (Y394) FITC and analysed by flow cytometry. **(A)** Phosphorylation of Lck (Y394) in unstimulated (closed symbols) and stimulated (open symbols) CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show the mean (±SEM) of eight experiments. **(B)** Phosphorylation of Lck (Y505) in unstimulated (closed symbols) and stimulated (open symbols) CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show the mean (±SEM) of eight (unstimulated cells) and six (stimulated cells) experiments.

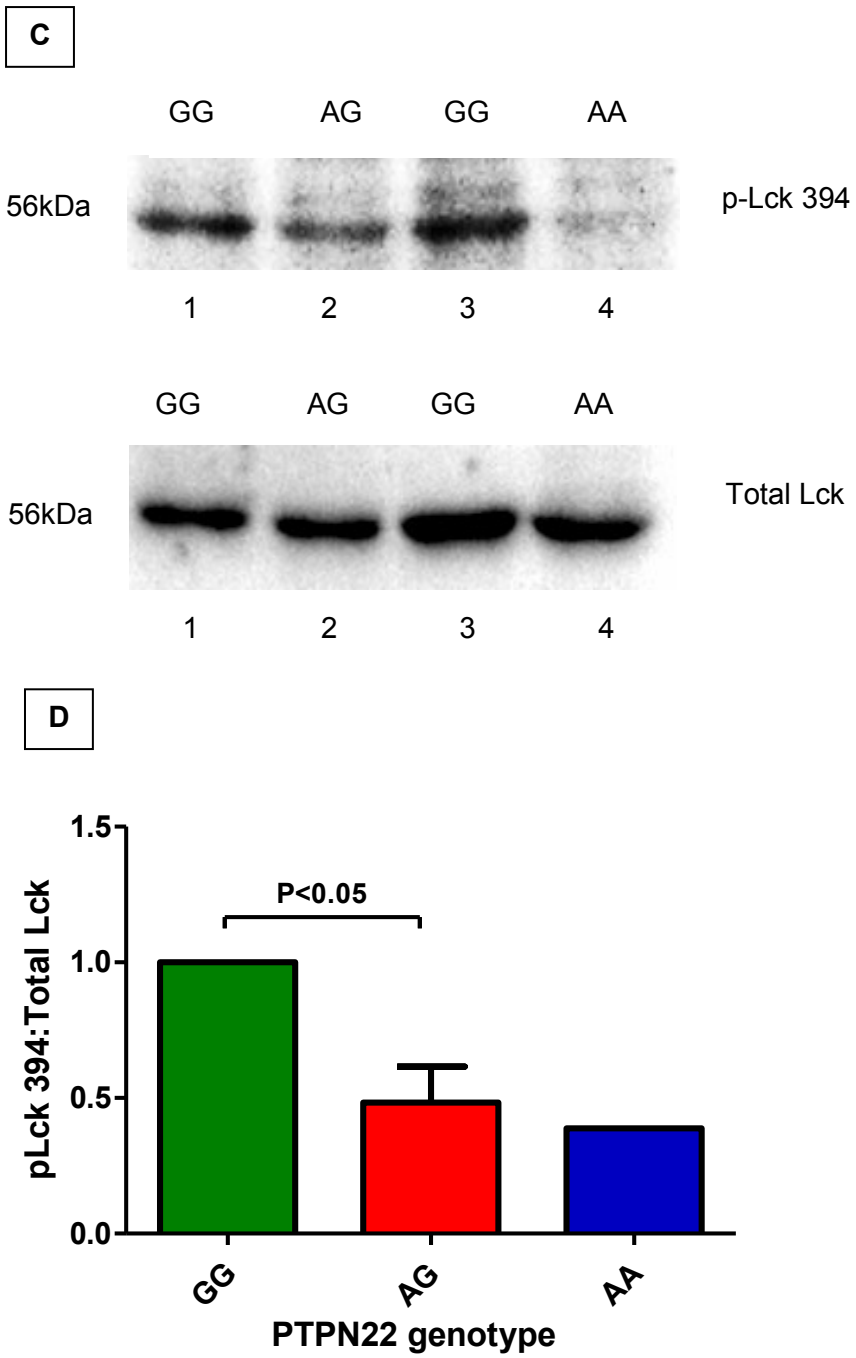


Figure 4.6 C+D Phosphorylation of the Lck kinase in CD4+ T cells isolated from healthy individual's control (GG), heterozygous (AG), and homozygous (AA) for the PTPN22 R620W variant assessed by western blotting. CD4+ T cells expressing the different forms of Lyp (GG, AG and AA) were isolated and prepared for western blotting. Samples were run on a 10% SDS gel and proteins transferred to a PVDF membrane. Blots were probed with a primary antibody to p-Lck 394 and then a horseradish peroxidise-linked secondary antibody. To re-probe for total Lck, membranes were stripped to remove any antibodies that had bound. Membranes were then probed using a primary antibody to total Lck and then a horseradish peroxidise-linked secondary antibody. **(C)** Representative image showing p-Lck 394 and total Lck protein. Results shown one of the five experiments performed. **(D)** Average ratio of p-Lck 394:total Lck. Results shown the mean (±SEM) of five experiments performed with age and sex matched GG and AG T cells. P<0.05, Paired t-test compared with control (GG) cells.

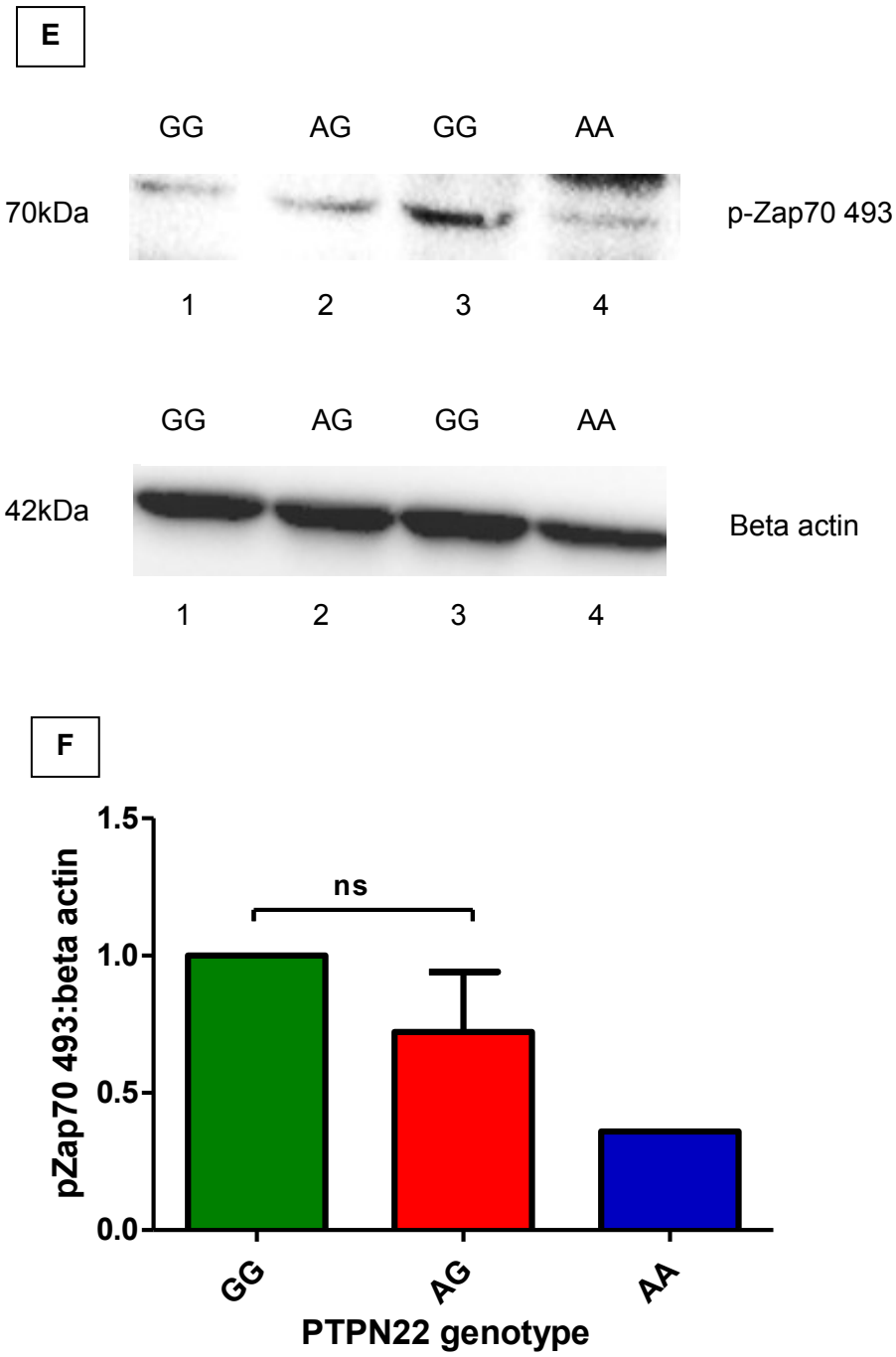


Figure 4.6 E+F Phosphorylation of the Zap-70 in CD4+ T cells isolated from healthy individual's control (GG), heterozygous (AG), and homozygous (AA) for the PTPN22 R620W variant assessed by western blotting. CD4+ T cells expressing the different forms of Lyp (GG, AG and AA) were isolated and prepared for western blotting. Samples were run on a 10% SDS gel and proteins transferred to a PVDF membrane. Blots were probed with a primary antibody to p-Zap70 493 and then a horseradish peroxidise-linked secondary antibody. To re-probe for beta actin, membranes were stripped to remove any antibodies that had bound. Membranes were then probed using a primary antibody to beta actin and then a horseradish peroxidise-linked secondary antibody. **(E)** Representative image showing p-Zap70 493 and beta actin. Results shown one of the four experiments performed. **(F)** Average ratio of p-Zap70 493:beta actin. Results shown the mean (±SEM) of four experiments performed with age and sex matched GG and AG T cells.

4.2.1.9 Proliferation of T lymphocytes isolated from healthy individuals expressing PTPN22 R620W

After directly investigating Lyp phosphatase activity and Lyp substrate activation status, we wanted to determine if changes in the activation of these proteins had any effect on overall function of T lymphocytes expressing R620W. An important functional read out of T cell activation is cytokine production, and also if the cytokines produced are pro inflammatory such as IFN-γ and TNF-α or anti-inflammatory such as IL-10. Changes in the type of cytokines produced by T cells are of importance and it has been suggested that the skewing of T cells to a pro-inflammatory Th1 phenotype could be a mechanism precipitating inflammatory disease (Firestein 2003). If the R620W variant caused alterations in TCR signalling which preferentially favoured a Th1 response, this could be a mechanism by which the variant increases an individual's risk of inflammatory diseases like RA.

To investigate this, CD4+ T cells were isolated from healthy controls, control (GG) and heterozygous (AG) for R620W. T cells were stimulated with anti-CD3 and anti-CD28 and after 48 hours of culture their proliferation was assessed. It has been shown previously that there is no difference in proliferation between CD4+ T cells with and without the R620W variant (Rieck et al. 2007). Thus we wanted to confirm that proliferation was not affected by R620W expression in our culture conditions, to ensure any changes in cytokine production were not due to different rates of proliferation. Proliferation was monitored by loading the cells with CFSE and analysing cells after 48 hours of culture. It was found that there was no difference in T cell proliferation when comparing GG and AG T cells (Figure 4.7), with the average proliferation index being very similar in cells of both genotypes (GG=2.20±0.21 and AG=2.24±0.49, Figure 4.7 B).

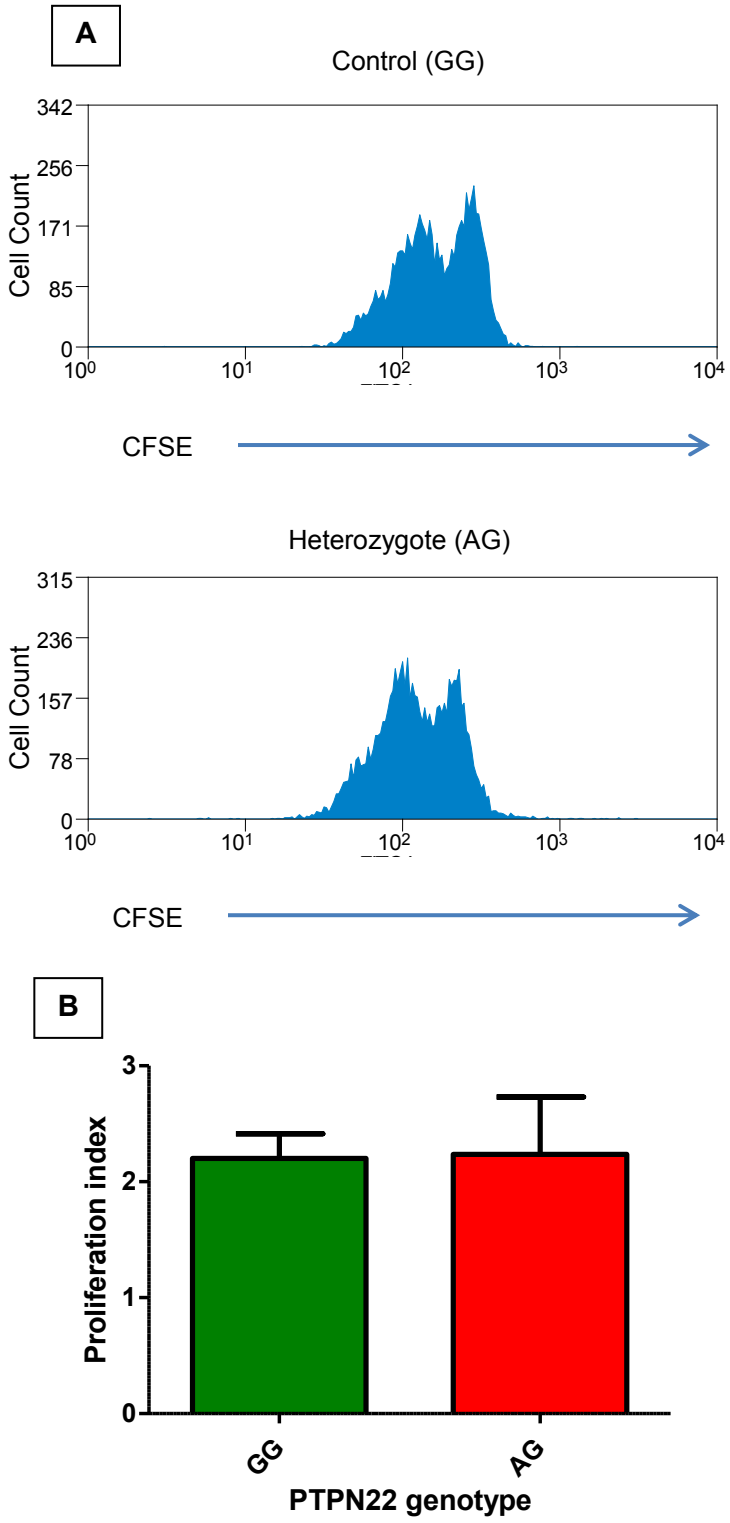
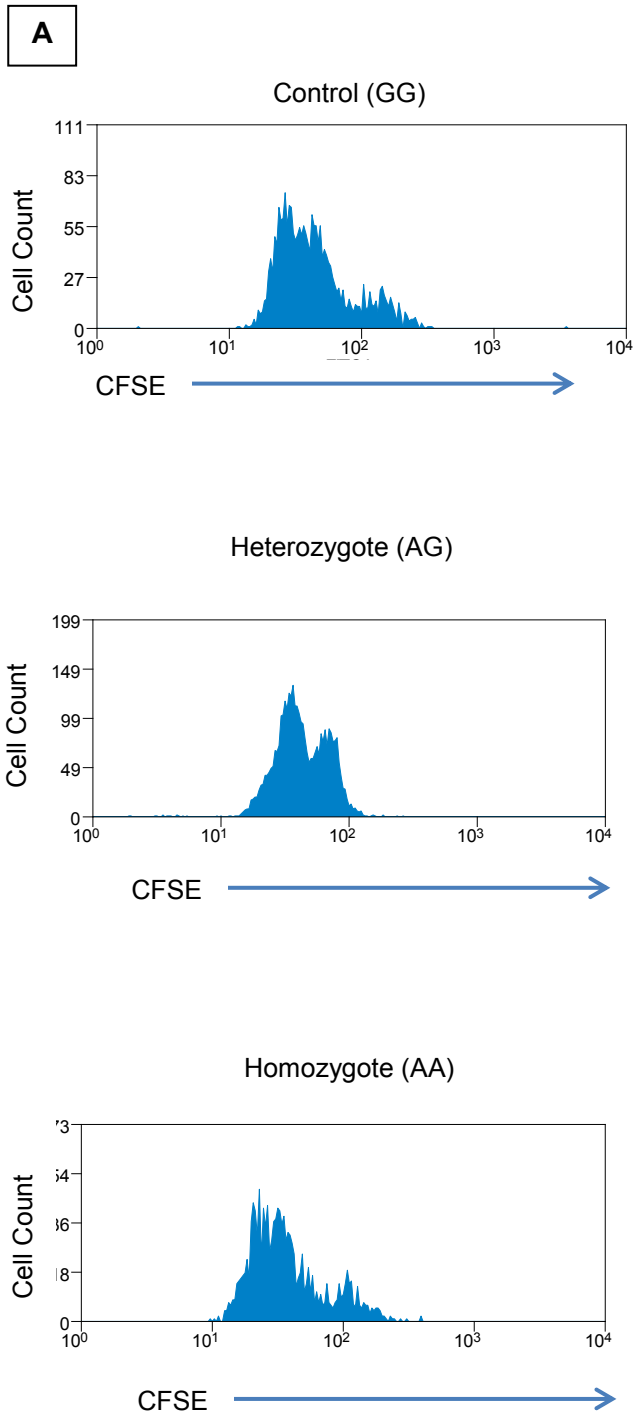


Figure 4.7 Expression of the PTPN22 R620W variant has no effect on CD4+ T cell proliferation in healthy individuals. CD4+ T cells expressing the different forms of Lyp (GG and AG) were isolated and loaded with 1μM CFSE. Cells were left overnight and then stimulated with plate bound anti-CD3 (3μg/ml) and anti-CD28 (5μg/ml) for 48 hours at 37°C. Cells were removed from culture and proliferation was assessed using flow cytometry. **(A)** Representative CFSE peaks obtained from one of eight separate experiments using control (GG) and heterozygous (AG) CD4+ T cells. **(B)** Average proliferation index obtained for control (GG) and heterozygous (AG) CD4+ T cells. Results represent the average number of peaks (±SEM) of eight separate experiments.

4.2.1.10 Proliferation of T lymphocytes isolated from rheumatoid arthritis patients expressing PTPN22 R620W

After confirming that expression of R620W had no effect on T cell proliferation in healthy individuals, the next line of investigation was to determine the effect of disease status on T cell proliferation. This was necessary to determine if expression of R620W had differential effects on T cell signalling in health and disease. It has been observed previously that T cells isolated from RA patients and patients with other autoimmune inflammatory diseases are largely hyporesponsive to simulation (Maurice et al. 1997). This is characterised by a down regulation of components of the TCR signalling complex, as well as decreased expression of signalling proteins such as Lck and Fyn (Jury et al. 2003). TNF-α is thought to be important in the pathogenesis of RA and it has been suggested that prolonged exposure of T cells to this cytokine could be a mechanism by which this T cell hyporesponsiveness occurs (Isomaki et al. 2001). In addition, the increase in oxidative stress levels observed in RA have been shown to structurally modify TCR signalling proteins (Cemerski et al. 2003), highlighting there are multiple mechanisms by which T cell proliferation and function could be reduced.

In order to investigate if R620W altered T cell proliferation in RA, CD4+ T cells were isolated from RA patients, control (GG), heterozygous (AG) and homozygous (AA) for the R620W variant. T cells were stimulated with anti-CD3 and anti-CD28 and after 48 hours of culture their proliferation was assessed. Proliferation was monitored by loading the cells with CFSE and analysing cells after 48 hours of culture. Similarly to CD4+ T cells isolated from healthy individuals, it was found that there was no significant difference in T cell proliferation when comparing GG, AG and AA T cells (Figure 4.8). However, there was a slight tendency for a higher average proliferation index in AA T cells (GG=1.61±0.07, AG=1.77±0.09 and AA=2.00±0.17, Figure 4.8 B).


A
Control (GG)
Cell Count
111
83
55
27
0
10^0
10^1
10^2
10^3
10^4
CFSE
Heterozygote (AG)
Cell Count
199
149
99
49
0
10^0
10^1
10^2
10^3
10^4
CFSE
Homozygote (AA)
Cell Count
0
10^0
10^1
10^2
10^3
10^4
CFSE

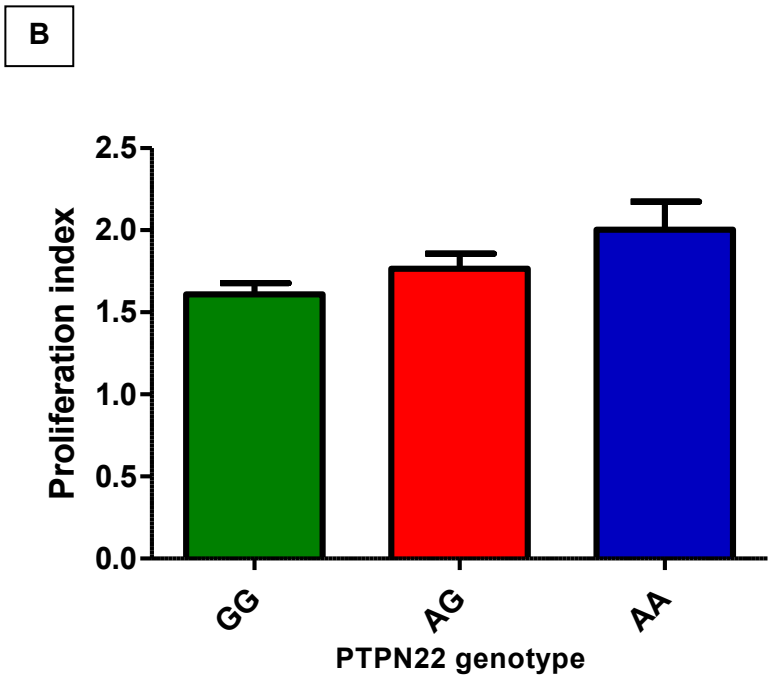


Figure 4.8 Expression of the PTPN22 R620W variant has no effect on CD4+ T cell proliferation in rheumatoid arthritis. CD4+ T cells expressing the different forms of Lyp (GG, AG and AA) were isolated and loaded with 1μM CFSE. Cells were left overnight and then stimulated with plate bound anti-CD3 (3μg/ml) and anti-CD28 (5μg/ml) for 48 hours at 37°C. Cells were removed from culture and proliferation was assessed using flow cytometry. **(A)** Representative CFSE peaks obtained from experiments using control (GG), heterozygous (AG) and homozygous (AA) CD4+ T cells. **(B)** Average proliferation index obtained for control (GG), heterozygous (AG) and homozygous (AA) CD4+ T cells. Results represent the average number of peaks (±SEM) of eight separate experiments using GG and AG T cells, and three separate experiments using AA T cells.

4.2.1.11 Changes in cytokine production by T lymphocytes isolated from healthy individuals expressing PTPN22 R620W

After confirmation that CD4+ T cell proliferation was unaffected by R620W in health and disease, it was investigated if cell phenotype and differentiation had been altered. A previous study has investigated the effects of the R620W variant on cytokine production in T cells and found there was decreased production of IL-10 by T cells isolated from healthy controls with the variant (Rieck et al. 2007). There were some trends observed such as decreased secretion of IL-4, but these did not reach statistical significance and there were no observed differences in secretion of IFN-γ or TNF-α. In this study, T cells were assessed after 24 hours of culture following stimulation using anti-CD3 and anti-CD28 coated beads. In our experiments we chose to take a slightly different approach. T cells were stimulated using plate-bound anti-CD3 and anti-CD28 and assessment of cytokine production was done after 48 hours of culture. In addition, the previous study detected cytokines by taking supernatant for use in enzyme-linked immune sorbent assay (ELISA) only. As well as taking samples of supernatant for use in ELISAs, T cells were treated with brefeldin A for 3 hours to prevent any cytokines produced during that time from being secreted. T cells were then stained with fluorescent conjugated antibodies to IL-4, IL-10, IL-17 and IFN-γ to assess short term cytokine production.

Using intracellular cytokine staining it was found that R620W had no effect on the percentage of IFN-γ secreting CD4+ T cells (Figure 4.9 A+C). There was a trend towards a decrease in the percentage of IL-4 producing cells in heterozygous T cells (0.13%±0.04) when compared to control T cells (0.3%±0.08) (Figure 4.9 B+C). There was a significantly decreased percentage of IL-10 producing T cells when comparing heterozygous (0.6%±0.1) to control (1.0%±0.2) and this was statistically significant (Figure 4.9 D+F). Lastly, the percentage of IL-17 secreting CD4+ T cells was unaffected by expression of the PTPN22 R620W variant (Figure 4.9 E+F). The effect of R620W on amount of cytokine secreted was then determined

by ELISA. It was observed that CD4+ T cells heterozygous for the variant secreted significantly more of the pro-inflammatory cytokines TNF-α (GG=232pg/ml±17 and AG=302pg/ml±12) and IFN-γ (GG=315pg/ml±47 and AG=475pg/ml±36) when compared with control CD4+ T cells (Figure 4.9 G+H). In contrast, there was no difference between the amount of IL-4, IL-10 and IL-17 secreted by CD4+ T cells of both genotypes (Figure 4.9 I-K).

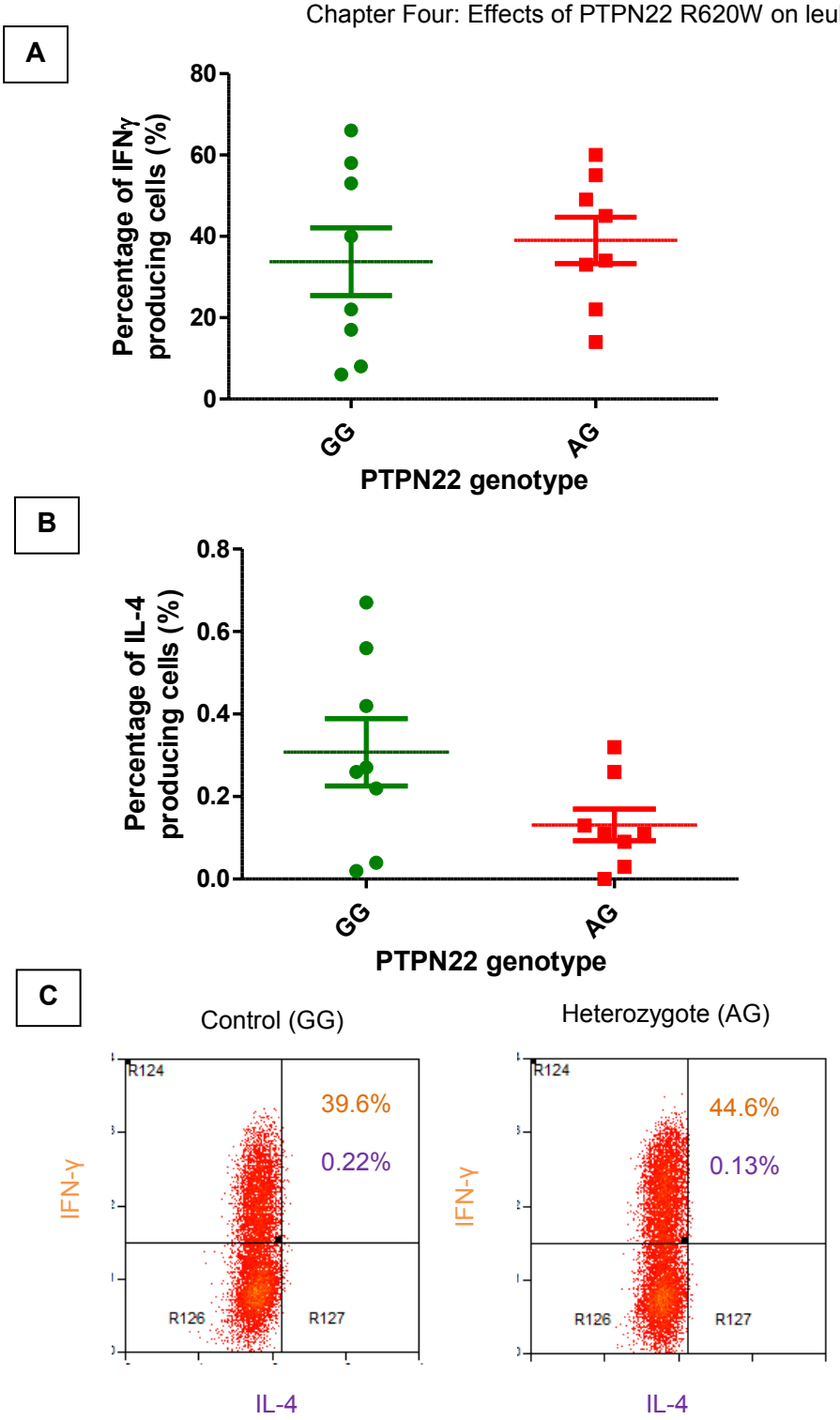


Figure 4.9 A-C Expression of the PTPN22 R620W variant has no effect on the percentage of IFN-γ and IL-4 secreting CD4+ T cells. CD4+ T cells expressing the different forms of Lyp (GG and AG) were isolated and left overnight in culture at 37°C. Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IFN-γ and IL-4 and assessed by flow cytometry. **(A)** Percentage of IFN-γ secreting CD4+ T cells expressing the different forms of Lyp (GG and AG). Results show the mean (±SEM) of eight separate experiments. **(B)** Percentage of IL-4 secreting CD4+ T cells expressing the different forms of Lyp (GG and AG). Results show the mean (±SEM) of eight separate experiments. **(C)** Representative flow cytometry plot of the percentage of IFN-γ and IL-4 secreting CD4+ T cells expressing the different forms of Lyp (GG and AG). Results show one of the eight experiments performed.

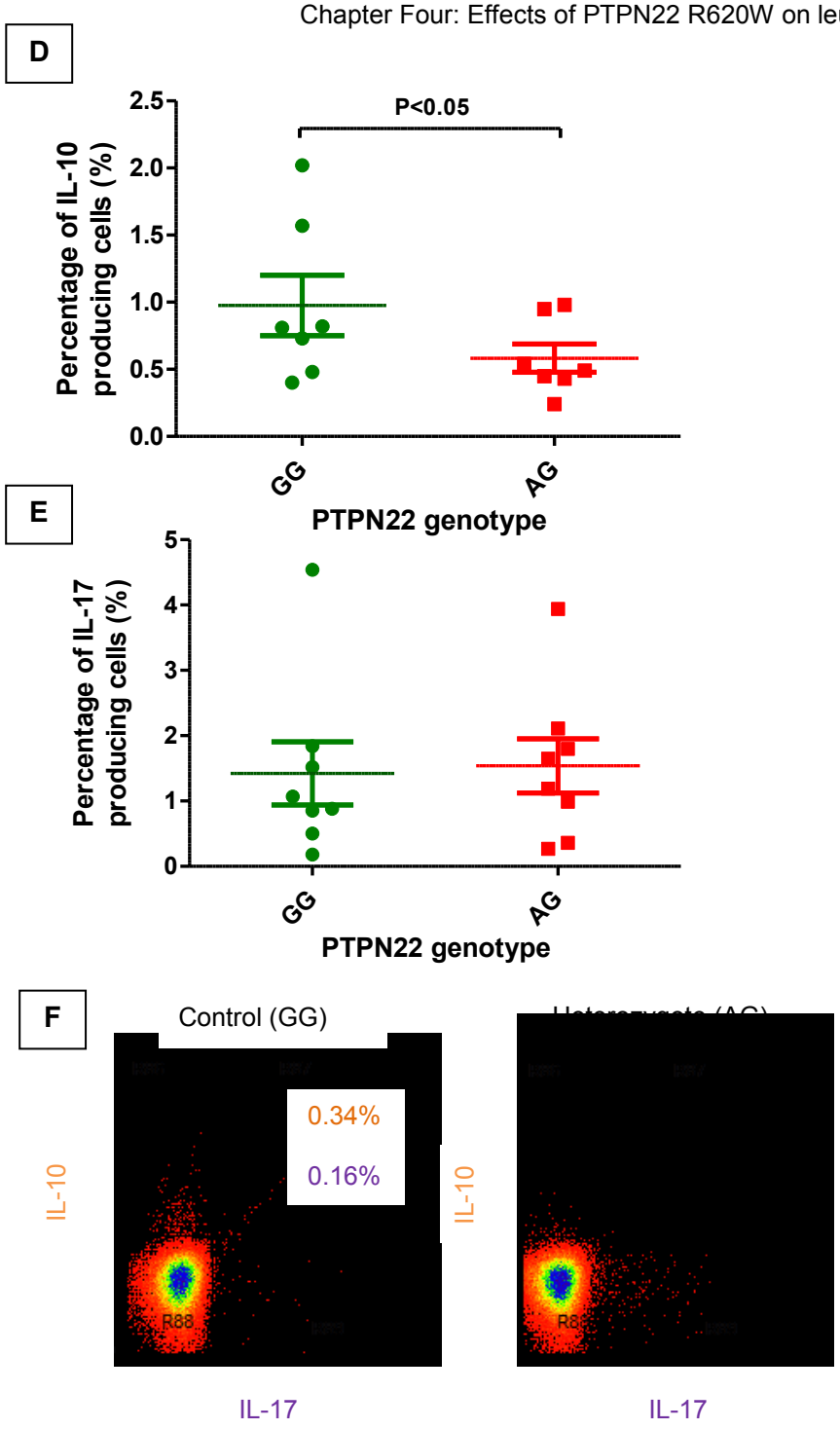
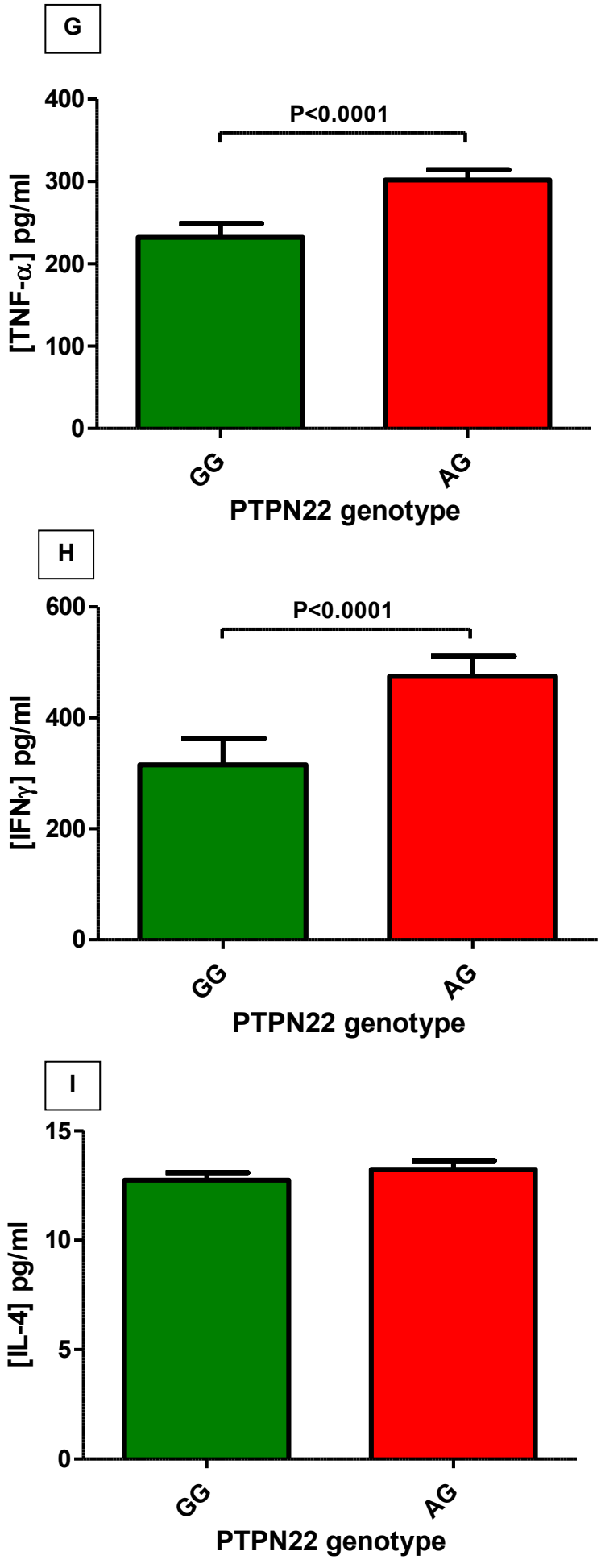


Figure 4.9 D-F Expression of the PTPN22 R620W variant decreases the percentage of IL-10 secreting CD4+ T cells and has no effect on the percentage of IL-17 secreting CD4+ T cells. CD4+ T cells expressing the different forms of Lyp (GG and AG) were isolated and left overnight in culture at 37°C. Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IL-10 and IL-17 and assessed by flow cytometry. **(D)** Percentage of IL-10 secreting CD4+ T cells expressing the different forms of Lyp (GG and AG). Results show the mean (±SEM) of eight separate experiments. **(E)** Percentage of IL-17 secreting CD4+ T cells expressing the different forms of Lyp (GG and AG). Results show the mean (±SEM) of eight separate experiments. **(F)** Representative flow cytometry plot of the percentage of IL-10 and IL-17 secreting CD4+ T cells expressing the different forms of Lyp (GG and AG). Results show one of the eight experiments performed. P<0.05, Paired t-test compared with control (GG) cells.


G
400
300
200
100
0
[TNF-α] pg/ml
P<0.0001
GG
AG
PTPN22 genotype
H
600
400
200
0
[IFNγ] pg/ml
P<0.0001
GG
AG
PTPN22 genotype
I
15
10
5
0
[IL-4] pg/ml
GG
AG
PTPN22 genotype

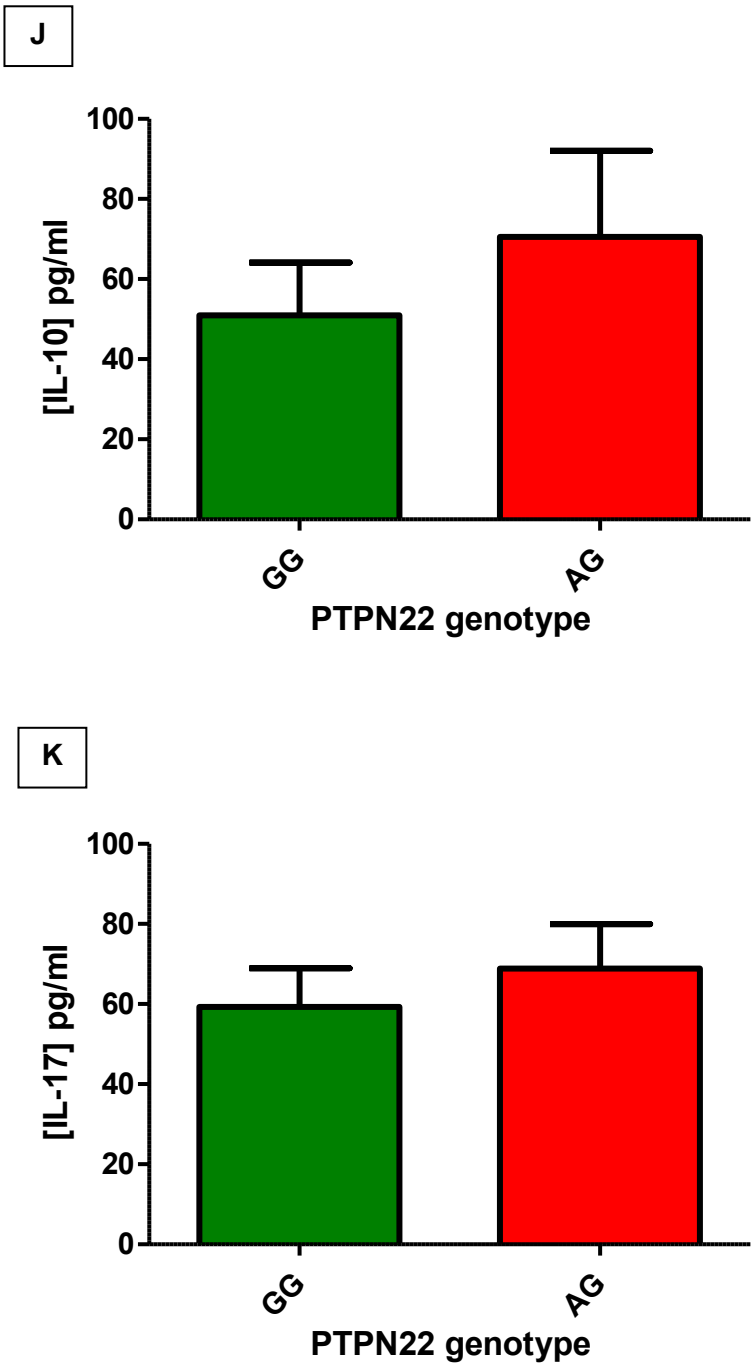


Figure 4.9 G-K Expression of the PTPN22 R620W variant increases the amount of IFN-γ and TNF-α produced by CD4+ T cells. CD4+ T cells expressing the different forms of Lyp (GG and AG) were isolated and left overnight in culture at 37°C. Cells were stimulated with plate bound anti-CD3 (3μg/ml) and anti-CD28 (5μg/ml) for 48 hours at 37°C. Supernatant samples were taken 48 hours after stimulation and assessed for the presence of **(G)** TNF-α **(H)** IFN-γ **(I)** IL-4 **(J)** IL-10 and **(K)** IL-17 using ELISA. Figures show the mean (±SEM) of eight separate experiments. P<0.0001, Paired t-test compared with control (GG) cells.

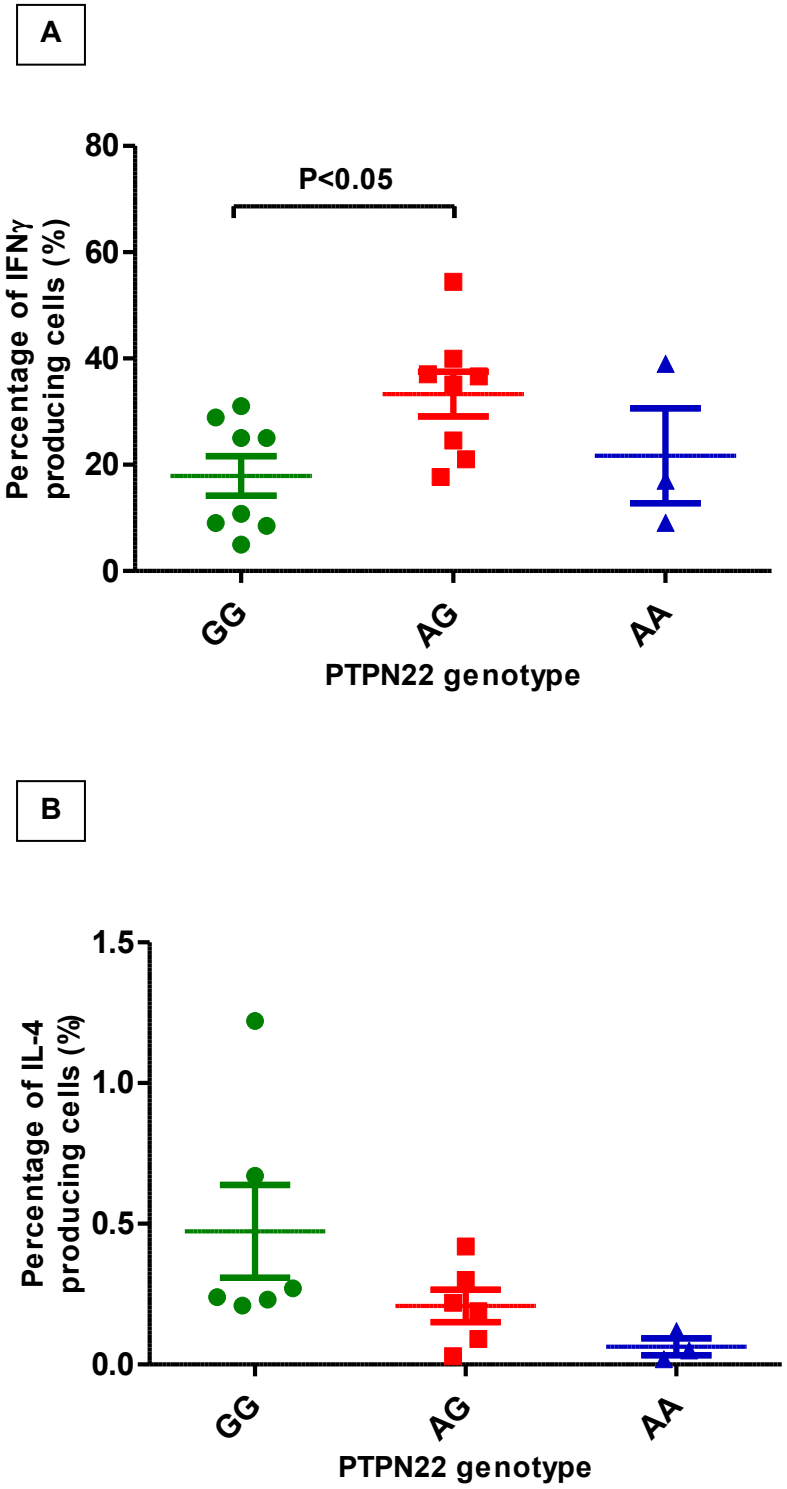
4.2.1.12 Changes in cytokine production by T lymphocytes isolated from rheumatoid arthritis patients expressing PTPN22 R620W

At present there have been no studies investigating the effect of PTPN22 R620W on cytokine production by CD4+ T cells in RA. However, studies carried out in healthy individuals have shown that T cells homozygous (AA) for the variant produce increased amounts of IFN-γ and decreased amounts of IL-17 suggesting a Th1 bias (Vang et al. 2013). Similarly we have observed that T cells isolated from healthy individuals heterozygous (AG) for the variant produced increased amounts of IFN-γ and TNF-α (Section 4.2.1.11), again supporting the hypothesis that the variant skews T cells towards a Th1 phenotype. To determine if this was also the case in RA, T cells were isolated from RA patients control (GG), heterozygous (AG), and homozygous (AA) for PTPN22 R620W. T cells were stimulated using plate-bound anti-CD3 and anti-CD28 and assessment of cytokine production was done after 48 hours of culture. This was carried out by taking supernatant for use in cytokine ELISAs and T cells were also stained with fluorescent conjugated antibodies to IL-4, IL-10, IL-17 and IFN-γ to assess short term cytokine production.

By using intracellular cytokine staining it was found that expression of R620W resulted in an increased percentage of IFN-γ secreting CD4+ T cells in patients with RA (Figure 4.10 A +C). The average percentage of control (GG) T cells secreting IFN-γ was 17.9±3.7%, which was statistically significantly increased to 33.3±4.2% in heterozygous (AG) T cells. There was a trend towards a decreased secretion of IL-4 secreting CD4+ T cells in AG patients (0.2±0.06%) when compared to GG patients (0.47±0.17%) (Figure 4.10 A+B). This trend also continued in homozygous (AA) T cells, which had the lowest percentage of IL-4 secreting T cells (0.06±0.03%). There was also a trend towards a decrease in the percentage of IL-17 secreting CD4+ T cells in AG and AA patients when compared to GG patients, (GG=1.46±0.23, AG=0.9±0.2% and AA=0.76±0.29%, Figure 4.10 E+F). Finally, it was observed that there was a statistically significant decrease in the percentage of IL-10

secreting CD4+ T cells in AG and AA patients (Figure 4.10 D+F). It was found that 0.53±0.09% of GG T cells secreted IL-10, which was significantly reduced in AG (0.29±0.04%) and AA (0.35±0.01%) T cells.

The effect of R620W on amount of cytokine secreted by T cells isolated from RA patients was then determined by ELISA. Similarly to healthy controls, it was observed that CD4+ T cells heterozygous and homozygous for the variant secreted significantly more of the pro-inflammatory cytokines TNF-α (GG=321pg/ml±43, AG=481pg/ml±33, and AA=499pg/ml±76) and IFN-γ (GG=235pg/ml±29, AG=363pg/ml±35, and AA=317pg/ml±54) when compared with control CD4+ T cells (Figure 4.10 G+H). Again, there was no difference between the amount of IL-4, IL-10 and IL-17 secreted by CD4+ T cells of all genotypes (Figure 4.10 I-K).


A
P<0.05
Percentage of IFNγ producing cells (%)
80
60
40
20
0
GG
AG
AA
PTPN22 genotype
B
Percentage of IL-4 producing cells (%)
1.5
1.0
0.5
0.0
GG
AG
AA
PTPN22 genotype

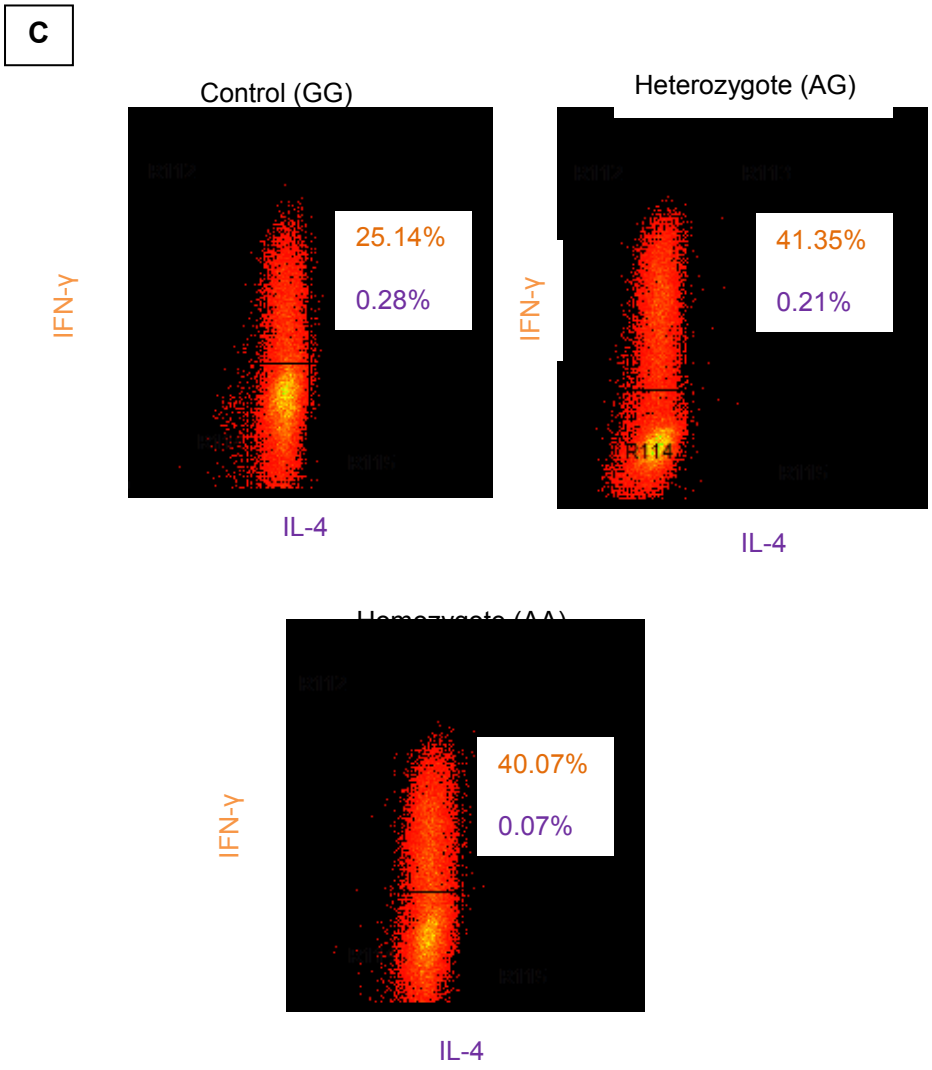
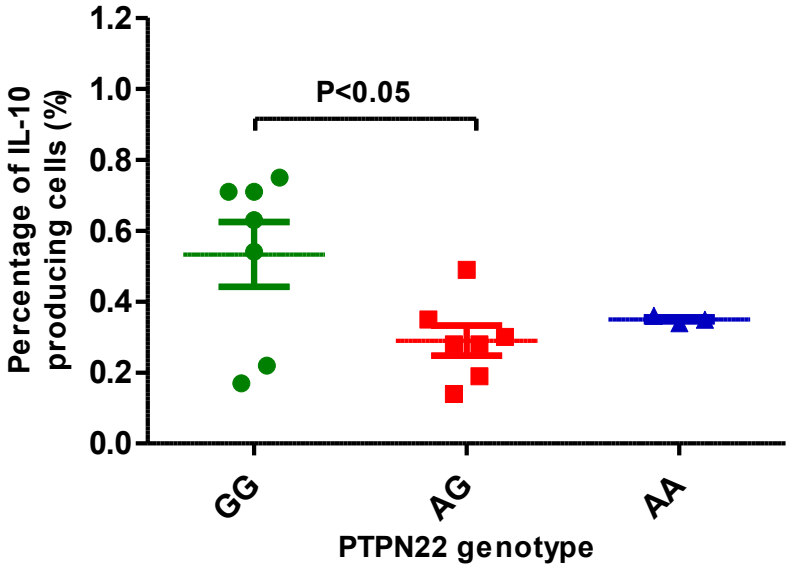
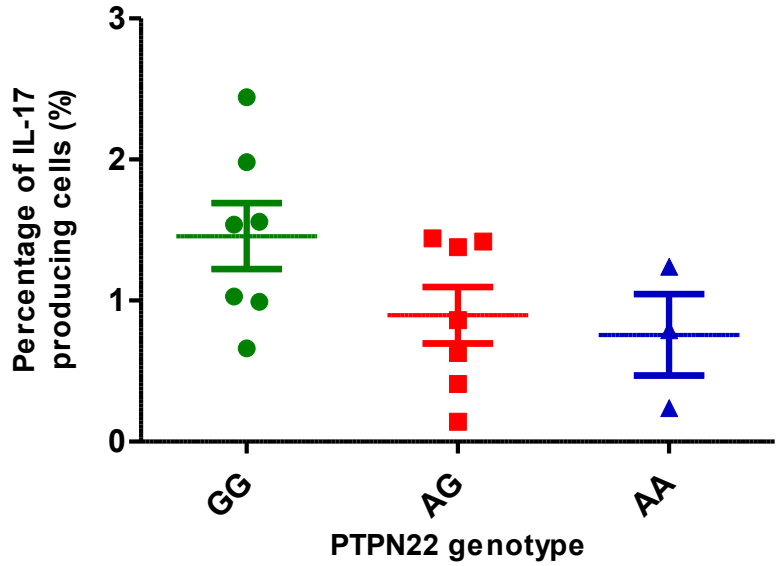


Figure 4.10 A-C Expression of the heterozygous form of the PTPN22 R620W variant increases the percentage of IFN-γ secreting CD4+ T cells in rheumatoid arthritis. CD4+ T cells expressing the different forms of Lyp (GG, AG and AA) were isolated from rheumatoid arthritis patients and left overnight in culture at 37°C. Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IFN-γ and IL-4 and assessed by flow cytometry. **(A)** Percentage of IFN-γ secreting CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show the mean (±SEM) of eight separate experiments for GG and AG, and three separate experiments for AA. **(B)** Percentage of IL-4 secreting CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show the mean (±SEM) of six separate experiments for GG and AG, and three separate experiments for AA. **(C)** Representative flow cytometry plot of the percentage of IFN-γ and IL-4 secreting CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show one of eight and six experiments performed with GG and AG, and three separate experiments for AA. $P<0.05$, Paired t-test compared with control (GG) cells.

D


1.2
1.0
0.8
0.6
0.4
0.2
0.0
P<0.05
Percentage of IL-10 producing cells (%)
GG
AG
AA
PTPN22 genotype

E


3
2
1
0
Percentage of IL-17 producing cells (%)
GG
AG
AA
PTPN22 genotype

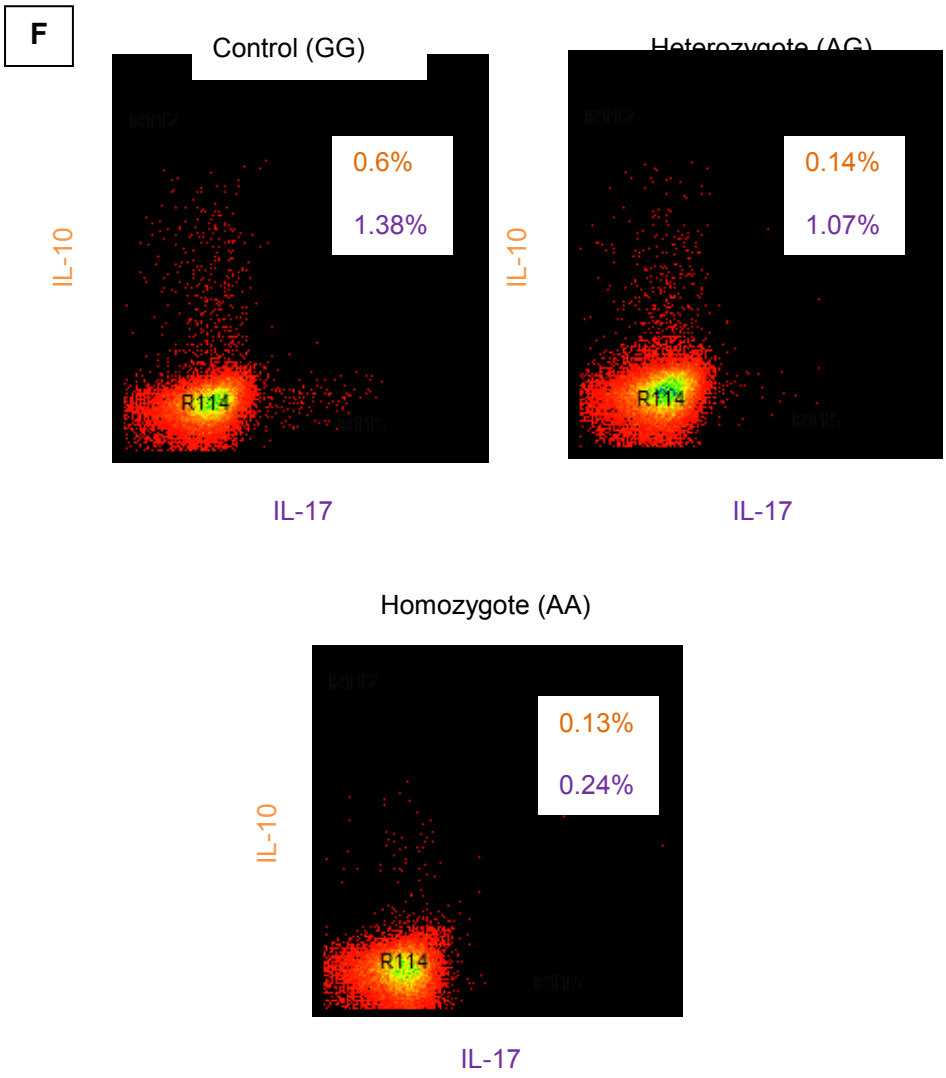
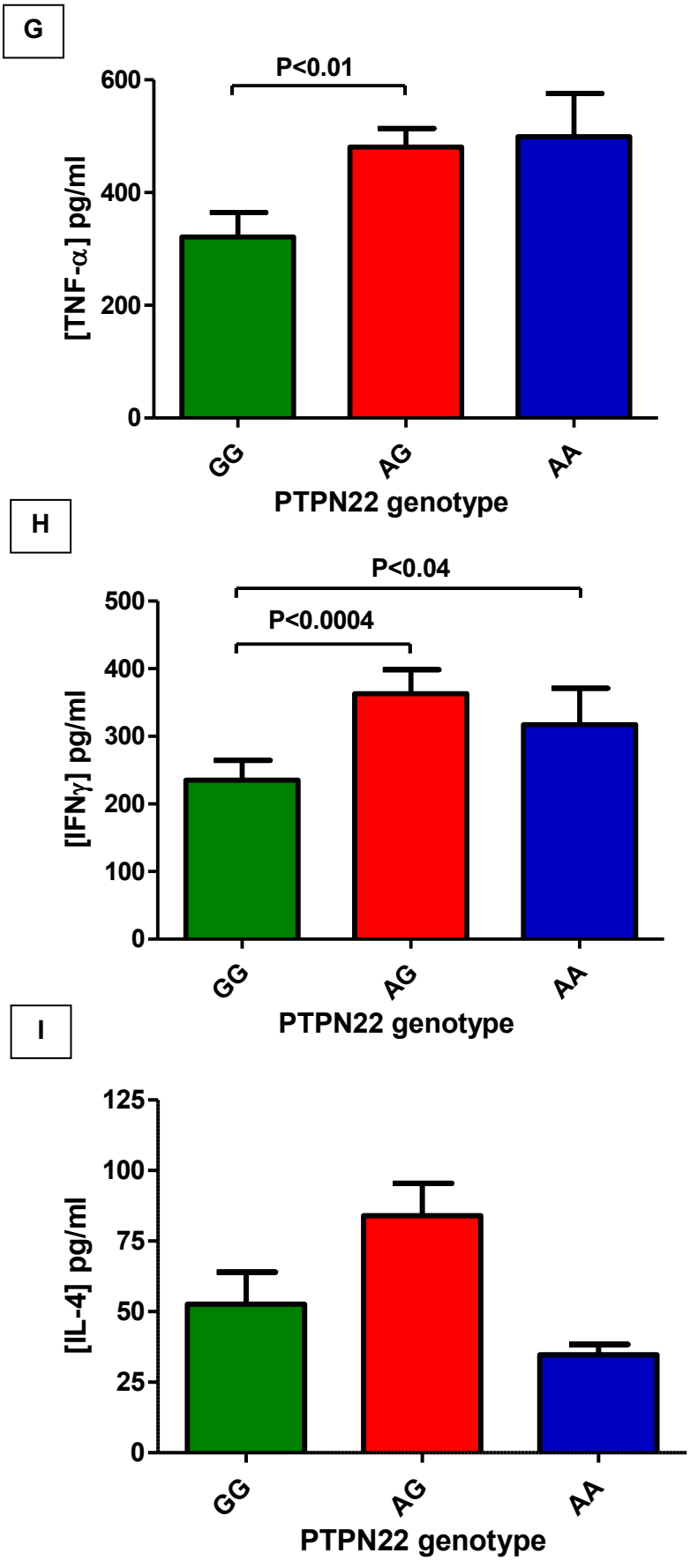


Figure 4.10 D-F Expression of the heterozygous form of the PTPN22 R620W variant decreases the percentage of IL-10 secreting CD4+ T cells in rheumatoid arthritis. CD4+ T cells expressing the different forms of Lyp (GG, AG and AA) were isolated from rheumatoid arthritis patients and left overnight in culture at 37°C. Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IL-10 and IL-17 and assessed by flow cytometry. **(A)** Percentage of IL-10 secreting CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show the mean (±SEM) of seven separate experiments for GG and AG, and three separate experiments for AA. **(B)** Percentage of IL-17 secreting CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show the mean (±SEM) of seven separate experiments for GG and AG, and three separate experiments for AA. **(C)** Representative flow cytometry plot of the percentage of IL-10 and IL-17 secreting CD4+ T cells expressing the different forms of Lyp (GG, AG and AA). Results show one of the seven experiments performed with GG and AG, and three separate experiments for AA. $P<0.05$, Paired t-test compared with control (GG) cells.


G
P<0.01
600
400
200
0
[TNF-α] pg/ml
GG
AG
AA
PTPN22 genotype
H
P<0.04
P<0.0004
500
400
300
200
100
0
[IFNγ] pg/ml
GG
AG
AA
PTPN22 genotype
I
125
100
75
50
25
0
[IL-4] pg/ml
GG
AG
AA
PTPN22 genotype

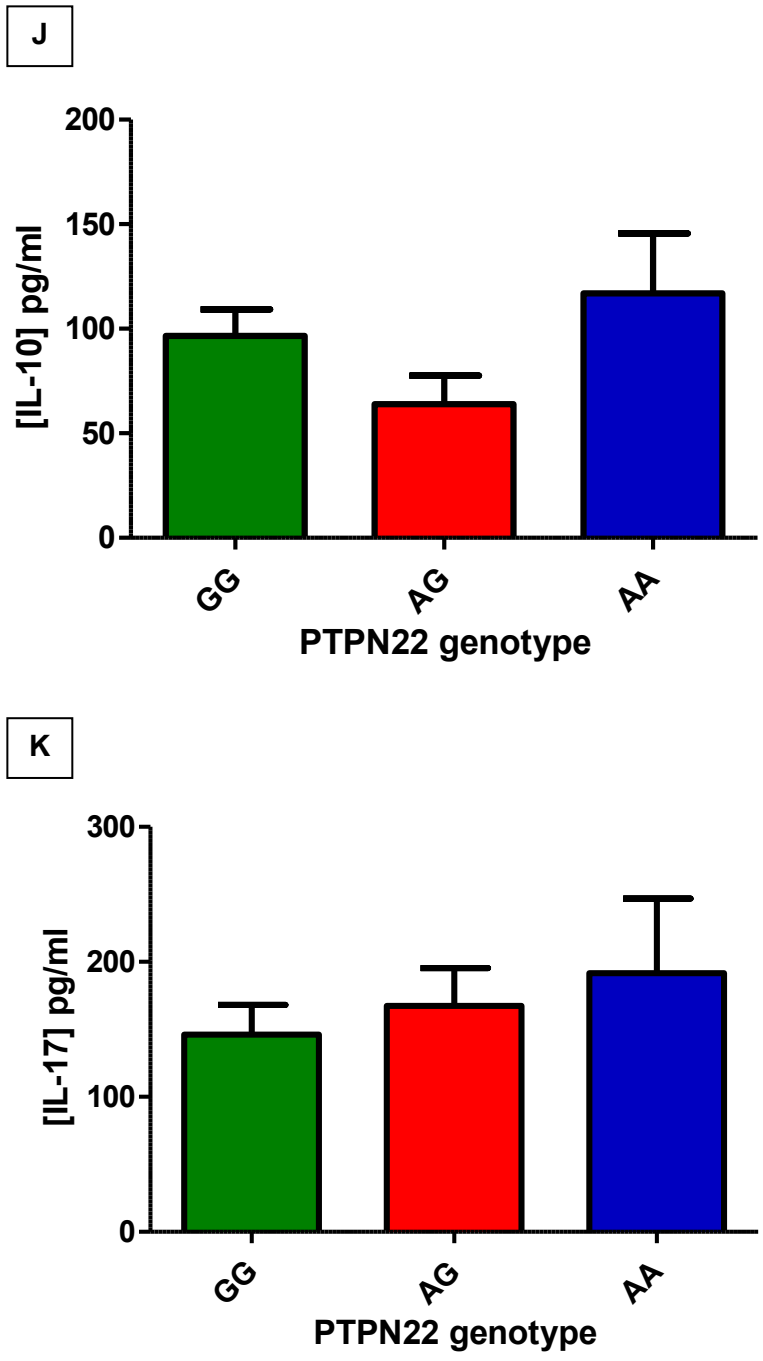


Figure 4.10 G-K Expression of the PTPN22 R620W variant increases the amount of IFN-γ and TNF-α produced by CD4+ T cells in rheumatoid arthritis. CD4+ T cells expressing the different forms of Lyp (GG, AG and AA) were isolated from rheumatoid arthritis patients and left overnight in culture at 37°C. Cells were stimulated with plate bound anti-CD3 (3μg/ml) and anti-CD28 (5μg/ml) for 48 hours at 37°C. Supernatant samples were taken 48 hours after stimulation and assessed for the presence of **(G)** TNF-α **(H)** IFN-γ **(I)** IL-4 **(J)** IL-10 and **(K)** IL-17 using ELISA. Figures show the mean (±SEM) of eight (GG), eight (AG) and three (AA) separate experiments. $P<0.04$, $P<0.01$ and $P<0.0004$, Paired t-test compared with control (GG) cells.

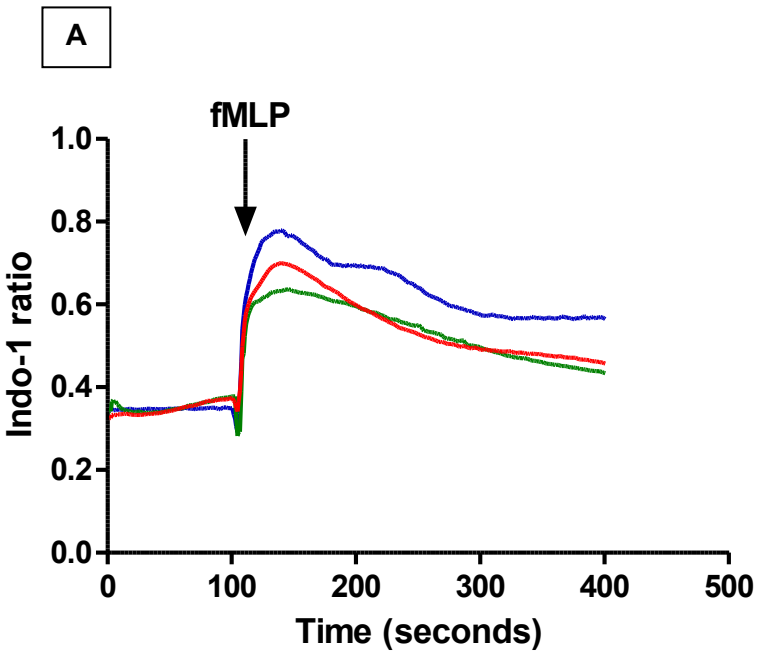
4.2.2 Effects of PTPN22 R620W on neutrophil signalling and function

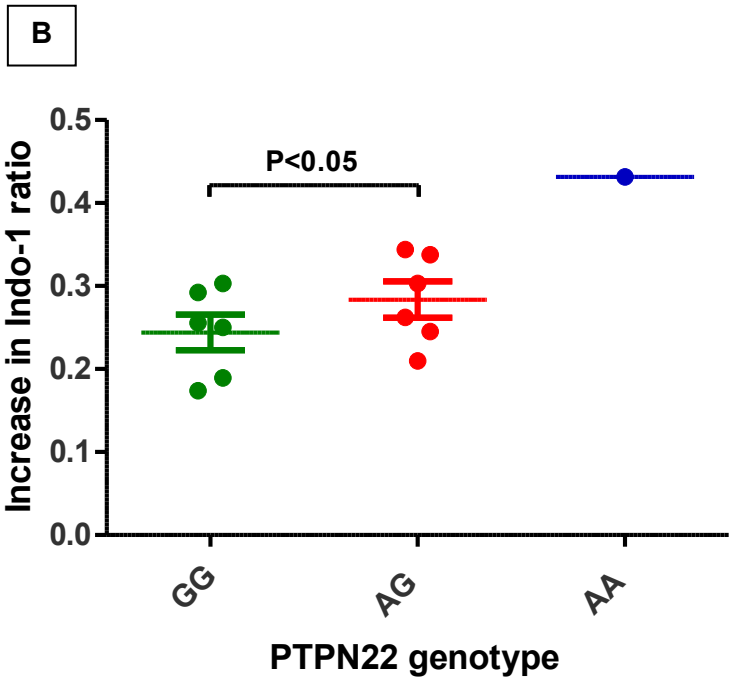
Previous work on PTPN22 R620W has largely focussed on T cells, given their importance in the pathogenesis of RA and other autoimmune inflammatory conditions (Firestein 2003). Indeed it has been shown in this thesis (Section 4.2.1) and by others that expression of R620W alters T cell function even in healthy individuals. However, no studies to date have investigated the role of Lyp and its variants in another type of leukocyte, the neutrophil. Many of the mechanisms used by neutrophils to combat infection can also cause significant tissue damage if neutrophil activation is prolonged or enhanced. For example, in the affected joints of RA patients neutrophils are found in large numbers, exhibit resistance to death and show increased effector functions (Wright et al. 2010). Interestingly it has been shown that the Lyp protein is highly expressed by neutrophils, but its function in this cell type is at present unknown (Chien et al. 2003). Given the current lack of knowledge of Lyp function in neutrophils, the effect of R620W on neutrophil function in healthy individuals and RA patients was investigated. To achieve this, neutrophils were isolated from individuals control (GG), heterozygous (AG) and homozygous (AA) for PTPN22 R620W and their signalling and function analysed.

4.2.2.1 Enhanced calcium signalling in neutrophils expressing PTPN22 R620W

As the function of Lyp in neutrophils is unknown, it was important to assess the effect of the Lyp variant on overall signalling through neutrophil surface receptors. This was done by adding N-formyl-Met-Leu-Phe (fMLP) to isolated neutrophils to mimic the presence of formylated peptides derived from bacteria, which would be present during an infection. fMLP binds to formyl peptide receptors (FPRs) on the neutrophil cell surface, which triggers further signalling and neutrophil activation. One of the processes resulting from FPR engagement is the release of Ca^{2+} from intracellular stores and later by an influx across the plasma membrane. Therefore, measuring overall Ca^{2+} release gives an indication of the strength and duration of signal induced by FPR activation. This was determined by loading neutrophils with the Ca^{2+} sensitive dye Indo-1 AM and measuring the change in Indo-1 ratio induced by the addition of fMLP.

It was found that when neutrophils isolated from healthy individuals heterozygous (AG) and homozygous (AA) for PTPN22 R620W were stimulated with fMLP, this caused increased Ca^{2+} release when compared to age and sex matched individuals without R620W (Figure 4.11 A+B). The average increase in Indo-1 ratio for control (GG) neutrophils was 0.24±0.02 and this increased to 0.28±0.02 in heterozygous (AG) neutrophils and further still to 0.43 in homozygous (AA) neutrophils. This increase in Ca^{2+} release was also observed in RA patients with R620W (Figure 4.11 C+D). When compared to healthy controls, Ca^{2+} release in all neutrophils isolated from RA patients was higher, but a stepwise increase was still observed with R620W. The average increase in Indo-1 ratio for RA control (GG) neutrophils was 0.39±0.04, which increased to 0.44±0.02 in the RA heterozygous (AG) neutrophils and further still to 0.56 in the RA homozygous (AA) neutrophils.


A
fMLP
1.0
0.8
0.6
0.4
0.2
0.0
Indo-1 ratio
0
100
200
300
400
500
Time (seconds)


B
P<0.05
0.5
0.4
0.3
0.2
0.1
0.0
Increase in Indo-1 ratio
GG
AG
AA
PTPN22 genotype

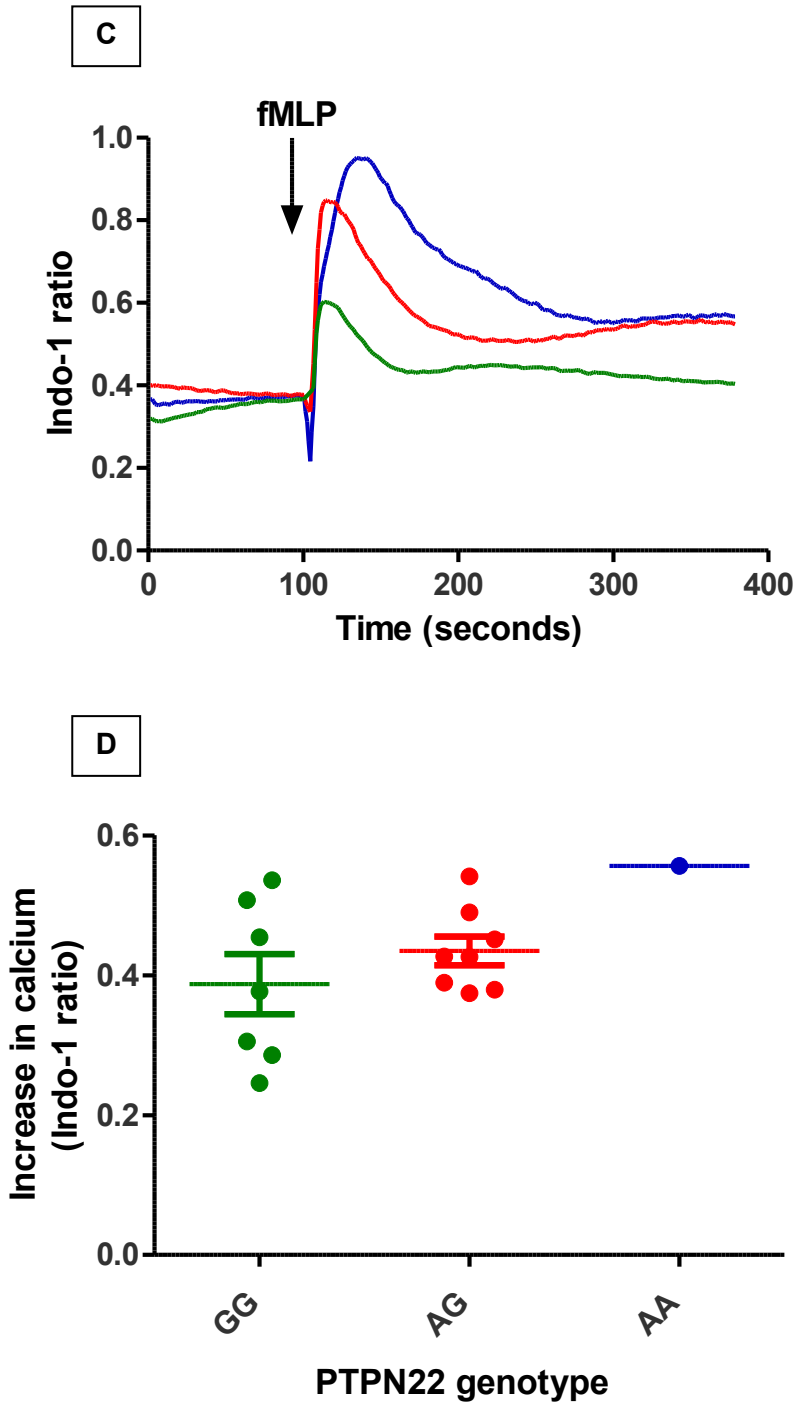


Figure 4.11 Increased calcium release by neutrophils heterozygous (AG) and homozygous (AA) for the PTPN22 R620W variant in response to fMLP. Neutrophils were isolated from healthy individuals and rheumatoid arthritis patients control (green), heterozygous (red) and homozygous (blue) for PTPN22 R620W. Cells were washed and loaded with the Indo-1 AM ester. Cells were washed again in HBSS and transferred to an acrylic cuvette. After a baseline was established, 50μM fMLP was added. **(A)** One representative measure of six experiments performed using neutrophils isolated from healthy individuals **(B)** Mean peak rise in Indo-1 AM ratio after stimulation of neutrophils isolated from healthy individuals **(C)** One representative measure of seven-eight experiments performed using neutrophils isolated from rheumatoid arthritis patients **(D)** Mean peak rise in Indo-1 AM ratio after stimulation of neutrophils isolated from rheumatoid arthritis patients. $P<0.05$ Wilcoxon matched-pairs signed rank test compared with control (GG) neutrophils.

4.2.2.2 Enhanced production of reactive oxygen species by neutrophils expressing PTPN22 R620W

Release of Ca^{2+} is an important mediator of other neutrophils effector functions in inflammation including NADPH oxidase activation and adhesion to endothelium (Pozzan et al. 1983, Foyouzi-Youssefi et al. 1997, Schaff et al. 2008). Given that R620W was shown to increase Ca^{2+} release it was hypothesised that other Ca^{2+} dependent neutrophil effector functions would also be affected. In order to investigate this, the ability of neutrophils to produce ROS in response to fMLP stimulation was tested. The NADPH oxidase is responsible for the catalysis of simple reactions that generate ROS in neutrophils. To do this, the multi component enzyme complex is assembled at the neutrophil plasma membrane where superoxide is produced following the addition of one electron to oxygen, using NADPH as the electron donor (Babior 1999).

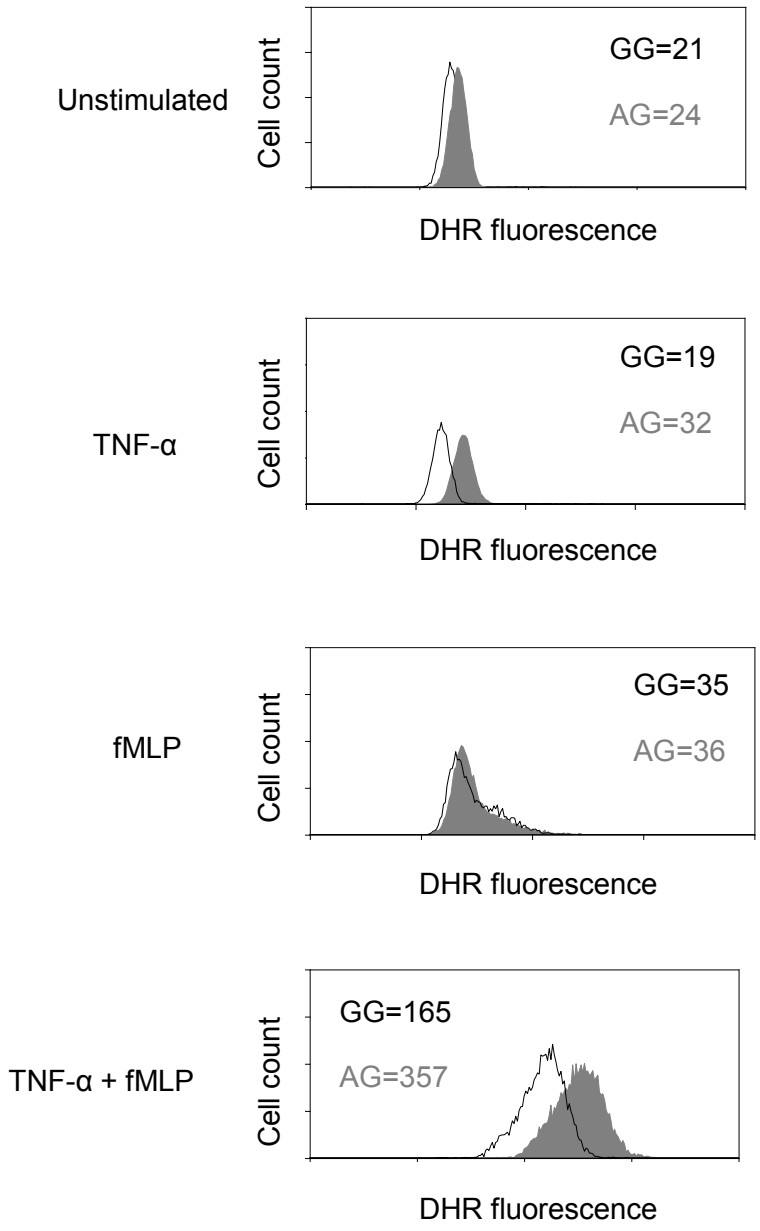
In order to assess if ROS production was affected by R620W, neutrophils were isolated from individuals control (GG) and heterozygous (AG) for R620W and incubated with dihydrorhodamine (DHR). DHR is a non-fluorescent dye which easily diffuses across cell membranes where it can be oxidised by ROS to cationic rhodamine 123 which is highly fluorescent, and thus can be used as an indicator of ROS production by neutrophils. Neutrophils were also incubated with TNF-α prior to addition of DHR, to investigate effects of TNF-α priming in these cells.

It was found that unstimulated neutrophils isolated from healthy individuals produced little ROS (Figure 4.12 A+B). When neutrophils were primed using TNF-α or stimulated using fMLP, either treatment alone did not result in a significant increase in ROS production by control (GG) or heterozygous (AG) neutrophils. However, when cells were primed with TNF-α and then stimulated using fMLP, a large increase in ROS production was observed which was significantly enhanced in heterozygous (AG) neutrophils (Figure 4.12 A-C). In GG neutrophils, the average DHR fluorescence in unstimulated cells was 69±13 which increased

to 233±36 when cells were primed with TNF-α and then stimulated with fMLP. The observed increase was much greater in AG neutrophils with the average DHR fluorescence in unstimulated cells being 155±66 which increased to 786±337 when cells were primed with TNF-α and then stimulated with fMLP. When this increase in DHR fluorescence was expressed as a percentage of GG neutrophils isolated from age and sex matched individuals, the AG neutrophils produced 308±111% more ROS when compared to GG neutrophils which were normalized to 100% (Figure 4.12 C).

These experiments were also conducted using neutrophils isolated from RA patients control (GG) and heterozygous (AG) for PTPN22 R620W. It was found that unstimulated neutrophils isolated from RA patients were slightly more active, with the average DHR fluorescence measured 173±54 in GG neutrophils and 87±28 in AG neutrophils. When neutrophils were primed with TNF-α and then stimulated with fMLP, similar findings to that of neutrophils isolated from healthy controls were observed (Figure 4.12 D-F). The observed increase was much greater in AG neutrophils, with the average DHR fluorescence in unstimulated cells being 87±28 which increased to 629±270 following priming and stimulation. In contrast the increase was much less in GG neutrophils, with the average DHR fluorescence in unstimulated cells being 173±54 which increased to 421±104. When this increase in DHR fluorescence was expressed as a percentage of GG neutrophils, the AG neutrophils produced 86±49% more ROS when compared to GG neutrophils which were normalized to 100% (Figure 4.12 F).

A


Unstimulated
Cell count
GG=21
AG=24
DHR fluorescence
TNF-α
Cell count
GG=19
AG=32
DHR fluorescence
fMLP
Cell count
GG=35
AG=36
DHR fluorescence
TNF-α + fMLP
Cell count
GG=165
AG=357
DHR fluorescence

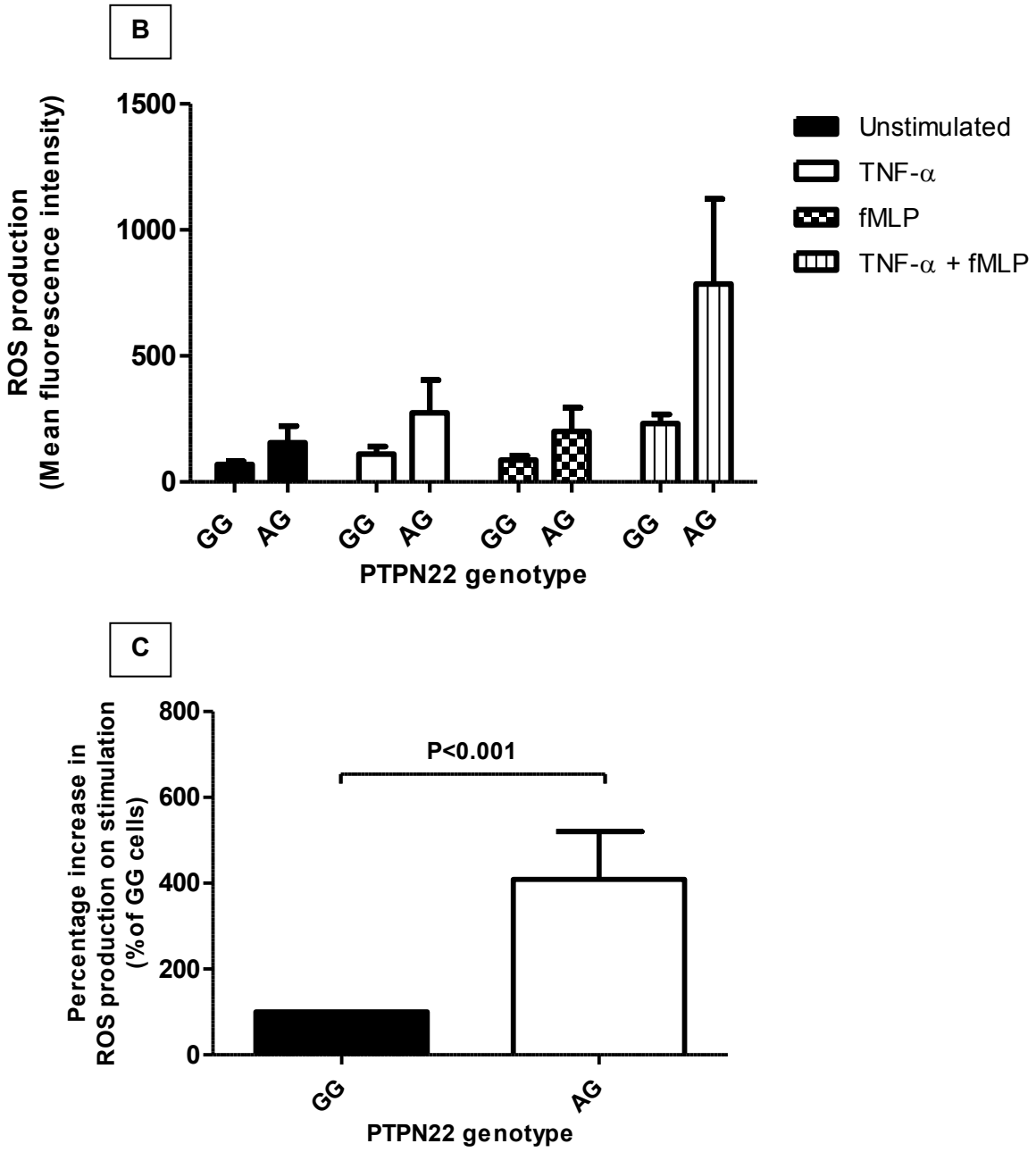
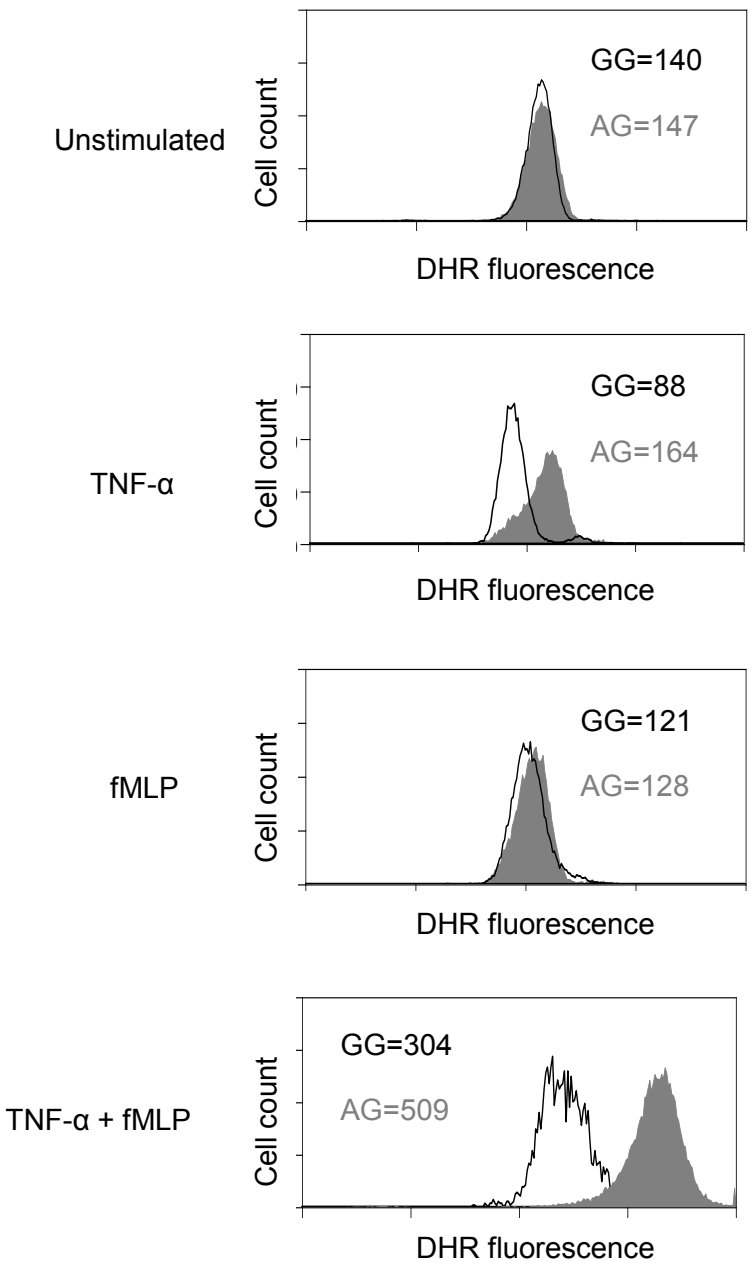


Figure 4.12 A-C Enhanced production of reactive oxygen species by neutrophils heterozygous (AG) for PTPN22 R620W isolated from healthy individuals. Neutrophils were isolated from age and sex matched healthy individuals control (GG) and heterozygous (AG) for PTPN22 R620W. Neutrophils were primed with 0.5μ TNF-α for 15 minutes at 37°C. Dihydrorhodamine (DHR) was added to cells for 5 minutes at 37°C and cells were then stimulated with 1μM fMLP for 5 minutes at 37°C. Cells were washed in ice-cold PBS and fixed using 2% paraformaldehyde overnight at 4°C. Reactive oxygen species production was assessed by measuring DHR fluorescence using flow cytometry. **(A)** One representative experiment of eight performed using control neutrophils (black solid line) and heterozygous neutrophils (grey shaded). **(B)** Average DHR fluorescence in unstimulated, TNF-α primed, fMLP stimulated, and primed and stimulated neutrophils. **(C)** Percentage increase in reactive oxygen species production in neutrophils primed with TNF-α and stimulated with fMLP, when compared to unstimulated neutrophils. Results are expressed as a percentage of control (GG) neutrophils of an age and sex matched individual. Results show the mean (±SEM) of eight separate experiments. P<0.001 Wilcoxon matched-pairs signed rank test compared with control (GG) neutrophils.

D


Unstimulated
Cell count
GG=140
AG=147
DHR fluorescence
TNF-α
Cell count
GG=88
AG=164
DHR fluorescence
fMLP
Cell count
GG=121
AG=128
DHR fluorescence
TNF-α + fMLP
Cell count
GG=304
AG=509
DHR fluorescence

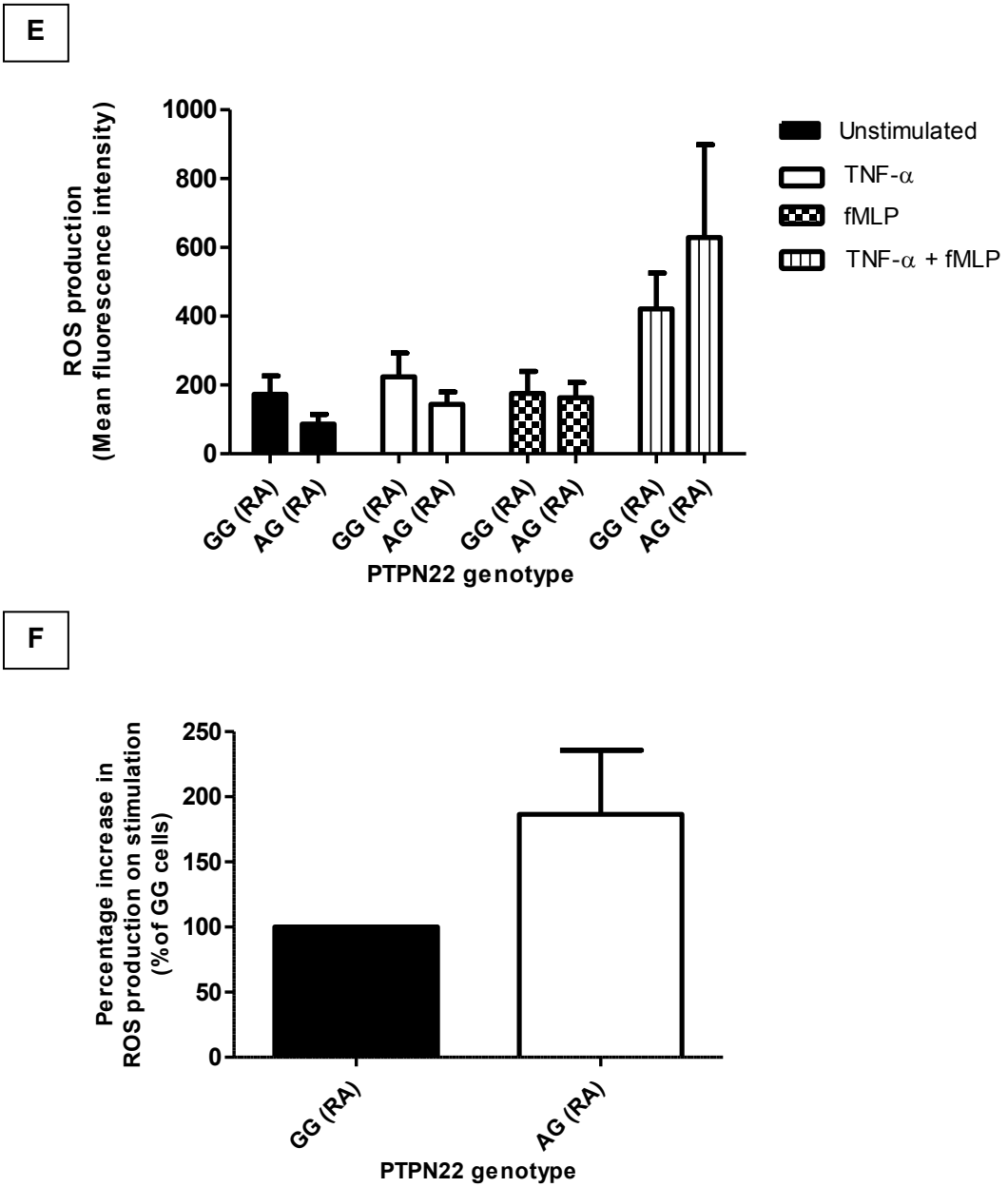
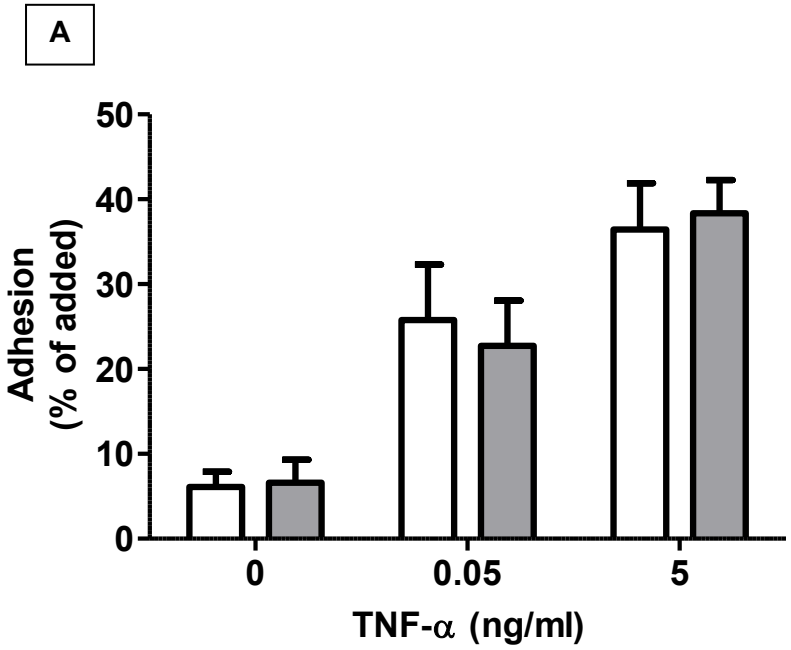


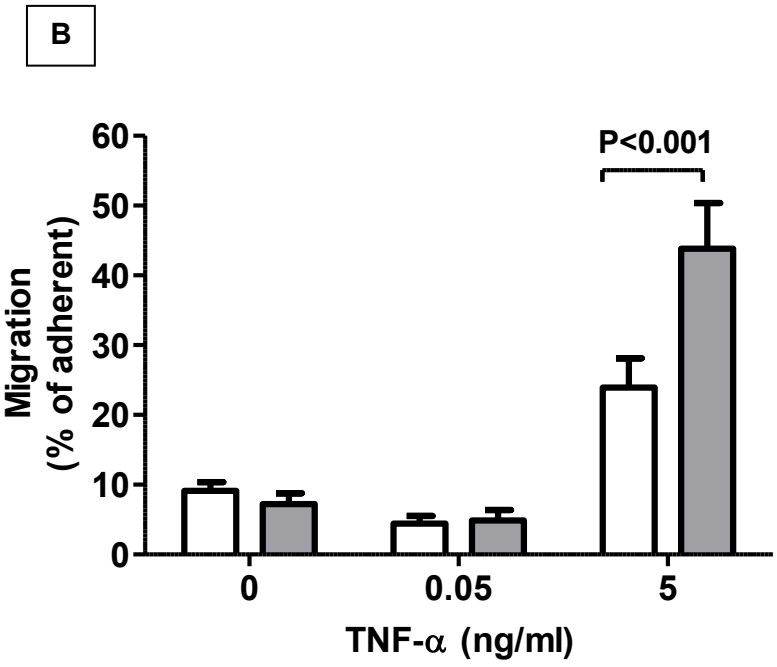
Figure 4.12 D-F Enhanced production of reactive oxygen species by neutrophils heterozygous (AG) for PTPN22 R620W isolated from rheumatoid arthritis patients. Neutrophils were isolated from rheumatoid arthritis patients control (GG) and heterozygous (AG) for PTPN22 R620W. Neutrophils were primed with 0.5µg TNF-α for 15 minutes at 37°C. Dihydrorhodamine (DHR) was added to cells for 5 minutes at 37°C and cells were then stimulated with 1µM fMLP for 5 minutes at 37°C. Cells were washed in ice-cold PBS and fixed using 2% paraformaldehyde overnight at 4°C. Reactive oxygen species production was assessed by measuring DHR fluorescence using flow cytometry. (A) One representative experiment of four performed using control neutrophils (black solid line) and heterozygous neutrophils (grey shaded). (B) Average DHR fluorescence in unstimulated, TNF-α primed, fMLP stimulated, and primed and stimulated neutrophils. (C) Percentage increase in reactive oxygen species production in neutrophils primed with TNF-α and stimulated with fMLP, when compared to unstimulated neutrophils. Results are expressed as a percentage of control (GG) neutrophils of an age and sex matched individual. Results show the mean (±SEM) of four separate experiments.

4.2.2.3 Increased migration of neutrophils expressing PTPN22 R620W

For neutrophils to carry out effector functions in tissues at a site of inflammation, first they must adhere and migrate through blood vessel walls (Kolaczkowska et al. 2013). The ability of neutrophils to adhere and migrate is an important determinant of their future functional capability, as neutrophils in the blood are largely inactive and require priming and recruitment (Wright et al. 2010). Changes to adhesion and migration of leukocytes could impact on the number of cells recruited to inflammatory sites, such as the synovial joints in RA (Firestein 2003). In order to determine the effect of R620W on neutrophil adhesion and migration, the ability of neutrophils to adhere and migrate through inflamed endothelium was assessed. To do this, human umbilical vein endothelial cells (HUVEC) were treated with TNF-α to model inflamed endothelium and then isolated neutrophils were added. The percentage of neutrophils that had adhered and migrated through the HUVEC was then assessed over time.

It was observed that neutrophil adhesion increased in a TNF-α dose dependent manner, independent of the PTPN22 genotype (Figure 4.13 A). It was found that neutrophils isolated from healthy individuals heterozygous (AG) for R620W were more migratory when compared to control (GG) neutrophils (Figure 4.13 B-D). Significantly more AG neutrophils transmigrated through EC treated with high dose TNF-α (5ng/ml) at 2 minutes when compared to neutrophils isolated from GG individuals (24±4% and 44±7% migrated for GG and AG neutrophils respectively, Figure 4.13 B-D). However, similar levels of migration were observed at 9 minutes (38±5% and 43±9% migrated for GG and AG neutrophils respectively). These results suggest that AG neutrophils are able to migrate more quickly through across inflamed endothelium when compared to GG neutrophils.


A
Adhesion (% of added)
50
40
30
20
10
0
0
0.05
5
TNF-α (ng/ml)


B
Migration (% of adherent)
P<0.001
60
50
40
30
20
10
0
0
0.05
5
TNF-α (ng/ml)

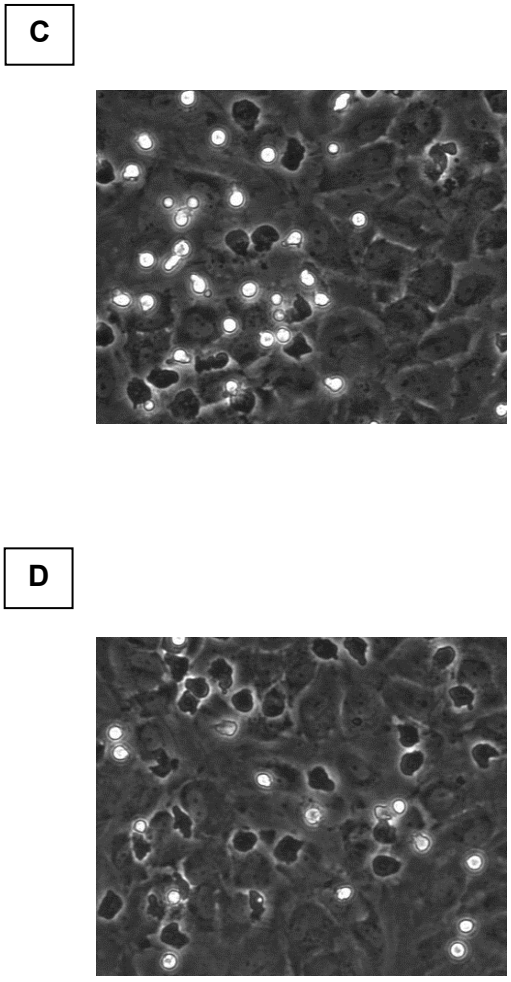


Figure 4.13 Increased migration of neutrophils heterozygous (AG) for PTPN22 R620W isolated from healthy individuals. HUVEC were treated in the presence or absence of TNF-α (0, 0.05, 5ng/ml) for 4 hours. Neutrophils were isolated from healthy individual's control (GG) and heterozygous (AG) for PTPN22 R620W and allowed to settle on the HUVEC monolayer for 6 minutes, after which non-adherent cells were removed by washing and neutrophil behaviour was analysed by phase contrast microscopy. **(A)** Neutrophil adhesion assessed at 2 minutes expressed as a percentage of total cells added. GG neutrophils (white bars) and AG neutrophils (grey bars) are shown. **(B)** Neutrophil transmigration was assessed at 2 and 9 minutes and expressed as a percentage of adherent cells that had migrated. ANOVA shows a significant effect of PTPN22 genotype ($p<0.05$) on neutrophil transendothelial migration. GG neutrophils (white bars) and AG neutrophils (grey bars) are shown. Representative micrographs of the behaviour of neutrophils isolated from **(C)** GG or **(D)** AG individuals on HUVEC treated with 5ng/ml TNF. Round phase bright cells are defined as those cells adherent to the surface of the endothelium. Dark, distorted phase dark cells are defined as cells that have migrated and are underneath the endothelium. Data are mean ± SEM from 6 (GG) and 7 (AG) independent experiments. $P<0.001$ by Bonferonni post test compared with control (GG) neutrophils. These experiments were done by Dr Helen McGettrick.

4.3 Discussion

In this chapter, the effects of PTPN22 R620W were investigated by measuring how expression of this genetic variant altered the signalling and function of neutrophils and T cells. The current literature was conflicting, with studies providing evidence for the variant being a gain (Vang et al. 2005) and loss (Hermiston et al. 2009) of function phosphatase in T cells. There was also debate as to the functional consequences of this change in the phosphatase activity of Lyp, with studies finding both an increase (Hermiston et al. 2009, Vang et al. 2013) and decrease (Vang et al. 2005, Rieck et al. 2007) in T cell signalling and responses. In contrast to this, nothing was known about the function of Lyp in neutrophils even though the protein is highly expressed (Chien et al. 2003).

By using the assay procedure described in Chapter 3 we were able to directly measure the phosphatase activity of Lyp in freshly isolated CD4+ T cells and showed that heterozygosity for R620W resulted in a decreased amount of Lyp protein, which had a higher intrinsic specific phosphatase activity (Figure 4.5). These results suggest that in fact all previous studies could be correct and that it is important to take into account the phosphatase activity and protein amount. For example, it was found in one study using Jurkat T cells transfected with the non-variant and variant Lyp that intrinsic phosphatase activity was higher (Vang et al. 2005). However, the amount of Lyp in this study is not comparable to that in a native T cell, as the transfection technique causes large amounts of protein to be produced. A study using primary CD4+ T cells isolated from RA patients has shown that expression of R620W indeed reduces the amount of Lyp protein present, but the activity of this protein was not measured (Zhang et al. 2011). Given this observed change in Lyp phosphatase activity the phosphorylation status of known Lyp substrates in T cells were measured. Flow cytometry for phosphorylation of the activating tyrosine residue of Lck kinase (Y394) revealed there to be no difference when comparing R620W and non-R620W T cells (Figure 4.6 A+B). However,

when this was assessed using immunoblotting it was found that phosphorylation of the activating tyrosine residue of Lck kinase (Y394) was significantly reduced in R620W expressing T cells (Figure 4.6 C+D). These conflicting results suggest that the effects of variant Lyp are not straightforward and that the location of different signalling proteins within T cells is of importance. For example, when measuring protein expression by immunoblotting the proteins have been denatured and separated from each other, whereas flow cytometry is carried out in whole cells with proteins in their native state and cellular location. In addition to Lck, the activating residue of Zap-70 (Y493) was also measured by immunoblotting and shown to be reduced in R620W expressing T cells (Figure 4.6 E+F).

The overall effects of these changes in Lyp phosphatase activity and Lyp substrate phosphorylation were then determined in healthy individuals and RA patients. It was observed that T cell proliferation was unaffected by R620W in both health and disease (Figure 4.7 and 4.8). This suggests that any changes in cytokine production by the T cells were not due to changes in proliferation rates. In healthy individuals it was found that expression of R620W did not alter the Th1/Th2 balance. This was determined using intracellular cytokine staining to measure the percentage of T cells secreting IFN-γ, IL-4, IL-10 and IL-17. The percentage of CD4+ T cells secreting IFN-γ, IL-4 and IL-17 were similar in R620W and non-R620W individuals, however there was a significant decrease in the percentage of IL-10 secreting R620W T cells (Figure 4.9). This was also observed in R620W T cells isolated from RA patients (Figure 4.10) suggesting this phenotype is maintained in disease. In addition, RA T cells expressing R620W also contained a higher percentage of IFN-γ secreting cells when compared to non-R620W RA T cells (Figure 4.10 A+C). This suggests that in RA patients with R620W a Th1 phenotype predominates, which could contribute to the pathogenesis of RA by increasing the generation of inflammatory T cells. When the quantity of cytokines produced were determined by ELISA, it was observed that T cells from healthy individuals and RA patients expressing R620W produced increased

amounts of the pro-inflammatory cytokines IFN-γ and TNF-α (Figure 4.9/10 G+H). It could be hypothesised based on these results that R620W promotes the generation of inflammatory T cells.

As the expression of Lyp has been reported to be high in neutrophils (Chien et al. 2003) the effect of R620W was also investigated in this cell type. It was found that expression of R620W resulted in increased Ca^{2+} release when neutrophils from healthy individuals and RA patients were stimulated with fMLP (Figure 4.11). It has been reported previously that Ca^{2+} release is greater in neutrophils isolated from the peripheral blood of patients with inflammatory diseases such as RA and our findings were similar (Davies et al. 1994). It was found that the highest Ca^{2+} release was by neutrophils isolated from RA patients heterozygous (AG) and homozygous (AA) for R620W (Figure 4.11). Increased Ca^{2+} release could be a mediator of increased neutrophil effector functions observed in inflammatory conditions and indeed it was found that R620W neutrophils produced more ROS when primed with TNF-α and stimulated with fMLP (Figure 4.12). R620W neutrophils were also found to be more migratory when compared to non-R620W neutrophils (Figure 4.13). Expression of R620W could explain why increased numbers of leukocytes such as neutrophils migrate into the joint and the increased ROS production could contribute to the joint destruction characteristic of RA. Given that these changes in neutrophil function are seen in both health and disease, this suggests that R620W alone does not cause pathogenic neutrophil function and that additional factors are required for these functions to contribute to disease development. These observations also highlight the importance of neutrophil priming by TNF-α, a key cytokine in the pathogenesis of RA (Brennan et al. 1992). Many of these functional alterations caused by R620W only occured in the presence of TNF-α, suggesting R620W neutrophils could respond differently to non-R620W neutrophils when presented with an inflammatory insult.

This chapter has described the effects of R620W in health and RA, by comparing its effects in two key types of leukocyte, the T cell and the neutrophil. Evidence has been presented which suggests that R620W T cells are inherently more inflammatory, which was illustrated by their ability to produce increased amounts of pro-inflammatory cytokines (as summarised in Table 4.2 A). Study has also shown that R620W neutrophils are more active, which was characterised by increased Ca^{2+} release, increased ROS production and enhanced migration through inflamed endothelium (as summarised in Table 4.2 B). Given that many of these observations have been made in healthy individuals and those with RA, this strongly reinforces that expression of R620W alone is not enough to induce the development of autoimmune inflammatory disease. Indeed it is known that complex diseases like RA result from interactions between a number of genetic and environmental factors (Scott et al. 2011). Based on this, the next chapter of work focuses on the effect of cigarette smoking on leukocyte signalling, with a view to identifying potential mechanisms by which R620W and cigarette smoking could interact to promote the development of RA. At present it has been shown that these two factors interact to increase risk of RA (Kallberg et al. 2007), but the biolgical mechanisms behind this are not yet known.

Table 4.2 Summary of the effects of PTPN22 R620W on CD4+ T cell and neutrophil function.
Tables show a summary of the results presented in Chapter Four examining the effects of PTPN22 R620W on **(A)** CD4+ T cell and **(B)** neutrophil signalling. Results are presented as the parameter assessed and the effect of PTPN22 R620W on this parameter. An arrow pointing upwards indicates an increase with PTPN22 R620W. An arrow pointing downwards indicates a decrease with PTPN22 R620W. A dash indicates no change with PTPN22 R620W.

A

Parameter assessed (CD4+ T cells)	Effect of PTPN22 R620W
Lyp phosphatase activity	↑
Lyp substrate phosphorylation	↓
Cell proliferation	—
Inflammatory cytokine production	↑

B

Parameter assessed (Neutrophils)	Effect of PTPN22 R620W
Calcium signalling	↑
Reactive oxygen species production	↑
Adhesion to endothelium	—
Migration across endothelium	↑

CHAPTER FIVE: EFFECTS OF CIGARETTE SMOKE ON LEUKOCYTE SIGNALLING

5.1 Introduction

Factors present within the environment are known to make a large contribution to an individual's risk of developing RA and other autoimmune inflammatory conditions (Liao et al. 2009). These factors are known to modulate specific components of the innate and adaptive immune responses, through their ability to alter the way immune cells respond to stimuli. Environmental factors commonly associated with increased risk of RA are previous infection, inadequate antioxidant intake, ageing and cigarette smoking (CS) (Liao et al. 2009). CS has long been associated with an increased risk of many human diseases and has widespread effects on the immune system (van der Vaart et al. 2004). Compounds contained within CS are known to activate inflammatory responses and ROS present within smoke can damage components of cells including their DNA and proteins (Borgerding et al. 2005). This detrimental effect of smoking on DNA integrity is thought to be a major mechanism by which smoking specifically increases the risk of cancer (Asami et al. 1997). Aside from direct DNA damage smoking also decreases the amount of circulating antioxidants, without which normal cellular functions cannot be maintained (Alberg 2002).

Glutathione (GSH) is the major circulating antioxidant and has a number of important functions (Droge et al. 1994), one of which is to prevent oxidative damage to cellular proteins. PTPs are particularly susceptible to damage via this mechanism and without adequate GSH these proteins can become irreversibly inactivated and no longer regulate signalling through immune cell receptors. Oxidants such as hydrogen peroxide (H_2O_2) are known to inactivate PTPs (Rider et al. 2003) but it is not known if CS has this same effect. Given that both H_2O_2 and CS deplete the antioxidant GSH, it could be hypothesised that these two agents could have effects on PTPs via a similar mechanism.

In Chapter 4 the effects of the genetic variant PTPN22 R620W on Lyp phosphatase activity and overall leukocyte function were examined. R620W and CS are known to interact via an

unknown mechanism to greatly increase the risk of RA (Costenbader et al. 2008). In this chapter, we investigated the hypothesis that this interaction occurs by these two factors modulating leukocyte signalling to promote inflammation and autoimmunity, which contribute to the development of RA. Similarly to Chapter 4, we wanted to determine the effects of CS on Lyp phosphatase activity and overall cell function. To do this a model of exposure to cigarette smoke extract (CSE) was used in which leukocytes in culture were exposed to CSE and their signalling and function in response to this investigated. For comparative purposes the effects of exposure to H_2O_2 on CD45 and Lyp phosphatase activity were studied to assess if the effects of CSE were similar. The effects of CSE exposure in T lymphocytes were extensively characterised by measuring TCR signalling parameters, cell viability, proliferation and cytokine production. Study was extended to investigate potential mechanisms by which CSE acts by exploring which component of CSE could be exerting specific effects on T lymphocytes. Previous work has shown that T cells can be less proliferative and secrete decreased amounts of cytokines when treated with CS (Lambert et al. 2005, Glader et al. 2006) and we wished to further investigate the mechanisms by which this could occur.

5.2 Results

A number of studies have assessed the impact of CS and CSE exposure on T lymphocyte function (Ouyang et al. 2000, Lambert et al. 2005, Vassallo et al. 2005, Robbins et al. 2008). To do this two main methods have been used, (1) isolation of T lymphocytes from current smokers and (2) in *vitro* exposure of T lymphocytes to CSE. The former is ideal for examining the outcome of smoking on resulting signalling in T lymphocytes, however the latter is more appropriate for studying the mechanisms by which these changes in signalling events may occur. Based on this we chose to use an *in vitro* model of CSE exposure with a view to investigating the mechanisms of CSE-induced functional changes. CSE was generated using a simple system as described in Chapter 2 (Section 2.2.8). Other studies which have used a similar method have produced mixed results. Some data show that exposure to CSE reduces T cell proliferation and cytokine production (Lambert et al. 2005, Glader et al. 2006), however these measures have been shown to be unchanged by others (Zavitz et al. 2008). It is possible these conflicting results could be explained by differences in CSE preparation method, experimental concentrations, cohorts, cell stimulation methods or culture conditions. Previous work has also shown that treating CD4+ T cells in an *in vitro* co-culture with DCs and CSE reduces their ability to produce IL-2, IL-1β, IFN-γ, and TNF-α, but increases their ability to produce the Th2 cytokine IL-4 (Ouyang et al. 2000, Vassallo et al. 2005). This implies that interactions between T lymphocytes and other immune cells in the presence of CSE are of importance and could affect their polarisation and differentiation.

5.2.1 Hydrogen peroxide decreases CD45 and Lyp activity in T lymphocytes

Oxidation of PTPs is an important regulatory mechanism which can render them inactive, having major consequences on upstream signalling pathways. Activity of the PTP is lost because an oxidised cysteine residue present in its active site cannot interact with the target substrate (Bottini et al. 2004). Before studying the effects of CSE on the activity of the CD45 and Lyp phosphatase, we wished to determine if these PTPs could be oxidatively inactivated by H_2O_2. This was necessary to determine the relative potency of CSE in comparison to H_2O_2 and to help investigation into if CSE could reduce the activity of PTPs by a similar oxidative mechanism. H_2O_2 was chosen as it can easily diffuse across the plasma membrane and instantly increase the level of intracellular ROS (Rhee 1999). As CD45 and Lyp are the two main PTPs regulating signalling through the TCR, it is of importance to assess if either one is more susceptible to regulation by oxidation. If one was more susceptible this could alter the balance between a PTP with a predominantly positive regulatory role (CD45) and a PTP with a negative regulatory role (Lyp), which could then have consequences on overall TCR signalling.

To investigate the effect of H_2O_2 on CD45 and Lyp phosphatase activity in T lymphocytes, Jurkat T cells and primary CD4+ T cells were exposed to a range of concentrations of H_2O_2 and their CD45 and Lyp phosphatase activity were measured. It was observed that exposure to H_2O_2 decreased the phosphatase activity of CD45 and Lyp in both Jurkat T cells and primary CD4+ T cells (Figure 5.1). The amount of Lyp protein bound during the assay procedure was not affected by treatment with H_2O_2 and the average amount of Lyp protein bound in untreated Jurkat T cells was 3ng±0.4, and in CD4+ T cells was 2.7ng±0.4. Although the protein amounts were similar, four times the amount of CD4+ T cells were used in each well when compared to Jurkat T cells. Lyp in Jurkat T cells was less susceptible to oxidation by H_2O_2 when compared to primary T cells (Figure 5.1 A + B). In Jurkat T cells treatment with 3.6mM±0.9 H_2O_2 resulted in a 50% reduction in the specific activity of Lyp. In contrast, a 50%

reduction in specific activity was observed with 384μM±86 H_2O_2 when measuring Lyp activity in primary CD4+ T cells.

The relative susceptibility to oxidation of CD45 and Lyp were measured and it was found that CD45 was much more susceptible to oxidation by H_2O_2 when compared to Lyp in Jurkat T cells and primary CD4+ T cells (Figure 5.1 C +D). The CD45 phosphatase had an IC50 of 1.9 mM±0.7 H_2O_2 and the Lyp phosphatase had an IC50 of 3.6mM±0.9 H_2O_2 in Jurkat cells. Both PTPs were more susceptible to oxidation in primary CD4+ T cells when compared to Jurkat T cells, but CD45 remained more susceptible than Lyp to oxidation with an IC50 for CD45 of 219μM±70 H_2O_2 and 384μM±86 H_2O_2 for Lyp.

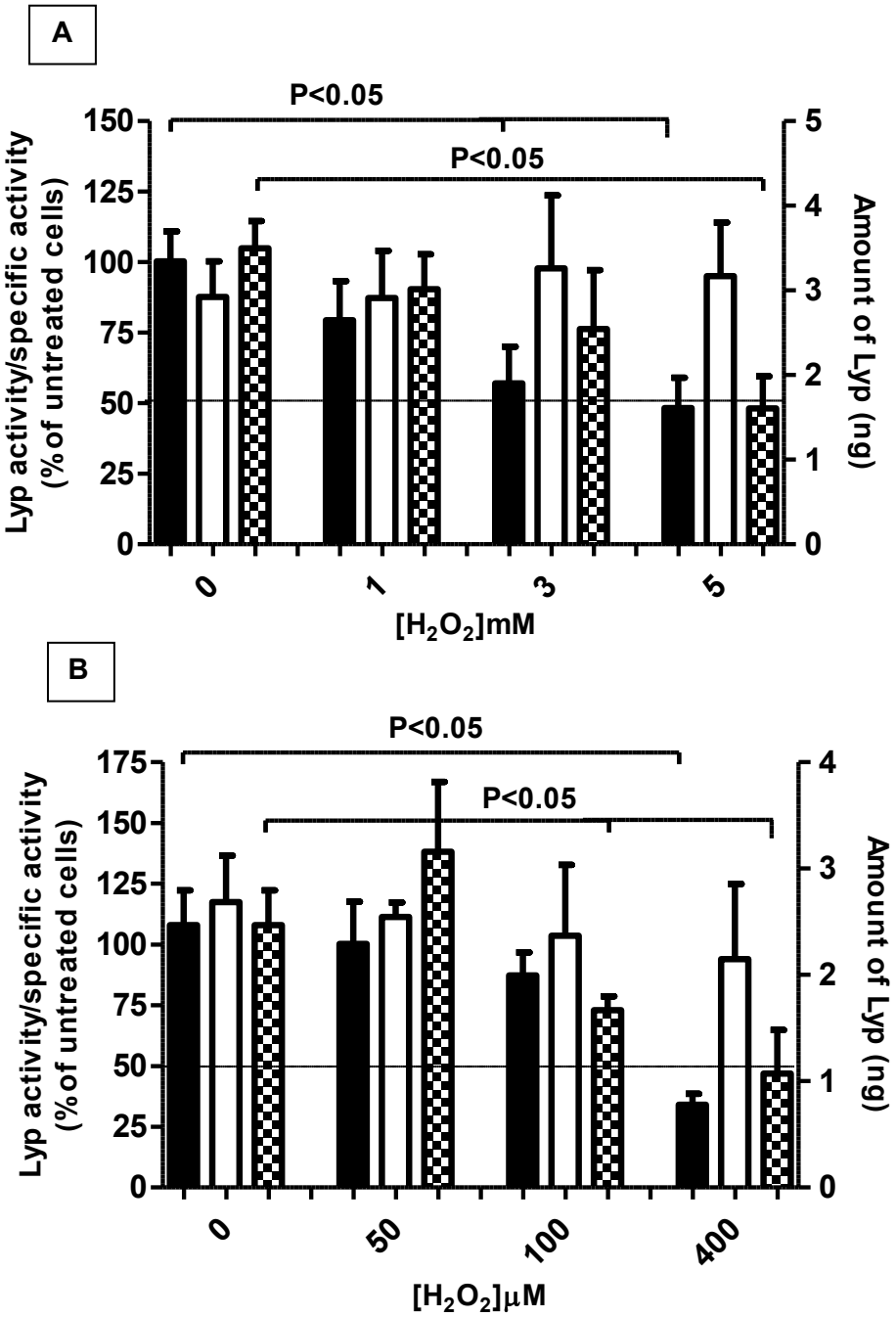


Figure 5.1 A+B H_2O_2 causes a decrease in the specific activity of the Lyp phosphatase in Jurkat T cells and peripheral blood CD4+ T cells. (A) Jurkat T cells (0.5×10^6) were exposed to 1mM, 3mM and 5mM H_2O_2 for 15 minutes as described in Chapter 2. After treatment, the specific activity of the Lyp phosphatase was measured. Data show the decrease in FI (black bars), and decrease in specific activity (checked bars) as a percentage of the untreated cells (exposed to 0mM H_2O_2). The actual amount of Lyp protein captured from each sample is also shown (white bars). All measures of activity were carried out in triplicate, and measures of amount carried out in duplicate. The graph shows the mean (±SEM) of three different experiments. **(B)** Whole CD4 + T cells (2.0×10^6) were freshly isolated from healthy volunteers and exposed to 50μM, 100μM and 400μM H_2O_2 before Lyp phosphatase activity was measured. Data show the decrease in FI (black bars), and decrease in specific activity (checked bars) as a percentage of the untreated cells (exposed to 0mM H_2O_2). The actual amount of Lyp protein captured from each sample is also shown (white bars). The graph shows the mean (±SEM) of results from three different donors. P<0.05, Mann Whitney non-parametric analysis when compared with untreated (0mM) T cells.

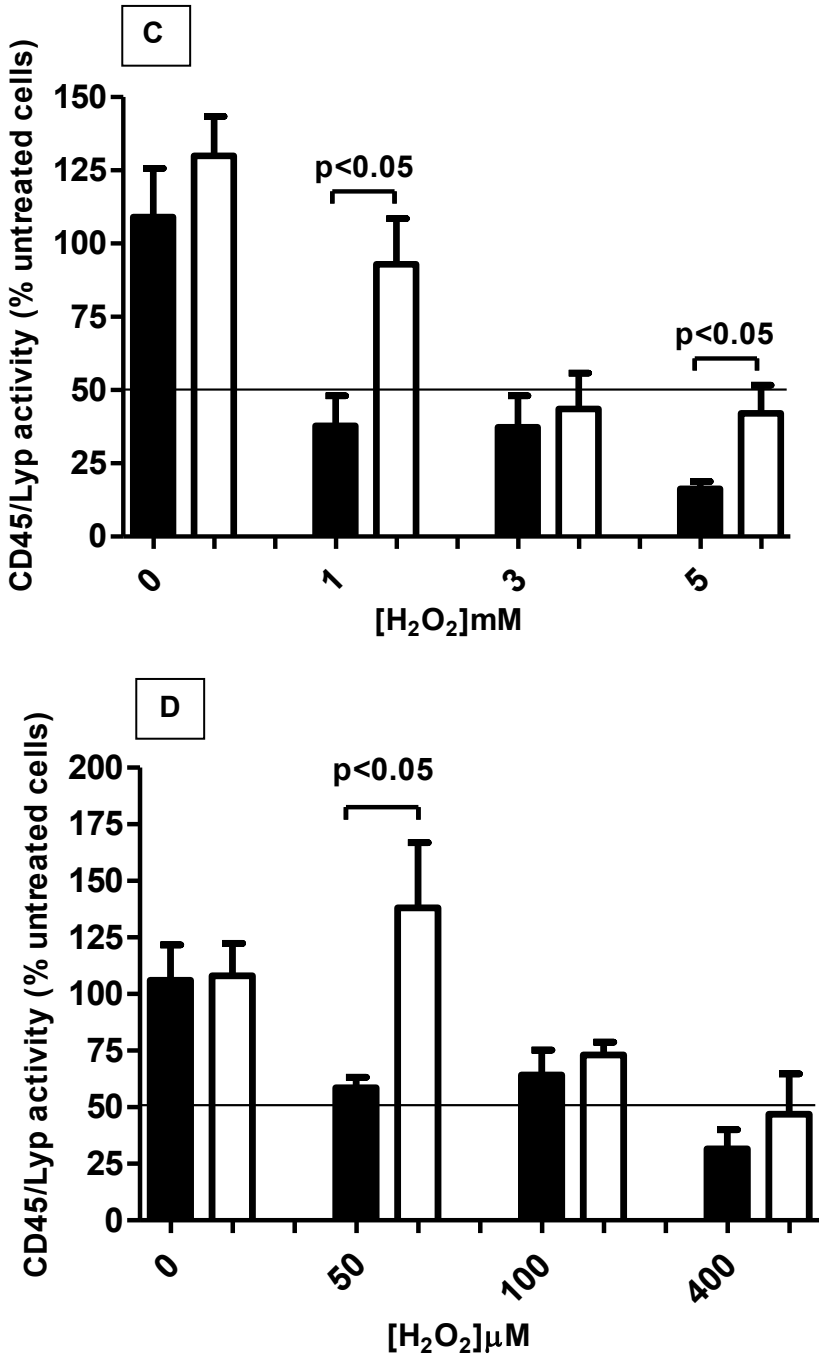


Figure 5.1 C+D The CD45 phosphatase is more susceptible to oxidation when compared to the Lyp phosphatase in Jurkat T cells and primary CD4+ T cells. (C) Jurkat T cells ($1.0x10^6$) were exposed to 1mM, 3mM and 5mM H_2O_2 for 15 minutes as described in Chapter 2. After treatment, the specific activity of the CD45 phosphatase and Lyp phosphatase was measured from the same cells ($0.5x10^6$ for CD45 and $0.5x10^6$ for Lyp). Data show the decrease in specific activity of CD45 (black bars) and Lyp (white bars) as a percentage of the untreated cells (exposed to 0mM H_2O_2). All measurements were carried out in triplicate and the graph shows the mean (±SEM) of three different experiments. P<0.05, Mann Whitney non-parametric analysis when comparing CD45 to Lyp **(D)** Whole CD4 + T cells ($2.5x10^6$) were freshly isolated from healthy volunteers and exposed to 50μM, 100μM and 400μM H_2O_2 before CD45 and Lyp phosphatase activity was measured ($0.5x10^6$ for CD45 and $2.0x10^6$ for Lyp). Data show the decrease in specific activity of CD45 (black bars) and Lyp (white bars) as a percentage of the untreated cells (exposed to 0mM H_2O_2). All measurements were carried out in triplicate and the graph shows the mean (±SEM) of results from three different donors. P<0.05, Mann Whitney non-parametric analysis when comparing CD45 to Lyp.

5.2.2 Cigarette smoke extract has no effect on T lymphocyte viability

CSE is known to have a number of effects on T cell function, for example a decrease in proliferative capacity and ability to produce a number of cytokines (Ouyang et al. 2000, Lambert et al. 2005). These observations could be explained by an increase in cell death induced by treatment with CSE. Given this possibility it was necessary to determine if the CSE generated by the method described in Chapter 2 (Section 2.2.8) had any effects on T cell survival. To fully characterise the effects of CSE on TCR signalling the Jurkat T cell line was used as availability of cells would not be an issue. Jurkat T cells were treated with different amounts of CSE (0%, 3% and 30%) and cultured for 3 days. After this time cells were stained with Annexin V and propidium iodide before being analysed by flow cytometry. Annexin V binds to phosphatidylserine which is only exposed on the outer plasma membrane of cells entering apoptosis and propidium iodide binds to DNA which is only exposed in dead cells when their membrane integrity is lost. These two stains together allow identification of viable cells which are negative for both markers.

It was found that treatment with CSE had no effect on the percentage of viable cells (Figure 5.2). The treatment used in further experiments would be 24 hours and even after 72 hours of treatment the cell viability was unaffected (0% CSE=75% viable cells, 3% CSE=78% viable cells and 30% CSE=75% viable cells). Given these results it was determined that the CSE preparation was suitable for use in further studies at these concentrations as it did not cause cell death.

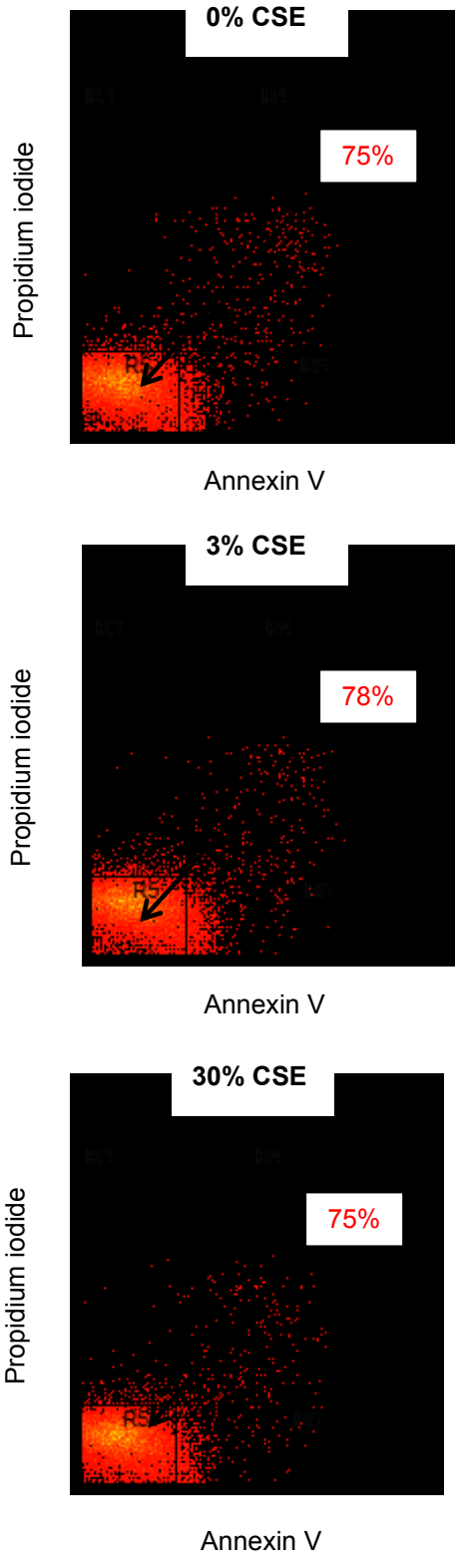


Figure 5.2 Treatment with cigarette smoke extract has no effect on T lymphocyte viability. Jurkat T cells were treated with cigarette smoke extract (0%, 3% and 30%) for 72 hours at 37°C. Cells were removed from culture and stained with Annexin V and propidium iodide. Cells were analysed by flow cytometry and the percentage of live cells determined (Gate R5, % in box indicated on each plot). Representative plots are shown from one of the three separate experiments performed.

5.2.3 Cigarette smoke extract decreases CD45 and Lyp phosphatase activity in T lymphocytes

Following confirmation that treatment with CSE did not affect T cell viability it was investigated if CSE could alter the activity of PTPs, including CD45 and Lyp. Due to the fact that CSE is a complex mixture of different compounds (Borgerding et al. 2005) it is difficult to predict how CSE will affect the activity of specific PTPs. It could be hypothesised that CSE could act via a similar mechanism to H_2O_2 to reduce PTP activity by oxidising the active site cysteine residue (Bottini et al. 2004). In support of this, large amounts of ROS have been shown to be present in CSE (Borgerding et al. 2005) which could contribute to its effects on the PTPs. To investigate the effects of CSE treatment on PTP activity Jurkat T cells were treated with CSE and their global PTP, CD45 and Lyp phosphatase activity measured.

It was found that treatment with CSE decreased the activity of all PTPs measured, with the CD45 and Lyp phosphatase both being affected (Figure 5.3). Globally PTPs were not as affected, for example treatment with 30% CSE decreased the activity of PTPs globally to 65±5.8% of the activity measured in untreated T cells. At a mild concentration of CSE (3%) the activity of CD45 and Lyp were equally reduced to 42±13% and 44±10% respectively. At a higher concentration of CSE (30%) the CD45 phosphatase was more susceptible to the effects of CSE treatment. With 30% CSE treatment CD45 phosphatase activity was 29±8.9% that of untreated T cells, whereas Lyp retained 41±6.7% of its activity. This suggests that lower concentrations of CSE equally affect CD45 and Lyp phosphatase activity, however when the concentration of CSE is increased the activity of Lyp is less affected when compared to CD45.

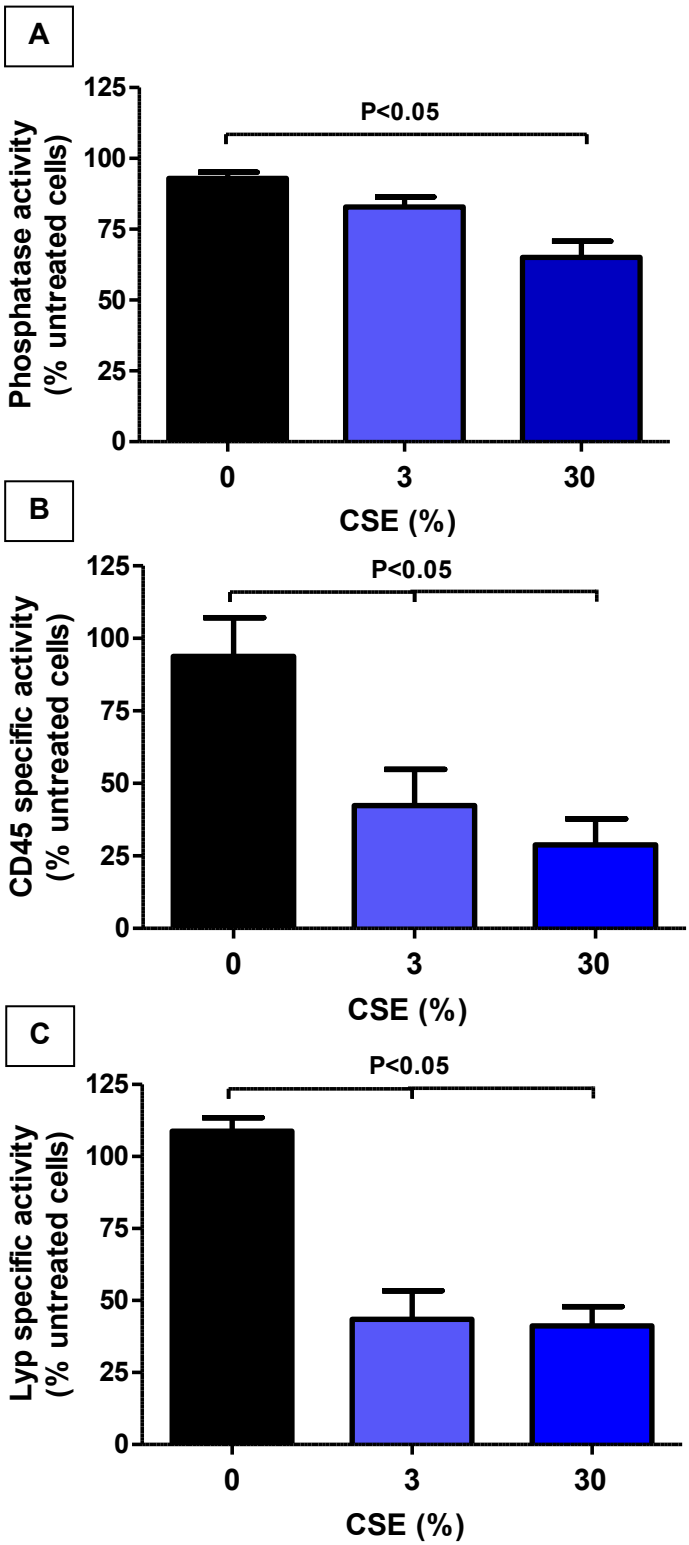


Figure 5.3 Treatment with cigarette smoke extract decreases the activity of protein tyrosine phosphatases including CD45 and Lyp in T lymphocytes. Jurkat T cells were exposed to cigarette smoke extract (0%, 3% and 30%) for 24 hours. After treatment, global protein tyrosine phosphatase was assessed, as well as the specific activity of the CD45 and Lyp phosphatase. Data show the percentage decrease in phosphatase activity of PTP's globally **(A)**, and the specific PTPs CD45 **(B)** and Lyp **(C)**. All measurements were taken in triplicate, and graphs show the mean (±SEM) of three different experiments. P<0.05, Mann Whitney non-parametric analysis when comparing untreated cells (0%) to treated cells (3% and 30%).

5.2.4 Cigarette smoke extract decreases the glutathione content of T lymphocytes

After observing that treatment of Jurkat T cells with CSE decreased the activity of the CD45 and Lyp phosphatase, the mechanism behind this observation needed to be examined. It is possible that CSE decreases the activity of these PTPs in a similar manner to H_2O_2 by oxidising their active site cysteine (Bottini et al. 2004). When considering the physiological roles of ROS such as H_2O_2, their concentration is usually maintained at a low level by the presence of adequate concentrations of the antioxidant GSH (Droge et al. 1994). Therefore if adequate GSH is present this can prevent oxidative inactivation of the PTP active site cysteine. Given that CSE is known to contain ROS which can deplete cells of GSH, it was investigated whether the CSE preparation used here could decrease the concentration of GSH in T cells. This was necessary as the CSE was generated in the laboratory and then stored at -80°C, which could have altered the composition of the extract. To do this, Jurkat T cells were treated with CSE, the cells lysed and their reduced GSH content was measured. It was found that the CSE preparation was able to significantly decrease the reduced GSH concentration in Jurkat T cells (Figure 5.4). Treatment with 3% CSE was able to decrease the reduced GSH content from 6.8±1μM in untreated cells to 3.1±0.3μM. The effects of CSE on GSH content appeared to be concentration dependent, as 30% CSE was able to decrease the reduced GSH content further to 1.4±0.4μM. Based on the fact that 3% CSE decreased GSH by around 50% and 30% CSE by around 80%, these concentrations of CSE were used in further experiments.

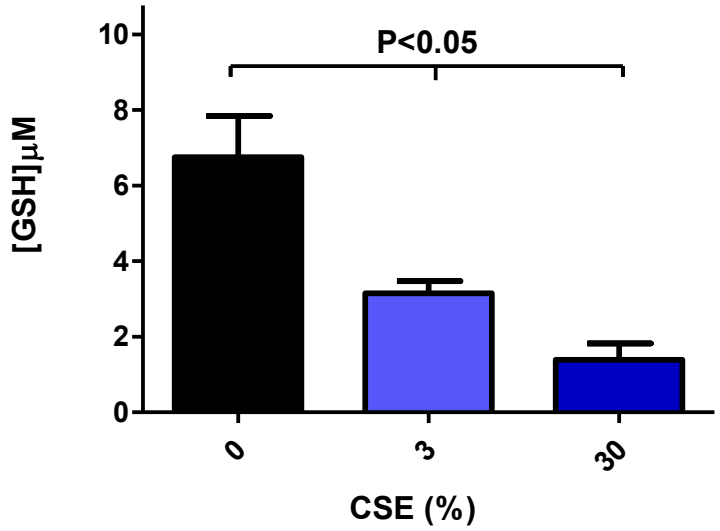


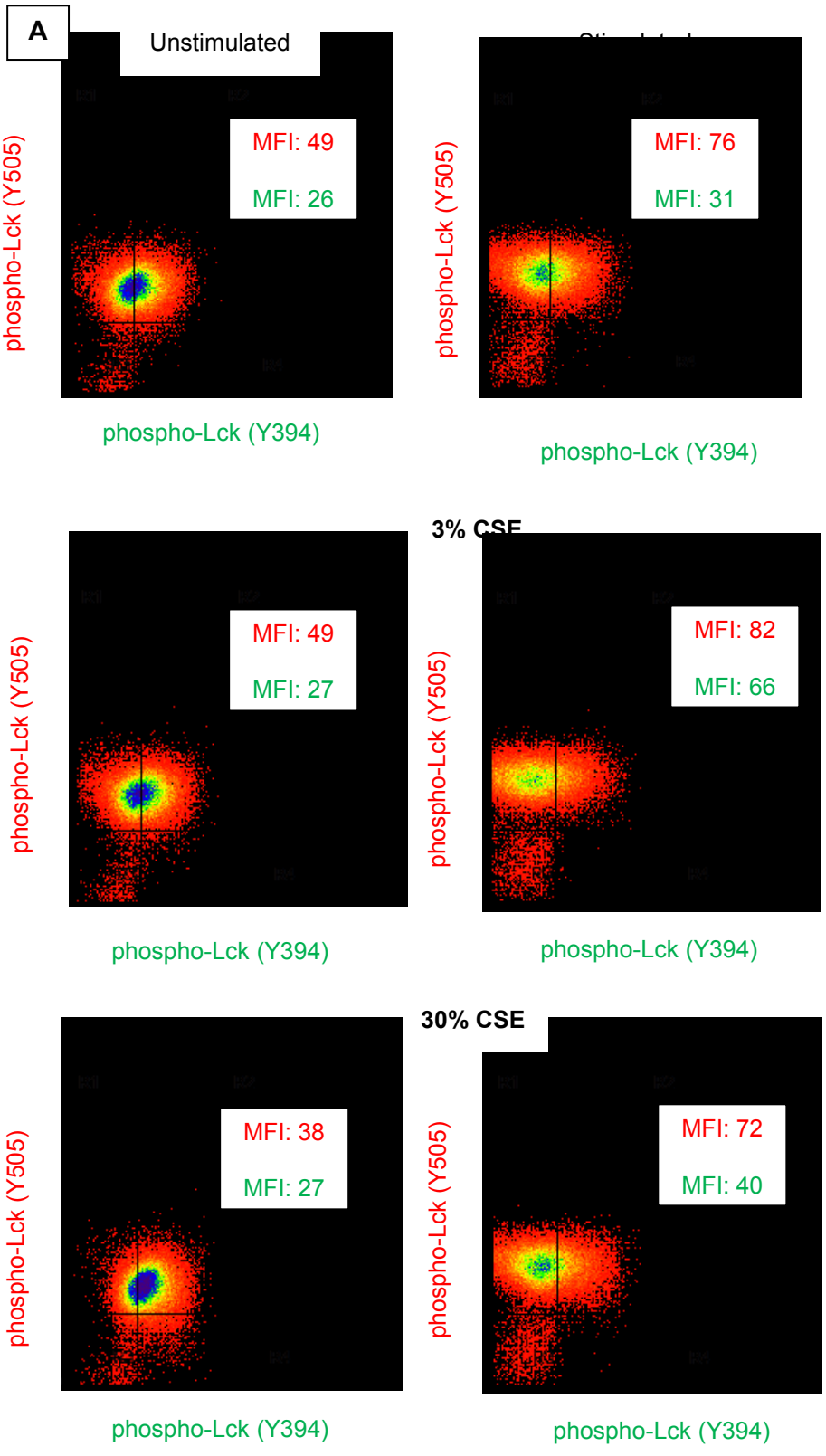
Figure 5.4 Treatment with cigarette smoke extract decreases the reduced glutathione content of T lymphocytes. Jurkat cells were treated with different concentrations of CSE (0%, 3% and 30%) for 24 hours at 37°C. Cells were removed from culture, lysed, and the reduced glutathione content measured. Readings were taken in triplicate and results show the mean (±SEM) of three separate experiments. P<0.05, Mann Whitney non-parametric analysis when comparing untreated cells (0%) to treated cells (3% and 30%).

5.2.5 Cigarette smoke extract alters Lck phosphorylation in T lymphocytes

The Src family kinase Lck is very important in early signal transduction through the TCR and its kinase activities are tightly regulated by phosphorylation processes. CD45 is known to dephosphorylate the inhibitory tyrosine residue (Y505) and the activating tyrosine residue (Y394) of Lck implicating a positive and negative regulatory role for this PTP (McNeill et al. 2007). In contrast Lyp has only been shown to dephosphorylate Y394, which confers only a negative regulatory role for Lyp (Wu et al. 2006). Based on this experiments were conducted to determine if CSE treatment changed the phosphorylation status of Lck kinase, which could be mediated by its effects on PTP activity. Jurkat T cells were treated with CSE and then stimulated using anti-CD3 and goat anti-mouse to cross link TCRs. Cells were fixed to maintain their phosphorylation status, stained with antibodies against the two phosphorylated tyrosine residues of Lck (Y394 and Y505) and then analysed by flow cytometry.

Treatment with CSE had no effect on the phosphorylation of Lck in unstimulated Jurkat T cells (Figure 5.5 A-C). This was true of both the activating residue Y394 (0% CSE=MFI 26±5, 3% CSE=MFI 27±8 and 30% CSE=MFI 27±6) and the inhibitory residue Y505 (0% CSE=MFI 49±7, 3% CSE=MFI 49±9 and 30% CSE=MFI 38±8). When cells were stimulated using anti-CD3 and goat anti-mouse to cross link receptors, there was some increase in Y505 phosphorylation when compared to unstimulated cells and there was not much change with CSE (0% CSE=MFI 25±8, 3% CSE=MFI 23±8, 30% CSE=MFI 32±9, % increase in Y505 phosphorylation on stimulation, Figure 5.5 C). In untreated cells, Y394 phosphorylation did not increase when cells were stimulated (MFI 2±2, % increase in Y394 phosphorylation on stimulation, Figure 5.5 B) however, a moderate increase in Y394 phosphorylation was observed when cells treated with CSE were subject to stimulation (3% CSE=MFI 39±8, 30% CSE=MFI 30±14, % increase in Y394 phosphorylation on stimulation, Figure 5.5 B).

A **0% CSE**


A
Unstimulated
MFI: 49
MFI: 26
phospho-Lck (Y505)
phospho-Lck (Y394)
MFI: 76
MFI: 31
phospho-Lck (Y505)
phospho-Lck (Y394)
3% CSE
MFI: 49
MFI: 27
phospho-Lck (Y505)
phospho-Lck (Y394)
MFI: 82
MFI: 66
phospho-Lck (Y505)
phospho-Lck (Y394)
30% CSE
MFI: 38
MFI: 27
phospho-Lck (Y505)
phospho-Lck (Y394)
MFI: 72
MFI: 40
phospho-Lck (Y505)
phospho-Lck (Y394)

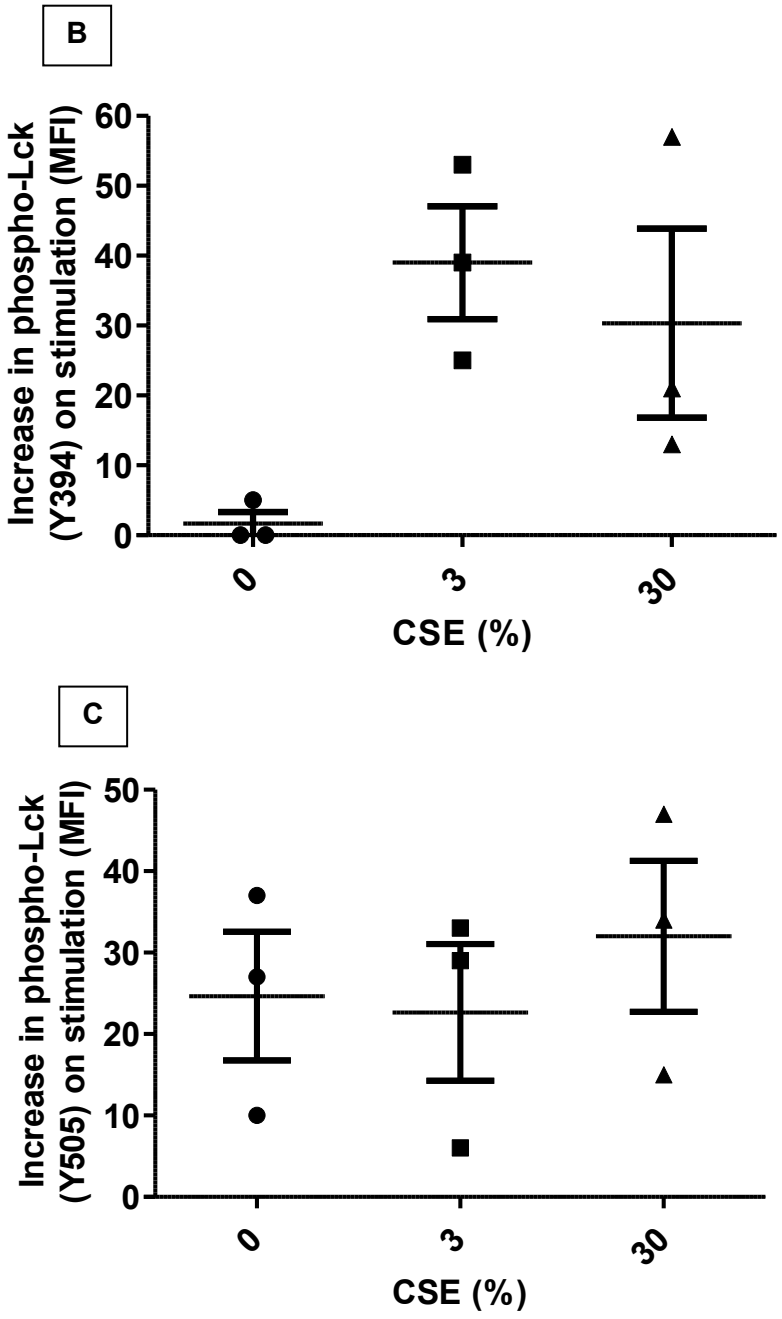
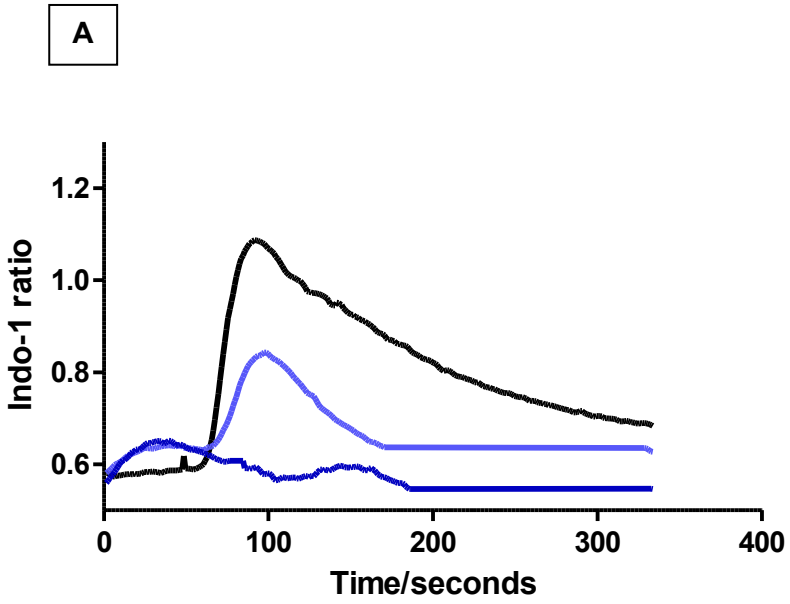


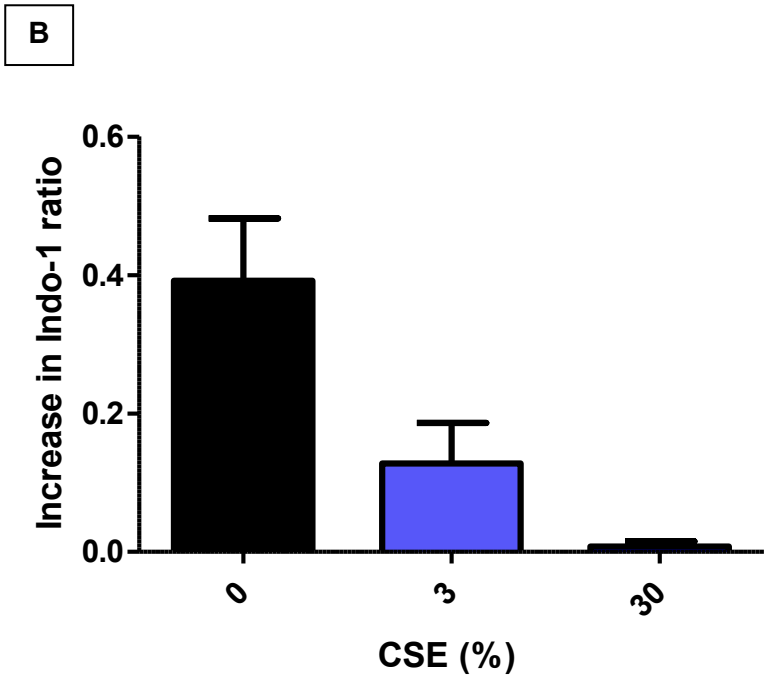
Figure 5.5 Cigarette smoke extract alters Lck phosphorylation in T lymphocytes. Jurkat cells were treated with different concentrations of CSE (0%, 3% and 30%) for 24 hours at 37°C. Cells were removed from culture and stimulated for 1 minute with 3µg/ml anti-CD3 and 4µg goat anti-mouse to cross link receptors. Cells were fixed and permeabalised to allow for intracellular staining. Cells were stained with antibodies to the phosphorylated residues of Lck: Lck (Y505) PE and Lck (Y394) FITC and analysed by flow cytometry. **(A)** Representative flow cytometry plot showing phosphorylation of Lck (Y394) and Lck (Y505) by measurement of mean fluorescence intensity in unstimulated and stimulated Jurkat T cells. **(B)** Increase in phosphorylation of Lck (Y394) in Jurkat T cells treated with CSE following stimulation **(C)** Increase in phosphorylation of Lck (Y505) in Jurkat T cells treated with CSE following stimulation. Results show the mean (±SEM) of three separate experiments.

5.2.6 Cigarette smoke extract decreases TCR-dependent calcium signalling in T lymphocytes

CSE was shown to have effects on specific proteins in T cells including changes in the activation of both PTPs (CD45 and Lyp) and PTKs (Lck), which could be mediated by the ability of CSE to decrease the reduced GSH content of T cells (Figures 5.3-5.5). In order to determine if these changes in protein activity had any effect on overall signalling through the TCR, experiments were performed to measure Ca^{2+} signalling in Jurkat T cells stimulated with anti-CD3, thapsigargin and ionomycin. Anti-CD3 stimulates T cells via binding to the TCR and inducing Ca^{2+} release. Thapsigargin induces Ca^{2+} release independent of the TCR and would provide evidence as to whether CSE affects TCR-dependent Ca^{2+} signalling only. If the response to thapsigargin is preserved this indicates that CSE has effects on part of the TCR signalling pathway, such as the PTPs and PTKs. Additionally, ionomycin was used as a positive control to determine if the Jurkat T cells were correctly loaded with the Ca^{2+} sensitive dye Indo-1 AM.

Treatment of Jurkat T cells with CSE decreased the Ca^{2+} signal in response to anti-CD3 (Figure 5.6 A+B), with untreated cells displaying an increase in Indo-1 ratio of 0.4±0.09 and cells treated with 30% CSE displaying an increase in Indo-1 ratio of 0.01±0.01. Responses to thapsigargin and ionomycin were preserved in untreated cells and cells treated with CSE (Figure 5.6 C+D). This indicates that only TCR-dependent Ca^{2+} signalling is affected by treatment with CSE.


A
1.2
1.0
0.8
0.6
Indo-1 ratio
0
100
200
300
400
Time/seconds


B
0.6
0.4
0.2
0.0
Increase in Indo-1 ratio
0
3
30
CSE (%)

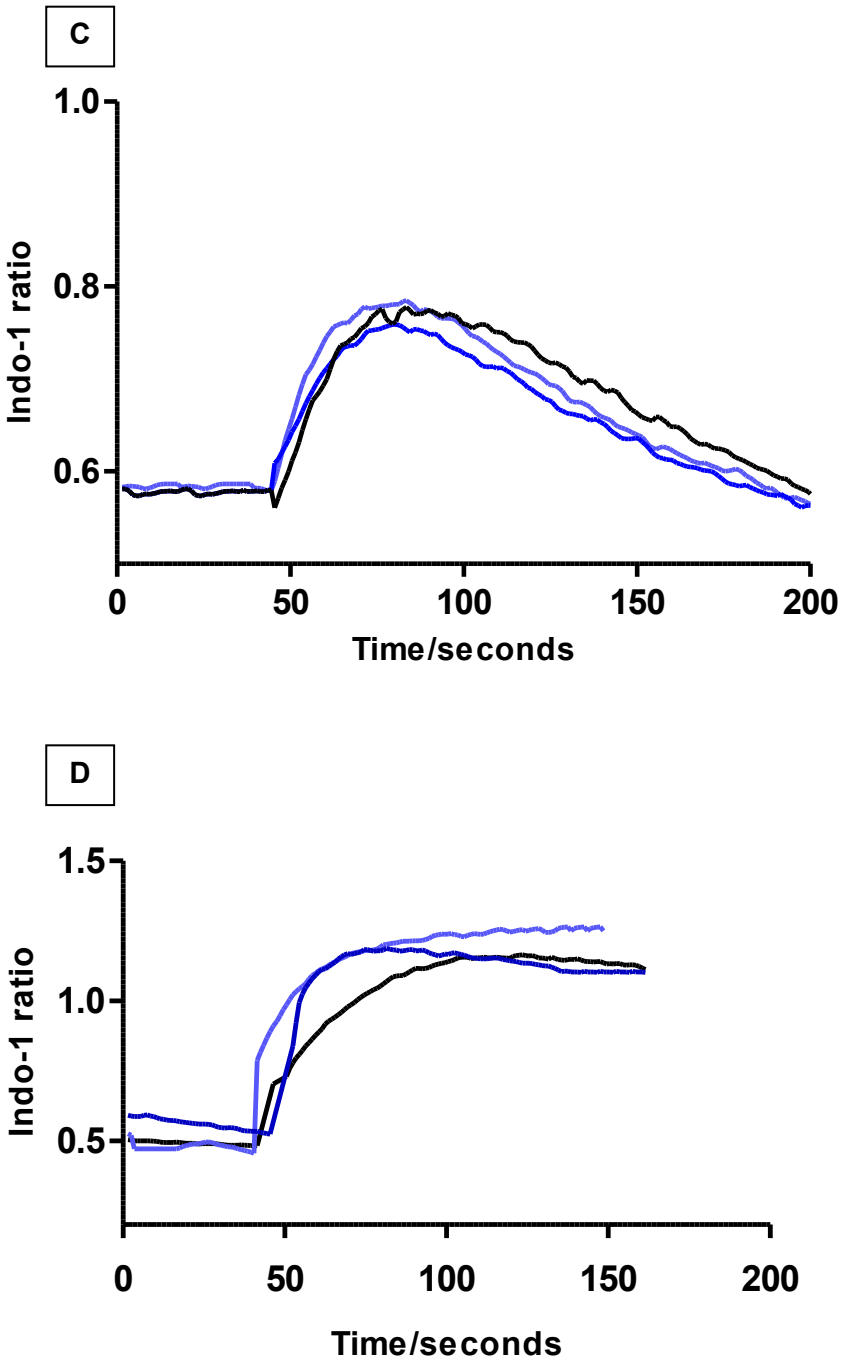


Figure 5.6 Treatment with cigarette smoke extract decreases T cell receptor dependent calcium signalling in T lymphocytes. Jurkat cells were treated with different concentrations of CSE (0%=black, 3%=light blue and 30%=dark blue) for 24 hours at 37°C. Cells were washed and loaded with the Indo-1 AM ester. Cells were washed again in HBSS and transferred to an acrylic cuvette. After a baseline was established, anti-CD3 (5µg/ml), Thapsigargin (50nM) or ionomycin (1µM) was added. **(A)** One representative measure of three experiments performed using anti-CD3 **(B)** Mean peak rise in Indo-1 AM ratio after stimulation with OKT3 from three experiments. **(C)** Mean peak rise in Indo-1 AM ratio after stimulation with thapsigargin from three experiments. **(D)** Mean peak rise in Indo-1 AM ratio after stimulation with ionomycin from three experiments.

5.2.7 Catalase treatment and filtration of cigarette smoke extract decreases efficacy

PTP activity was shown to be decreased when Jurkat T cells were treated with CSE (Figure 5.3), however the mechanism behind this effect was unknown. It was hypothesised that this effect of CSE on PTP activity could be due to the ability of CSE to deplete GSH which was also observed during further investigations (Figure 5.4). Despite this observation, other possible mechanisms of action of CSE need to be explored. To do this it was chosen to look at the effect of fractionation of the extract and also using the enzyme catalase to remove any H_2O_2 present in the extract.

The effect of removal of high molecular weight compounds and particulates was carried out to determine the role of these in the efficacy of CSE. The aqueous CSE used in these experiments contained both water soluble components and particulate matter (PM), both of which are thought to be important in the efficacy of CSE (Edmiston et al. 2009, Lemaitre et al. 2011). The extract was passed through a filter to remove particulates and chemicals >3000 in molecular weight, cells were then treated with particulate-free solution and their global PTP activity measured. Removing PM reduced the ability of the aqueous CSE to decrease global PTP activity in Jurkat T cells (Figure 5.7 A). When cells were treated with 30% CSE global PTP activity was only 65±5.8% that of untreated cells. After treating with filtered 30% CSE global PTP activity was 81±4.7% that of untreated cells, indicating that PM has a predominant role in the efficacy of CSE but the soluble components also have some affect.

Catalase is an enzyme responsible for catalysing the decomposition of H_2O_2 to oxygen and water and protects cells from oxidative damage. If the decrease in PTP activity induced by CSE treatment was due to the presence of H_2O_2, combined treatment with catalase should decrease the efficacy of CSE treatment. Jurkat T cells were treated with CSE and two types of catalase, (1) non-pegylated catalase, which had a short half-life and cannot adequately cross the plasma membrane and (2) pegylated catalase, which has a long half-life and can

easily cross the cell plasma membrane (Beckman et al. 1988). These two types of catalase were chosen to determine if ROS are already present in the CSE or if CSE treatment induces ROS production by T cells. Both types of catalase significantly decreased the ability of CSE to reduce PTP activity (Figure 5.7 B). Without catalase CSE caused a statistically significant reduction in PTP activity, but addition of catalase almost completely prevented this. When T cells were treated with 3% CSE, PTP activity was 68.6±4% that of untreated T cells whereas when catalase was introduced the PTP activity remained high at 87.3±6.7% that of untreated T cells.

The fact that removal of PM and use of catalase decrease the efficacy of CSE suggests that its effects may occur via multiple mechanisms and large molecular weight components of the extract could be important in inducing the production of ROS by T cells treated with CSE.

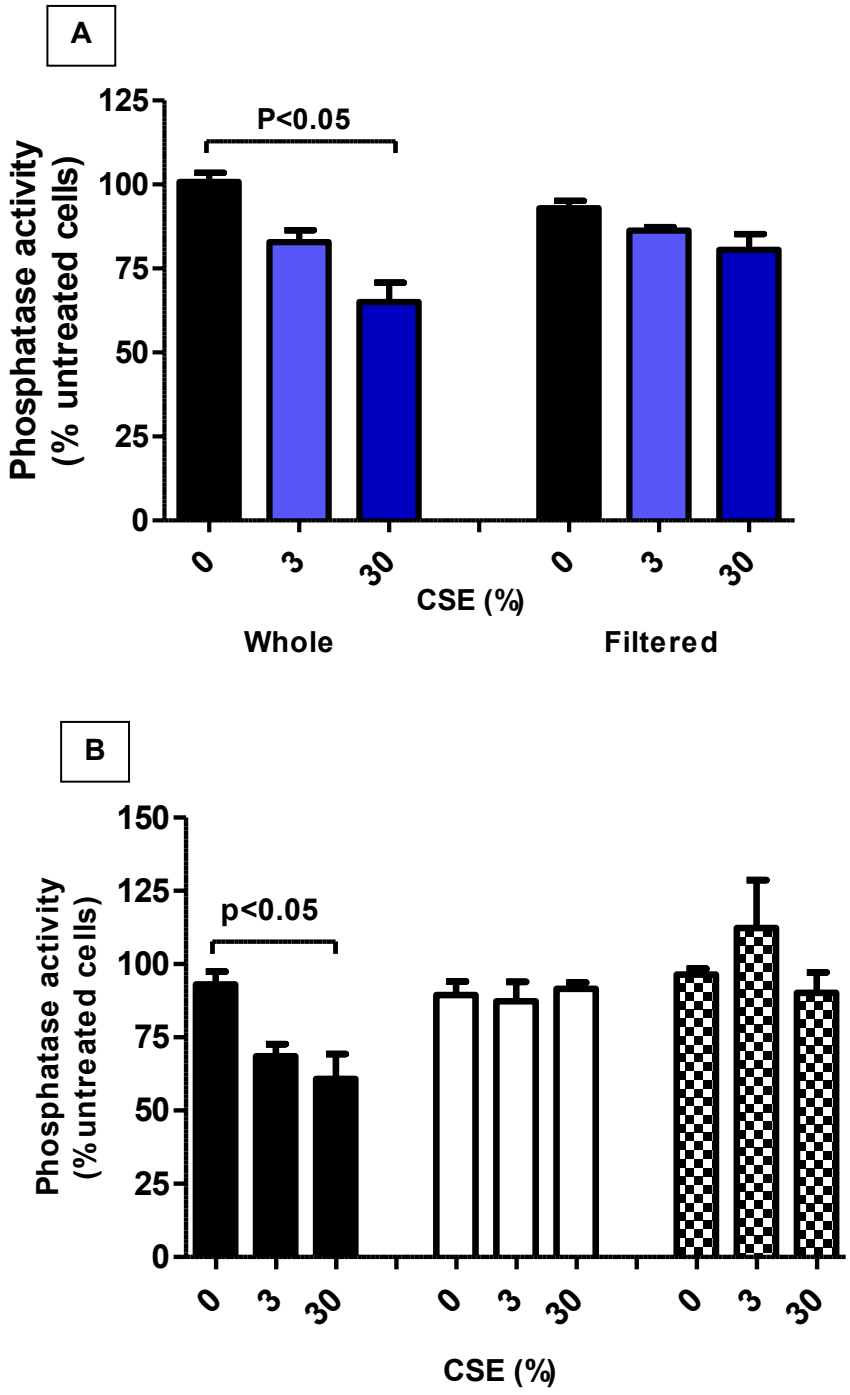


Figure 5.7 Catalase and filtration of cigarette smoke extract reduces its ability to decrease the activity of protein tyrosine phosphatases in T lymphocytes. To remove particulate matter, cigarette smoke extract (CSE) was filtered using a >3000mw filter. Jurkat T cells were treated with CSE for 24 hours at 37°C. To determine the effect of catalase and pegylated catalase, 1μM was added at the time of CSE treatment. After treatment, cells were removed from culture and their global protein tyrosine phosphatase assessed. **(A)** The effect of filtration on the efficacy of CSE. **(B)** The effect of catalase on the efficacy of CSE. Data show the global protein tyrosine phosphatase activity in untreated cells (black bars), cells treated with non-pegylated catalase (white bars), and cells treated with pegylated catalase (checked bars). Readings were taken in triplicate and results show the mean (±SEM) of three separate experiments. P<0.05, Mann Whitney non-parametric analysis when comparing untreated cells (0%) to treated cells (3% and 30%).

5.2.8 Cigarette smoke extract induces reversible oxidation of free SH groups

Oxidation of the PTP active site cysteine residue is an important mechanism by which PTP activity is regulated and studies have shown that the CD45 and Lyp phosphatase are both susceptible to regulation via this method (Rider et al. 2003, Holmes 2006, Bayley et al. 2013). Oxidation to cysteine sulfenic acid is reversible, however if oxidation beyond this occurs it is thought to result in irreversible inactivation of the PTP active site (Takakura et al. 1999). The occurrence of irreversible oxidation has been implicated in a number of disease pathologies and can be somewhat prevented if adequate concentrations of the antioxidant GSH are present (Jaswal et al. 2003). In addition, a decrease in GSH levels can also be observed in healthy smokers (Alberg 2002) illustrating that smoking can modify GSH levels. Given that treatment of T cells with CSE decreases the activity of the CD45 and Lyp phosphatase and depletes the antioxidant GSH, it was hypothesised that components of the CSE could directly oxidise PTPs in a similar manner to H_2O_2. This hypothesis was tested by treating Jurkat T cells with CSE and then subjecting the cells to an assay procedure which labelled reversibly oxidised cysteine residues or 'SH groups' of proteins.

Treatment with CSE increased the level of reversible oxidation of free SH groups (Figure 5.8). This can be seen by the increased intensity of protein bands observed on the membrane when Jurkat T cells were treated with 3% and 30% CSE. Most prominent bands were seen between 36kDa and 64kDa and less prominent bands were also observed at around 98kDa. These results suggest that indeed treatment with CSE could induce oxidation of PTPs in Jurkat T cells.

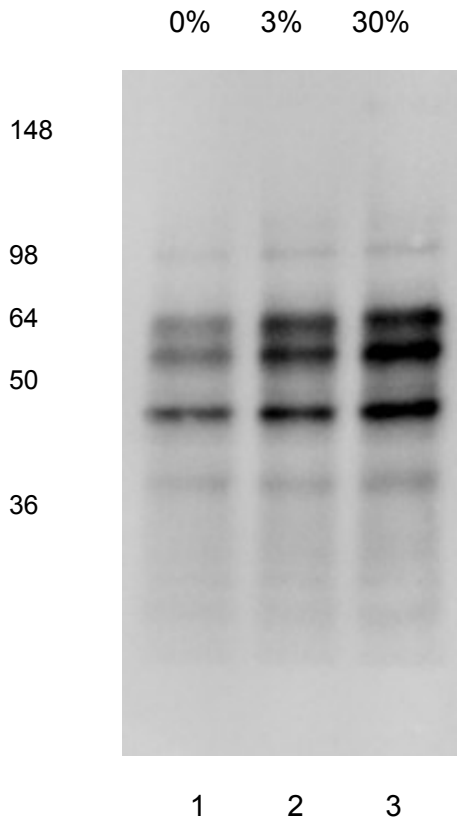


Figure 5.8 Treatment with cigarette smoke extract increases reversible oxidation of free SH groups in T lymphocytes. Jurkat T cells were treated with CSE for 24 hours at 37°C. Cells were removed from culture and subject to a cysteinyl labelling procedure. Biotinylated IAP probes were used to identify reversibly oxidised free SH groups which were then purified using streptavidin-sepharose beads. The beads were run on a 10% SDS-PAGE gel and to detect overall reversible oxidation of free SH groups the membrane was probed with a streptavidin-HRP linked antibody. The image shown is one representative of the three separate experiments performed.

5.2.9 Cigarette smoke extract has no effect on T lymphocyte proliferation

A number of studies have investigated the effects of CSE exposure and smoking on T cell proliferation, with some finding a reduction in proliferation (Glader et al. 2006, Mortaz et al. 2009) and others observing no difference (Zavitz et al. 2008). Due to these contradictions in current literature, the ability of T cells to proliferate in the CSE preparations used in these experiments was assessed. This was carried out by loading naïve and total CD4+ T cells with CFSE and treating them with CSE for 24 hours. Following CSE treatment, T cells were stimulated with anti-CD3 and anti-CD28 and their proliferation was assessed at 2 and 5 days after stimulation.

Treatment with CSE had no effect on the proliferation of naïve and whole CD4+ T cells (Figure 5.2.9). There was little proliferation of naïve T cells detected 2 days after stimulation (Figure 5.2.9 A) regardless of treatment with CSE. It was observed that 5 days after stimulation naïve T cells had replicated an average of 4 times (Figure 5.2.9 B) which was consistent in untreated cells and cells treated with CSE. Populations of whole CD4+ T cells proliferated more rapidly regardless of CSE treatment (Figure 5.9 C). After 5 days total CD4+ T cells had divided an average of 4 times (Figure 5.9 D) which again was unaffected by treatment with CSE. When the proliferation index was calculated (Figure 5.9 E+F) this also showed that there was no effect of CSE treatment on T cell proliferation. After 5 days of stimulation the proliferation index was similar in whole CD4+ T cells regardless of CSE treatment (0% CSE=3.25±0.08, 3% CSE=2.99±0.56, 15% CSE=3.29±0.32 and 30% CSE =3.36±0.49). These results confirm that the concentrations of CSE used in these experiments have no effect on the ability of naïve and total CD4+ T cells to proliferate.

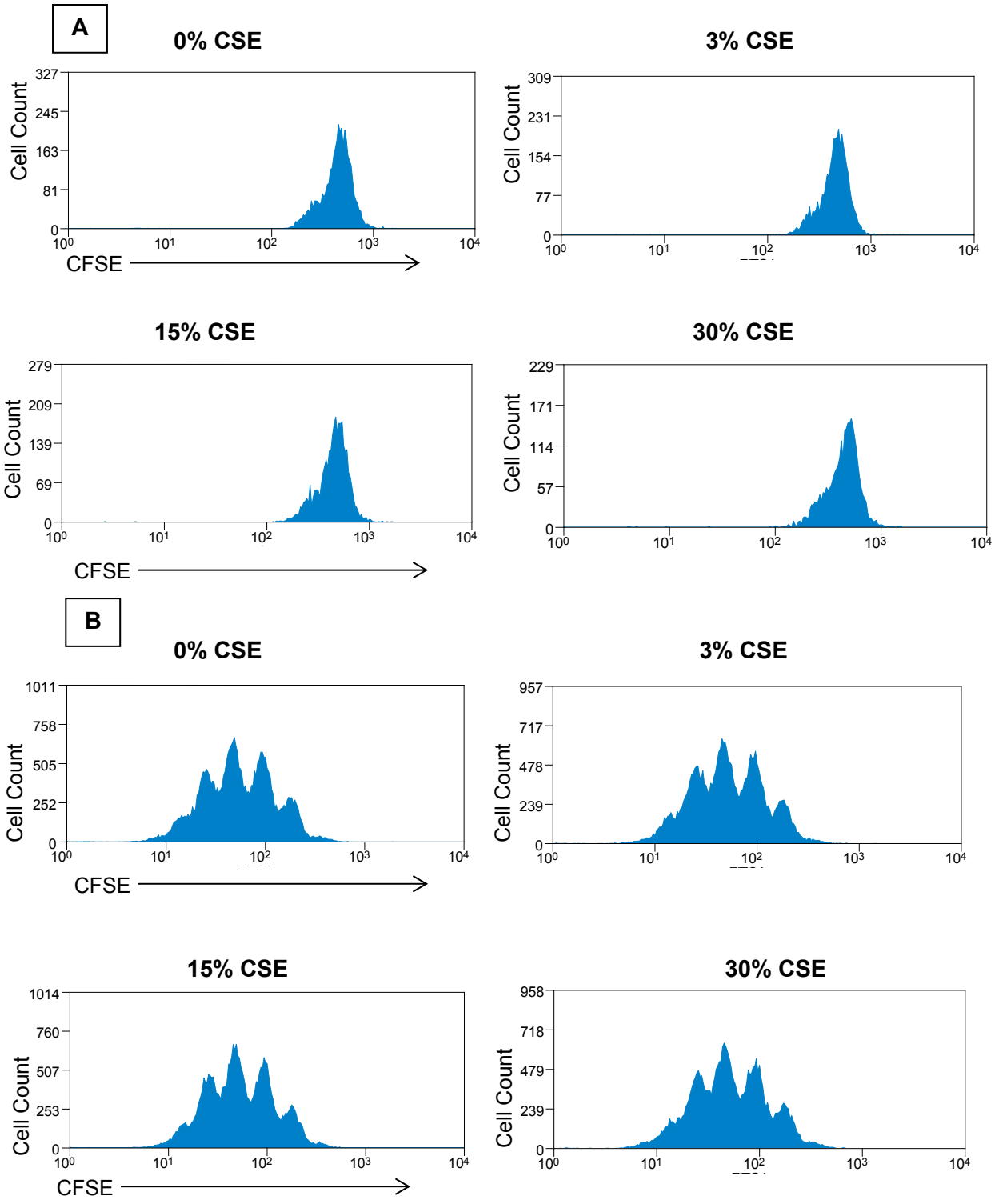


Figure 5.9 A+B Treatment with cigarette smoke extract has no effect on the proliferation of naïve CD4+ T cells. Naïve CD4+ T cells were isolated and loaded with 1μM CFSE. Cells were treated for 24 hours with CSE and then stimulated with plate bound anti-CD3 (3μg/ml) and anti-CD28 (5μg/ml). Cells were removed from culture 2 **(A)** and 5 **(B)** days after stimulation and assessed for proliferation using flow cytometry. Figures show CFSE peaks obtained from one of three experiments performed on Day 2 and one of two experiments performed on Day 5.

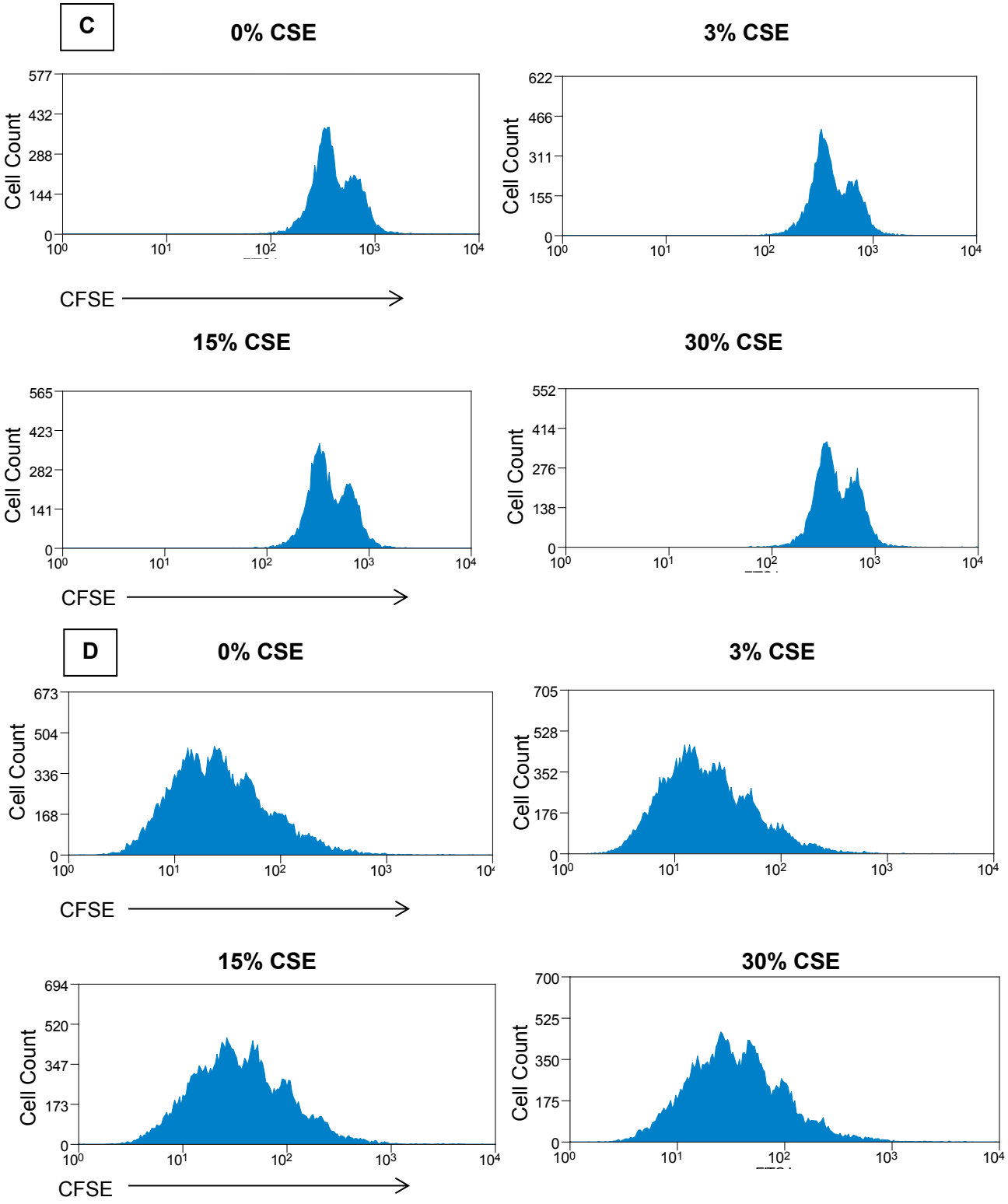


Figure 5.9 C+D Treatment with cigarette smoke extract has no effect on the proliferation of whole CD4+ T cells. Whole CD4+ T cells were isolated and loaded with 1μM CFSE. Cells were treated for 24 hours with CSE and then stimulated with plate bound anti-CD3 (3μg/ml) and anti-CD28 (5μg/ml). Cells were removed from culture 2 **(C)** and 5 **(D)** days after stimulation and assessed for proliferation using flow cytometry. Figures show CFSE peaks obtained from one of eight experiments performed on Day 2 and one of three experiments performed on Day 5.

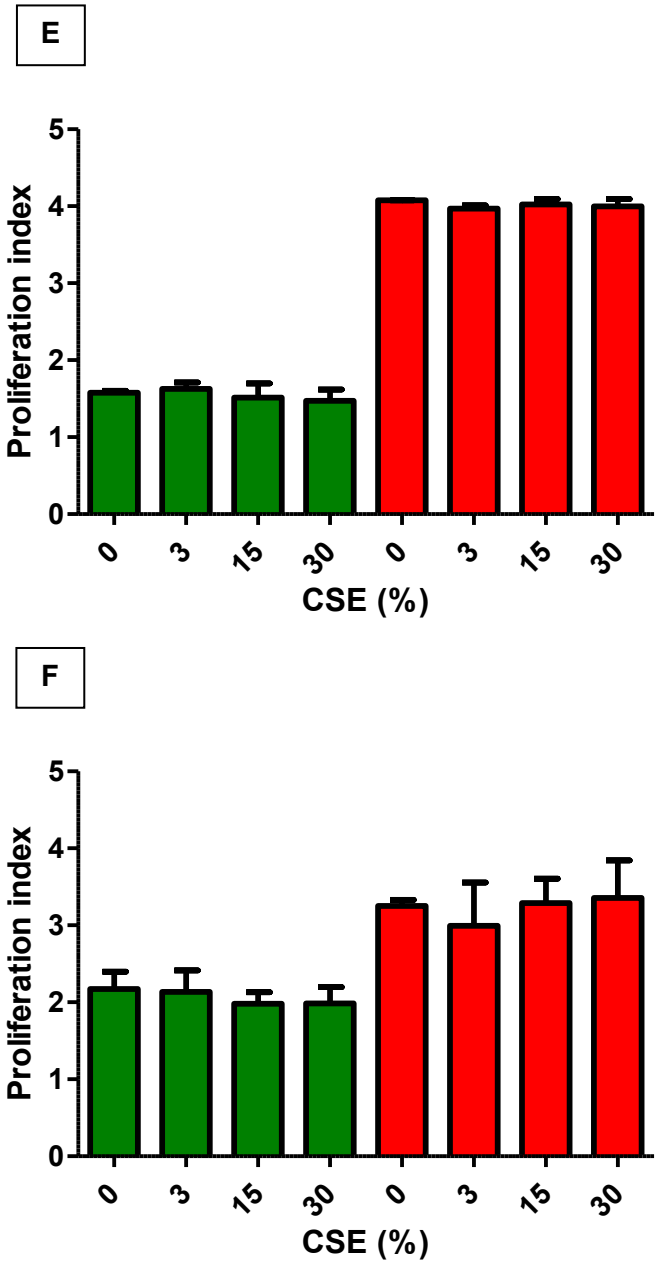


Figure 5.9 E+F Treatment with cigarette smoke extract has no effect on the proliferation index of naïve and whole CD4+ T cells. Naïve and whole CD4+ T cells were isolated and loaded with 1μM CFSE. Cells were treated for 24 hours with CSE and then stimulated with plate bound anti-CD3 (3μg/ml) and anti-CD28 (5μg/ml). Cells were removed from culture 2 (green bars) and 5 (red bars) days after stimulation and assessed for proliferation using flow cytometry. Graphs show the average proliferation index obtained for naïve CD4+ T cells **(E)** and whole CD4+ T cells **(F)**. Figure **(E)** shows the average proliferation index obtained from one of three experiments performed on Day 2 and one of two experiments performed on Day 5 with naïve CD4+ T cells. Figure **(F)** shows the average proliferation index obtained from one of eight experiments performed on Day 2 and one of three experiments performed on Day 5.

5.2.10 Cigarette smoke extract alters cytokine production by T lymphocytes

Previous work has shown that treating CD4+ T cells in an *in vitro* co-culture with DCs and CSE reduces their production of IL-2, IL-1β, IFN-γ, and TNF-α but increases their output of the Th2 cytokine IL-4 (Ouyang et al. 2000, Vassallo et al. 2005). Another study observed that when PBMC were exposed to CSE in the vapour phase they showed decreased production of IL-2, GMCSF, IL-1β, IL-6, IFN-γ, IL-8 and TNF-α (Lambert et al. 2005). Similar findings have been reported *in vivo* using a mouse model of cigarette smoke exposure (Robbins et al. 2008). However, there is a study which reported there to be no difference in the production of IFN-γ by T cells taken from smoke-exposed mice and PBMC taken from human smokers (Zavitz et al. 2008). Given these opposing observations the production of cytokines by CD4+ T cells treated with the CSE preparation was assessed. T cells were stimulated using plate-bound anti-CD3 and anti-CD28 and assessment of cytokine production was done after 48 hours of culture. Supernatant samples were taken at this time and production of IL-4, IL-10, IL17, TNF-α and IFN-γ were measured by ELISA. As well as this, T cells were treated with brefeldin A for 3 hours to retain any cytokines produced inside the cells. T cells were then stained with fluorescent conjugated antibodies to IL-4, IL-10, IL-17 and IFN-γ to assess short term cytokine production.

Using intracellular cytokine staining it was found that treatment of CD4+ T cells with CSE decreased the percentage of IFN-γ and IL-4 secreting cells, whereas the percentage of IL-10 and IL-17 secreting cells were unaffected (Figure 5.10 A-F). Treatment with 30% CSE decreased the percentage of IFN-γ producing T cells to 25±6%, compared to 34±8% in untreated cells. The percentage of IL-4 secreting cells was similarly affected, with 0.3±0.08% of untreated cells secreting IL-4 compared with 0.08±0.02% of cells treated with 30% CSE.

The effect of CSE on the cytokines secreted was also determined by ELISA. CSE decreased the amounts of TNF-α, IL-4 and IL-17 secreted by T cells (Figure 5.10 G, I and K). IL-17 was

not affected until a concentration of 30% CSE was used, however IL-4 and TNF-α secretion were decreased even with treatment using 3% CSE. Treatment with 3% CSE decreased the secretion of TNF-α to 207±17pg/ml compared to 232±17pg/ml in untreated cells. There were no significant differences in the secretion of IL-10 and IFN-γ when T cells were treated with CSE, however some trends were observed (Figure 5.10 H and J). When cells were treated with 15% CSE there was an increase in IFN-γ and IL-10 production which was not seen at other concentrations of CSE (0% CSE: IFN-γ=315±47pg/ml, IL-10=58±14pg/ml and 15% CSE: IFN-γ=393±46pg/ml and IL-10=76±17pg/ml).

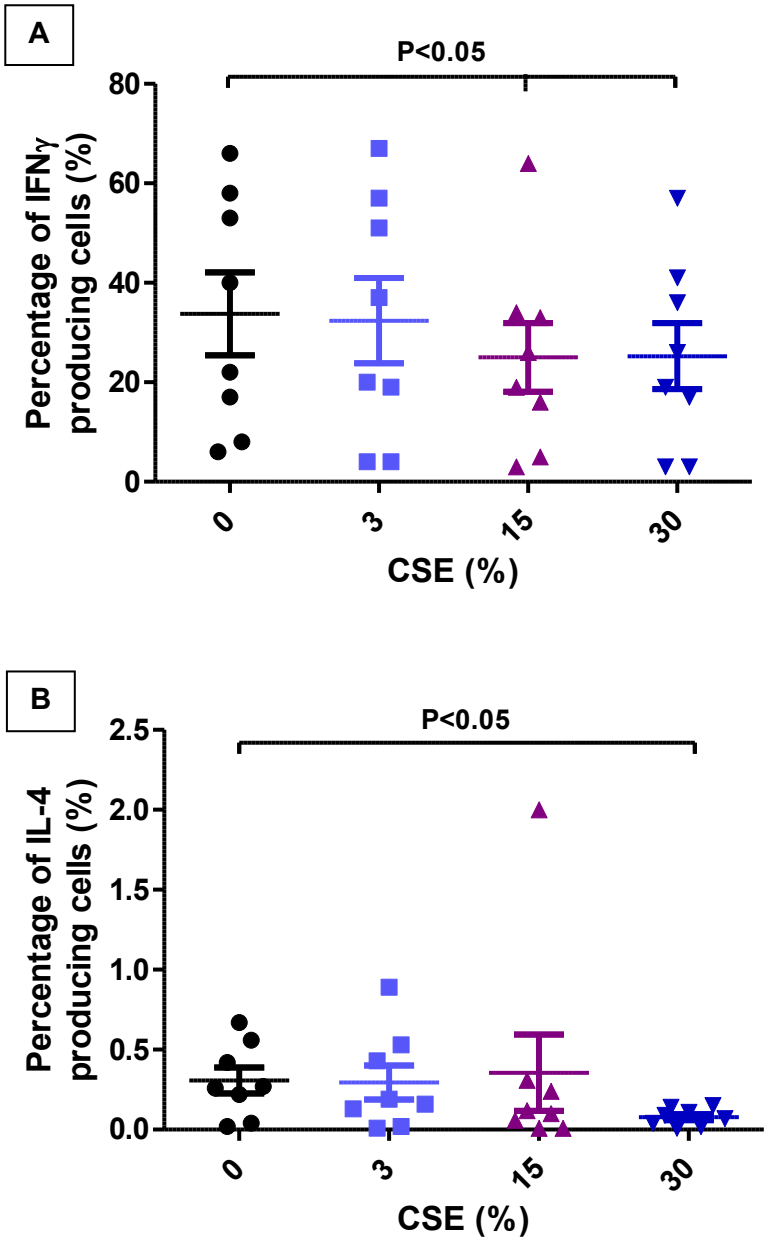


Figure 5.10 A+B Cigarette smoke extract decreases IFNγ and IL-4 production by T lymphocytes. CD4+ T cells were isolated and left overnight in culture at 37°C with different concentrations of cigarette smoke extract (CSE) (0%, 3% and 30%). Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IFN-γ and IL-4 and assessed by flow cytometry. Effects of CSE treatment on the percentage of CD4+ T cells secreting **(A)** IFN-γ and **(B)** IL-4. Results show the mean (±SEM) of eight separate experiments. P<0.05, Paired t-test compared with untreated (0% CSE) cells.

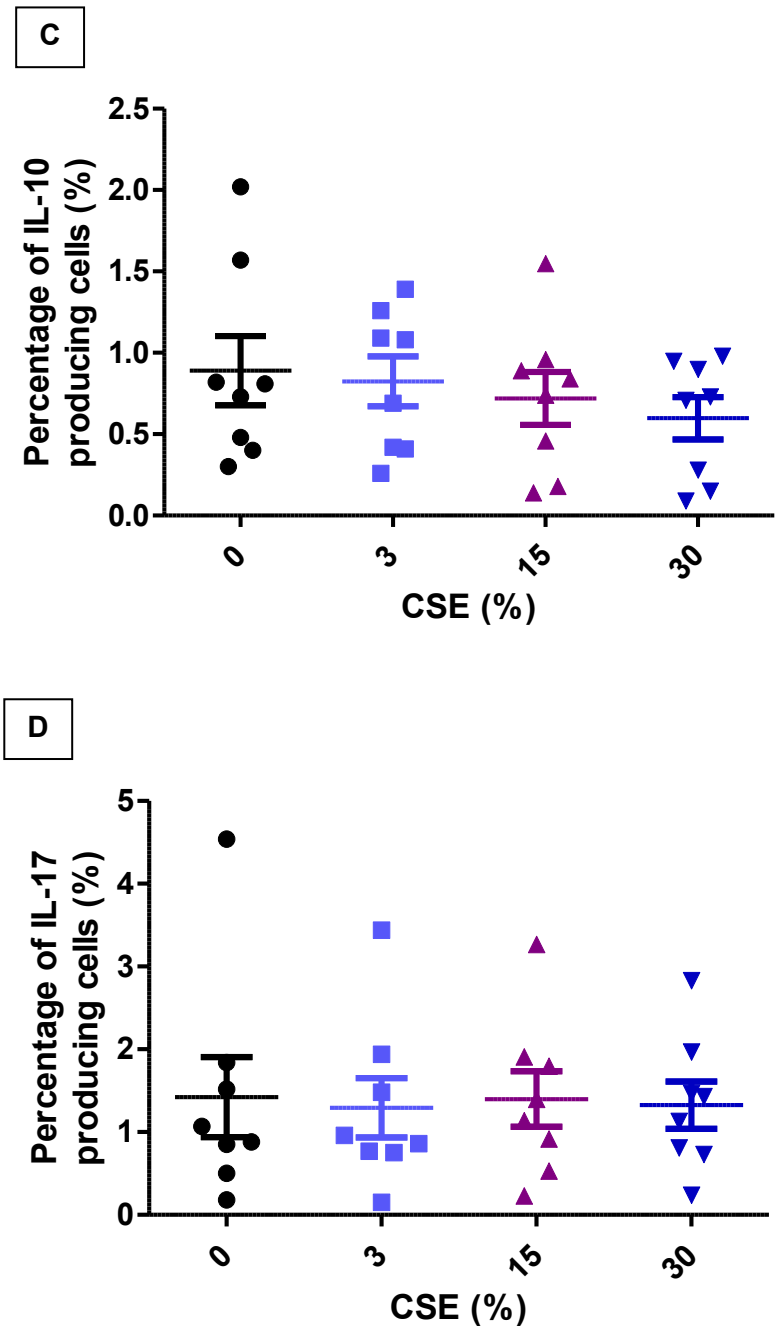


Figure 5.10 C+D Cigarette smoke extract has no effect on IL-10 and IL-17 production by T lymphocytes. CD4+ T cells were isolated and left overnight in culture at 37°C with different concentrations of cigarette smoke extract (CSE) (0%, 3% and 30%). Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IL-10 and IL-17 and assessed by flow cytometry. Effects of CSE treatment on the percentage of CD4+ T cells secreting **(C)** IL-10 and **(D)** IL-17. Results show the mean (±SEM) of eight separate experiments.

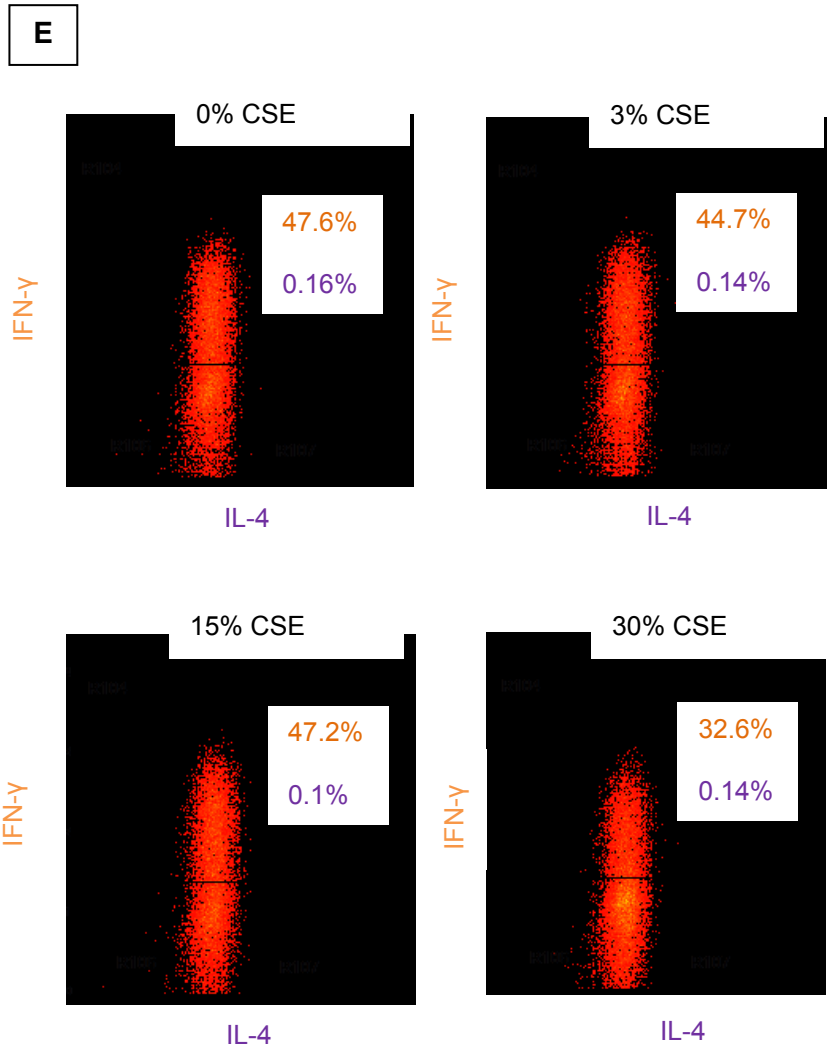


Figure 5.10 E Cigarette smoke extract alters cytokine production by T lymphocytes. CD4+ T cells were isolated and left overnight in culture at 37°C with different concentrations of cigarette smoke extract (CSE) (0%, 3% and 30%). Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were treated with 1mg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IFN-γ and IL-4 and assessed by flow cytometry. Figure shows a representative flow cytometry plot from one of eight experiments performed.

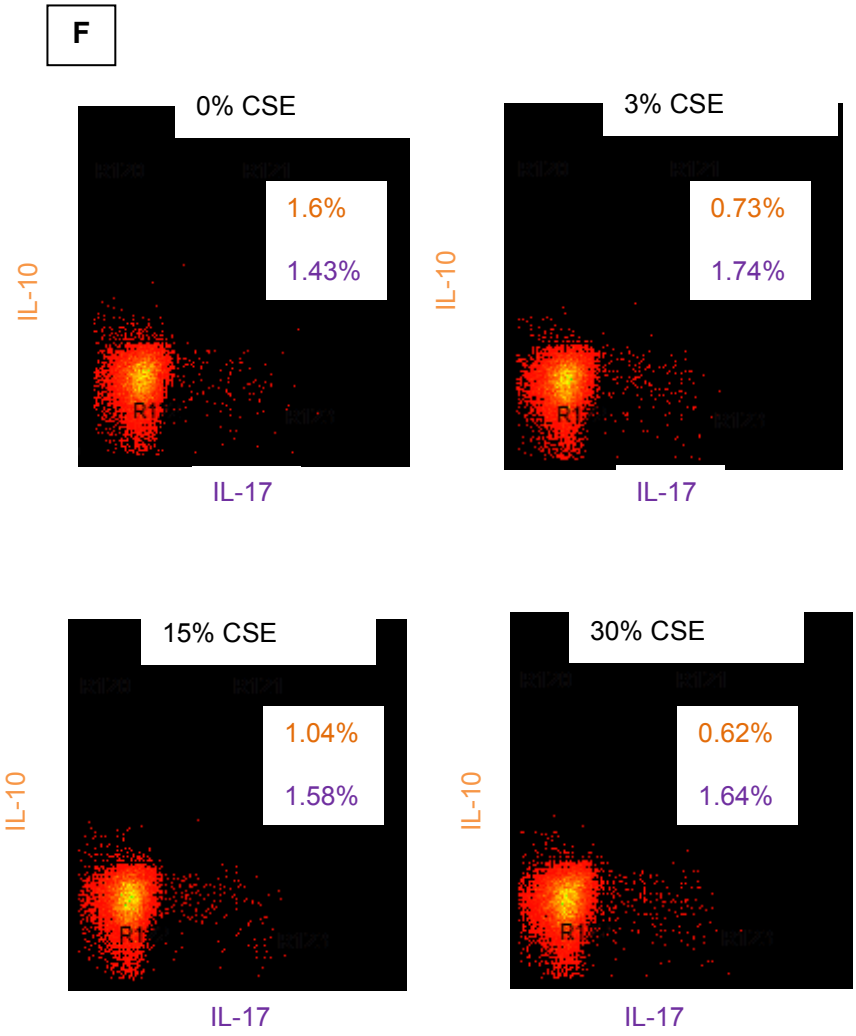
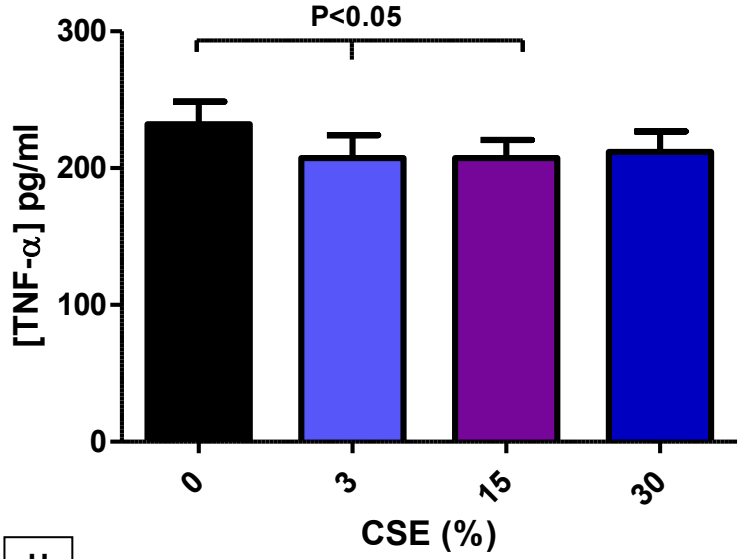
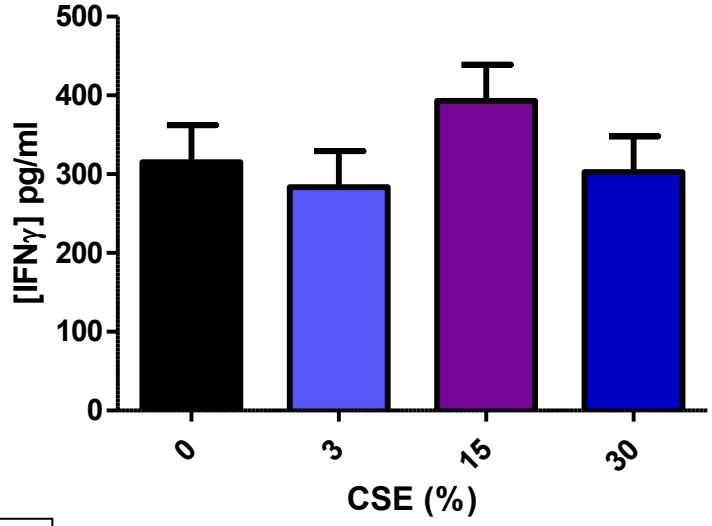


Figure 5.10 F Cigarette smoke extract alters cytokine production by T lymphocytes. CD4+ T cells were isolated and left overnight in culture at 37°C with different concentrations of cigarette smoke extract (CSE) (0%, 3% and 30%). Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Cells were treated with 1mg/ml brefeldin A for 3 hours and then removed from culture. Cells were treated with 100µg/ml brefeldin A for 3 hours and then removed from culture. Cells were stained with fluorescent conjugated antibodies to IL-10 and IL-17 and assessed by flow cytometry. Figure shows a representative flow cytometry plot from one of eight experiments performed.

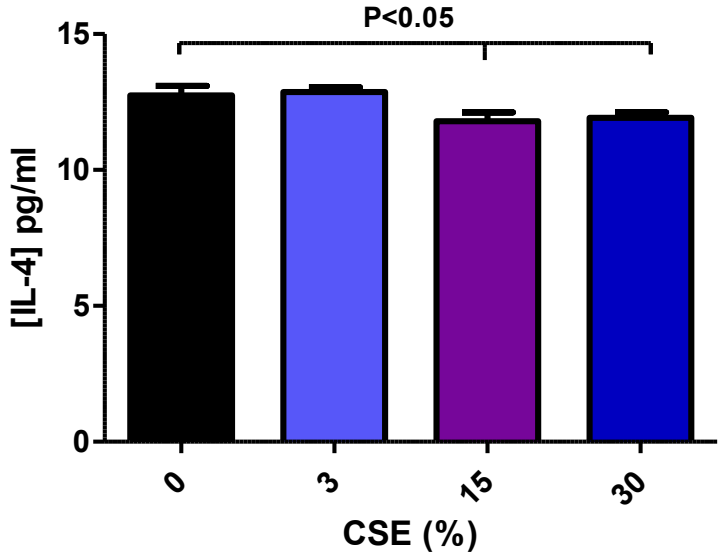
G


P<0.05
300
200
100
0
[TNF-α] pg/ml
0
3
15
30
CSE (%)

H


500
400
300
200
100
0
[IFNγ] pg/ml
0
3
15
30
CSE (%)

I


P<0.05
15
10
5
0
[IL-4] pg/ml
0
3
15
30
CSE (%)

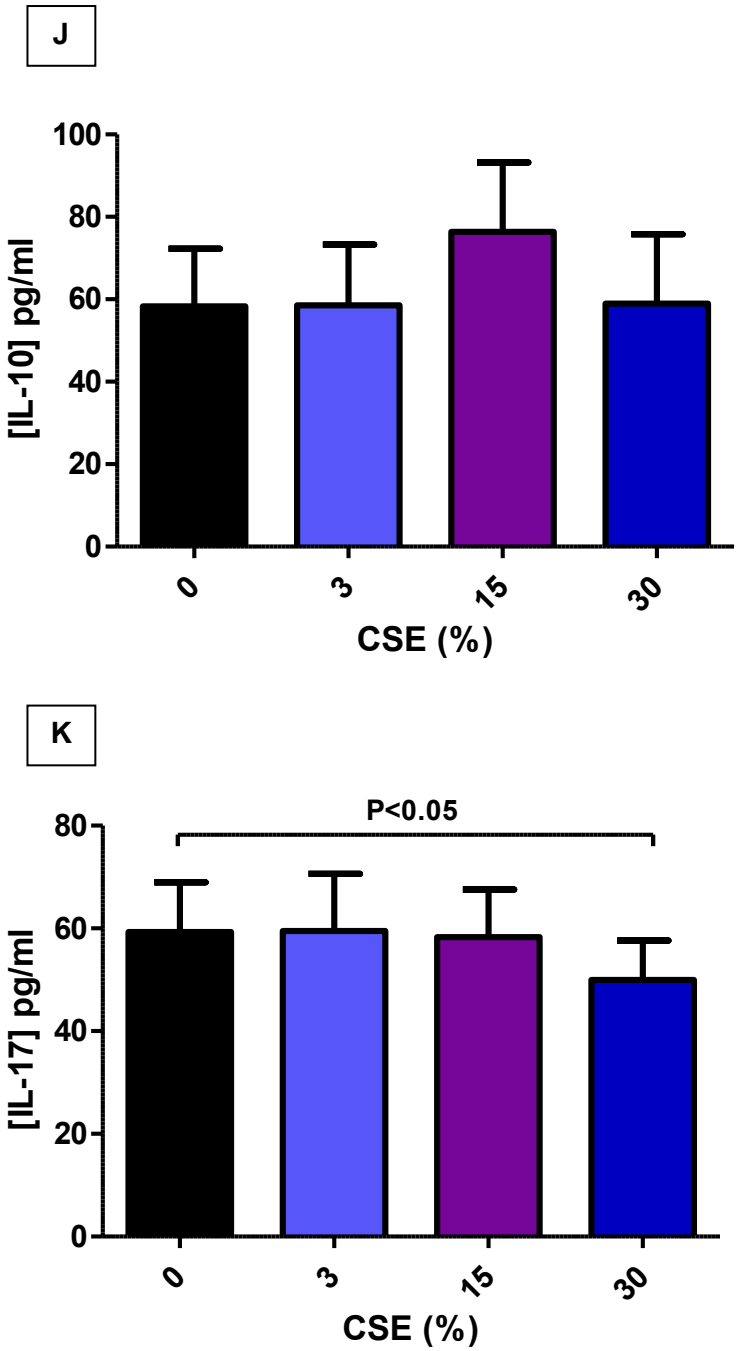


Figure 5.10 G-K Cigarette smoke extract alters cytokine production by T lymphocytes. CD4+ T cells were isolated and left overnight in culture at 37°C with different concentrations of cigarette smoke extract (CSE) (0%, 3% and 30%). Cells were stimulated with plate bound anti-CD3 (3µg/ml) and anti-CD28 (5µg/ml) for 48 hours at 37°C. Supernatant samples were taken 48 hours after stimulation and assessed for the presence of **(G)** TNF-α **(H)** IFN-γ **(I)** IL-4 **(J)** IL-10 and **(K)** IL-17 using ELISA. Figures show the mean (±SEM) of eight separate experiments. P<0.05, Paired t-test compared with untreated (0% CSE) cells.

5.3 Discussion

This chapter investigated the effects of oxidation and CS on T cell signalling using oxidation by H_2O_2 and an *in vitro* model of smoking using CSE exposure. It is well known that cysteine residues found within the active site of PTPs such as Lyp can be oxidised by H_2O_2, however all PTPs display a different susceptibility to oxidation via this method (Meng et al. 2002). These differences could be explained by protective elements of the PTP structure, or by their location and accessibility within the cell. CD45 and Lyp are two of the three main PTPs controlling TCR signalling and given their very different structures and locations it was hypothesised that their susceptibility to oxidation would also be different. This is of importance as oxidation is a key mechanism regulating the activity of PTPs and changes to oxidative regulation can have dramatic effects on TCR signalling (Rider et al. 2003). The oxidation of CD45 and Lyp in T cells was directly assessed using the method described in Chapter 3, and it was found that CD45 was much more susceptible to oxidative regulation when compared to Lyp (Figure 5.1). This could result in an overall reduced signal propagated through the TCR since if a large proportion of the activating effects of CD45 are removed, the negative regulation of signalling by Lyp would predominate. Increased levels of protein oxidation can be seen in RA (Jaswal et al. 2003) and increased oxidation of CD45 compared to Lyp could explain why RA T cells appear hyporesponsive (Cemerski et al. 2003).

As smoking is a risk factor for RA (Sugiyama et al. 2010) it was hypothesised that exposure of T cells to CSE would induce changes to PTP activity, possibly by oxidation. Analysis of smoke extracts has shown that they contain a wide range of compounds including ROS (Borgerding et al. 2005) and thus oxidation of PTPs by CSE represented a plausible mechanism by which smoking could alter T cell signalling. Exposure of T cells to CSE caused a reduction in the activity of PTPs overall and also a reduction in the phosphatase activities of CD45 and Lyp specifically (Figure 5.3). In contrast to H_2O_2 alone, CSE appeared to equally affect CD45 and Lyp suggesting the mix of compounds found in CSE could have

more complex effects on these PTPs. ROS were thought to be of importance in mediating the effects of CSE, as when T cells were treated with CSE and catalase the effects of the CSE were significantly reduced (Figure 5.7). This reduction in CSE efficacy occurred when two different types of catalase were used, (1) non-pegylated, which cannot enter cells, and (2) pegylated, which can diffuse across cell membranes. The fact that both types of catalase were equally as effective at reducing the effects of CSE suggests that ROS could be present both inside and outside the T cell membrane. At least some of the oxidation was shown to be reversible, which was determined by labelling reversibly oxidised cysteine residues or SH groups which are contained within the active site of the PTPs (Figure 5.8). Furthermore, there was a significant decrease in the cellular reduced GSH content again suggesting oxidation was taking place (Figure 5.4). This has also been observed *in vivo* even in "healthy" human smokers (Alberg 2002) showing that CS promotes a pro-oxidant environment. Further experiments showed that the particulate matter (PM) component of the extract was important for efficacy. When PM was removed by filtration the effects of CSE on PTP activity were significantly reduced (Figure 5.7). The PM component of CS is an important mediator of many of the pathological effects of smoking in the lungs and has been shown to induce oxidative stress and promote tissue injury by disrupting iron metabolism (Ghio et al. 2008).

A decrease in CD45 and Lyp phosphatase activities were somewhat reflected in the phosphorylation status of the activating tyrosine residue of Lck kinase (Y394), which was increased upon stimulation of T cells exposed to CSE (Figure 5.5). This effect on Lck phosphorylation was subtle when compared to the large increase in phosphorylation observed when T cells are exposed to H_2O_2 (Rider et al. 2013) again suggesting that CSE could have much more widespread and subtle effects on TCR signalling, not only mediated by PTPs and PTKs. There was a decrease in the overall signal propagated through the TCR which was illustrated by a decrease in TCR-dependent Ca^{2+} signalling (Figure 5.6). Ca^{2+}

signalling induced by ionomycin or thapsigargin independent of the TCR was unaltered in T cells treated with CSE (Figure 5.6). This highlights that the intrinsic Ca^{2+} release mechanisms are still intact in CSE-treated T cells but CSE treatment specifically alters signalling dependent upon TCR engagement.

Exposure of primary CD4+ T cells to CSE decreased the percentage of IFN-γ and IL-4 secreting cells, whilst having no effect on the percentage of IL-10 and IL-17 secreting cells (Figure 5.10). The amount of TNF-α, IL-4 and IL-17 secreted was reduced in cells treated with a high concentration of CSE (30%) however there was no difference in the amount of IL-10 and IFN-γ (Figure 5.10). These mixed results do not indicate that CSE treatment favours certain types of T cell subsets, for example Th1. Most changes to cytokine production were observed only when a high concentration of CSE was used (30%) and may represent a generalised depression of overall cytokine production. Previous studies have reported similar findings however the observations of decreased cytokine production have also been accompanied by a depression in other T cell effector functions such as proliferation (Lambert et al. 2005, Glader et al. 2006). In our experiments it was found that T cell proliferation was unaffected by CSE treatment (Figure 5.9). These conflicting findings may be a result of different methods of CSE preparation and length of exposure times. For example, in our experiments T cells were pre-incubated for 24 hours with CSE and then stimulated using anti-CD3 and anti-CD28 for 48 hours while still in the presence of CSE.

This chapter has addressed how CS and oxidation may contribute to T cell dysfunction in RA and other diseases in which suppressed levels of antioxidants are apparent. This was carried out by measuring TCR signalling parameters in T cells treated *in vitro* with CSE and revealed significant changes to proteins involved in the TCR signalling pathway. Overall CSE decreased signalling mediated through the TCR, possibly through an oxidative mechanism. Oxidation of CD45 and Lyp also occurred when T cells were treated with the oxidant H_2O_2 which was used to mimic an inflammatory insult in which large amounts of ROS would be

generated. Overall, CSE had major effects on the proteins controlling TCR signalling, but these changes were not reflected in overall cytokine production by T cells.

CHAPTER SIX: GENERAL DISCUSSION

6.1 Introduction

The association of the genetic variant PTPN22 (R620W) and cigarette smoking (CS) with an increased risk of autoimmune inflammatory diseases such as RA has been known for a long time (Vessey et al. 1987, Bottini et al. 2004) A large proportion of previous studies have concentrated on the effects of R620W on T cell function, given the importance of this cell type in the development of autoimmune diseases including RA (Cope et al. 2007). It has been suggested that expression of R620W alters the structure and function of the Lyp protein, which would then have upstream effects on the regulation of signalling through the TCR. CS is known to interact with R620W to further increase an individual's risk of RA via an unknown mechanism (Kallberg et al. 2007). This present study has examined how expression of the variant Lyp protein and CS affect the phosphatase activity of Lyp and the subsequent consequences for CD4+ T cell activation and function. In addition, the effect of variant Lyp on neutrophil function was also investigated, based on evidence of high expression of Lyp in neutrophils (Chien et al. 2003) and the lack of knowledge of Lyp function in this cell type. Neutrophils can represent a high percentage of leukocytes found in the inflamed joints of RA patients (Mohr et al. 1981) and thus this cell type could be key to initiation or maintenance of inflammation in the joint.

6.2 Summary of results

Expression of R620W had a number of effects on TCR signalling and overall T cell activation and function. Expression of the variant Lyp via R620W was associated with a decreased amount of Lyp protein which had an increased specific phosphatase activity in B and T lymphocytes (Figure 4.2 and 4.5). These changes in protein expression and activity were reflected in the phosphorylation status of the Lyp substrates Lck (Y394) and Zap70 (Y493), which were found to be less phosphorylated in T lymphocytes expressing the Lyp variant (Figure 4.6). The Lyp variant had no effect on CD4+ T cell proliferation in healthy individuals

and RA patients (Figure 4.7 and 4.8) however significant changes in cytokine production were observed (Figure 4.9 and 4.10). In healthy individuals expression of variant Lyp was associated with a decreased percentage of IL-10 producing cells and increased secretion of the pro-inflammatory cytokines IFN-γ and TNF-α (Figure 4.9). Findings were similar in RA patients with T cells expressing variant Lyp containing a smaller percentage of IL-10 producing cells and an increased percentage of IFN-γ producing T cells (Figure 4.10). Similarly to healthy individuals, Lyp variant expressing T cells isolated from RA patients also produced increased amounts of IFN-γ and TNF-α (Figure 4.10). Taken together, these results suggest that expression of the Lyp variant could contribute to the development autoimmune inflammatory diseases by promoting the generation of inflammatory T cells.

The Lyp variant also induced significant changes to the activation and function of neutrophils in healthy individuals and RA patients. It was found that the Lyp variant enhanced a number of neutrophil effector functions, including increased migration across inflamed endothelium, increased Ca^{2+} release and increased production of ROS following priming with the pro-inflammatory cytokine TNF-α (Section 4.2.2). Overall these results suggest that expression of the Lyp variant could increase the speed of migration of neutrophils to sites of inflammation and once at the site, be capable of exhibiting enhanced effector functions. This could be beneficial in the quick and effective destruction of invading pathogens, but if these functions are maintained over a longer time period this could promote pathological levels of inflammation.

Finally the effects of CS on TCR signalling and function were investigated to determine possible mechanisms by which this environmental risk factor could synergise with the genetic variant R620W to promote the occurrence of autoimmunity. Overall, it was found that exposure of T lymphocytes to cigarette smoke extract (CSE) decreased TCR signalling (Section 5.2). This was characterised by a decrease in TCR-dependent Ca^{2+} signalling and a suppression of cytokine production at higher concentrations of CSE (Figure 5.6 and 5.10). It

was found that CSE treatment decreased the activity of PTPs globally and also the specific phosphatase activity of CD45 and Lyp (Figure 5.3). The mechanism responsible for this reduced PTP activity could be oxidative as CSE treatment decreased the reduced glutathione (GSH) content of T cells and the efficacy of the extract was significantly reduced if T cells were treated with catalase to remove ROS (Figure 5.4 and 5.7). In addition, reversible oxidation of cysteine residues or SH groups was also induced by CSE treatment (Figure 5.8) further strengthening the hypothesis that the changes to PTP activity could be due to oxidation.

6.3 Development of the Lyp phosphatase assay

As part of this study, a method that enabled the measurement of the amount of Lyp protein and the Lyp phosphatase activity was developed. These two measures were then used to calculate the specific Lyp phosphatase activity to enable a more detailed analysis of Lyp function. This was advantageous when trying to identify how genetic and environmental factors could specifically and possibly subtly alter Lyp function. The development of this assay was essential in order to take forward investigations for this study, as other methods available at the time had considerable drawbacks (Section 3.1). A number of studies had used immunoprecipitation techniques to measure Lyp phosphatase activity (Cohen et al. 1999, Orru et al. 2009, Fiorillo et al. 2010) however these assays did not measure the activity and amount of Lyp protein simultaneously and also measurements in primary cells were often lacking. Therefore, these assays could not take into account any changes in binding affinity of Lyp to the antibody used in the immune purification procedure, which may be caused by modifications to the Lyp protein such as oxidation. For example, if a modification to Lyp prevented the particular antibody epitope used in the method from recognising the modified Lyp protein this would be measured as a decrease in activity, whereas in fact this is due to less Lyp binding during the assay procedure. The assay protocol developed here overcomes this problem by allowing measurement of Lyp activity and amount to account for

these differences. This proved useful in determining the effects of R620W on Lyp phosphatase activity as differences in Lyp activity were subtle, but the amount of protein bound in R620W cells was considerably reduced (Figure 4.2 and 4.5). Without this assay, these detailed analyses of Lyp function could not have been determined.

Another significant advantage of this assay over previous methods is its simplicity to set up and its applicability to other PTPs. This assay for measuring specific Lyp phosphatase activity was based on a previous method used for measuring specific CD45 phosphatase activity (Rider et al. 2003). With changes to the fluorescent substrate used in the assay, it was found that the immunocapture based method could easily be used for measuring Lyp phosphatase activity. Most laboratories have the facilities to carry out colorimetric and fluorimetric assay measurements and thus this method could be replicated by other research groups for measuring the activity of other PTPs expressed by immune cells. Furthermore, results obtained here show that the assay can be used to detect Lyp phosphatase activity in a number of cells lines (Jurkat, U937, and HL60) as well as primary T cells and it would be anticipated that other types of primary immune cells could be used, for example macrophages and neutrophils.

However as with all methods there are also disadvantages to the Lyp phosphatase assay. Although the number of cells needed for the assay is low ($1.0x10^6$ Jurkats and $2.0x10^6$ CD4+ T cells) it is not likely that this assay could be used to investigate the function of Lyp in small leukocyte subsets. For example, it would be quite expensive to obtain enough cells to compare the activity of Lyp in naïve and memory CD4+ T cells from the same donor. This could be of interest as loss of Lyp in memory T cells seems to have significant effects, whereas naïve T cells appear unaffected (Hasegawa et al. 2004). In addition, although the assay allows measurement of activity and amount from the same cell lysate samples, this is not carried out in the same well during the procedure. This problem was first encountered in the CD45 phosphatase assay where the reducing agent dithiothreitol (DTT) was found to

interfere with binding of the horseradish peroxidise-linked secondary antibody used in the detection of amount. DTT is required for full detection of Lyp phosphatase activity as it reduces the Lyp active site cysteine residue which may have been reversibly oxidised during preparation of the cells for the assay. To avoid this interference, the measures of Lyp activity and amount must be carried out in separate wells. This increases the number of cells required for the assay and is not ideal, especially when using cells isolated from clinical samples which may be limited. This could be overcome by trying an alternative reducing agent in the assay such as tris (2-carboxyethyl) phosphine (TCEP) which may not affect antibody binding.

6.4 PTPN22 R620W in lymphocytes – a gain or loss of function?

Ever since the discovery of R620W in 2004, there has been constant debate as to whether the mutation causes a gain or loss of function Lyp phosphatase (Vang et al. 2005, Zikherman et al. 2009). There is currently strong evidence for both a loss and gain of function, as determined by direct measurement of Lyp phosphatase activity as well as cell functional parameters. Despite this, many studies to date have a number of caveats which we have attempted to address in this study. For example, to our knowledge the phosphatase activity of Lyp in freshly isolated leukocytes from healthy individuals has never been determined. By measuring the Lyp phosphatase activity in CD4+ T cells freshly isolated from healthy individuals control (GG) and heterozygous (AG) for R620W we have shown that in fact the measured phosphatase activity of Lyp is comparable. However, the amount of Lyp protein present is significantly decreased in AG T cells resulting in an overall higher specific phosphatase activity (Figure 4.5).

A single study using leukocytes isolated from vasculitis patients showed that Lyp phosphatase activity was increased in R620W expressing individuals, although the assay used in this study was unable to detect Lyp phosphatase activity in non-R620W individuals

(Cao et al. 2012). Furthermore, it was found that the amount of Lyp protein was similar regardless of R620W genotype. This is different to what we have observed in healthy controls, which could be for a number of reasons. In this study using patients, the method used to measure Lyp phosphatase activity was similar to our method, as it was also based on immunocapture of the Lyp protein. However, this method did not appear as sensitive as the one we have developed, as it was unable to detect any Lyp phosphatase activity in patients without the R620W variant (Cao et al. 2012). This could be due to the fact that our assay uses the fluorescent substrate DiFMUP, whereas the assay used in this study detects phosphatase activity using p-NPP which has been shown to be much less sensitive (Huang et al. 1992). Another point to consider is that the samples used in this study were taken from patients with an autoimmune inflammatory disease, and no samples from healthy controls were used. The aetiology and development of vasculitis is complicated and may cause alterations in leukocyte signalling which could also influence the activity of phosphatases such as Lyp. An important experiment to confirm our results would be to carry out the Lyp phosphatase assay developed during this study using an alternative capture antibody. This would be necessary to confirm if R620W does indeed result in a decreased amount of Lyp protein, or whether the particular antibody used here has a decreased binding affinity for the variant Lyp protein. This would clarify the results and would provide further support for the gain of function Lyp phosphatase already observed in this work. Overall, it is unclear from this study and the single study in vasculitis patents if R620W induces a gain of Lyp phosphatase activity with no detectable difference in Lyp protein level.

Important observations regarding the effects of R620W on Lyp phosphatase activity and overall cell signalling have been made using Jurkat T cells transfected with the R620W mutant (Vang et al. 2005, Zikherman et al. 2009). When Jurkat T cells were transfected with different concentrations of the mutant R620W it was found that the resulting variant Lyp protein was much more efficient at inhibiting the transcription factor NFAT/AP1 when

compared to the non-mutant (Vang et al. 2005). Mutant Lyp protein from these cells was also shown to have increased phosphatase activity, which was measured by the ability of Lyp to dephosphorylate a peptide modelled on Lck (Vang et al. 2005). Another group used a similar transfection procedure but also overexpressed Csk, the binding partner of Lyp in T cells (Zikherman et al. 2009). By using this technique it was found that T cells with R620W displayed increased phosphorylation of Erk and increased Ca^{2+} mobilisation (Zikherman et al. 2009). Although Lyp phosphatase activity was not measured directly, these functional measures suggest that R620W increases TCR signalling.

These two studies in particular highlight the complexity of the effects of this genetic variant on its resulting protein product and furthermore how this alters protein-protein interactions to affect cell signalling. Overall these differing observations could be due to different methods of T cell stimulation, different functional read outs used as measures of TCR signalling or unknown additional effects of transfecting immortalised cell lines. The advantage of the results presented here is that Lyp phosphatase activity was measured directly in CD4+ T cells freshly isolated from healthy individuals, removing the confounding factors of transfection, stimulation method, disease status and use of immortalised cell lines.

In summary, when considering both data from this thesis and that currently available in the literature, a reasonable conclusion could be that R620W does indeed result in a gain of function Lyp phosphatase but its effects are subtle due to Lyp protein degradation and changes to its availability within cells. This idea of a subtle effect of R620W is feasible, given that the R620W variant is widely expressed in healthy controls (Table 4.1.2.1) and there has been evidence that it may be under positive selection (Gomez et al. 2005). This positive selection of the variant is thought to be advantageous by increasing immunity to bacterial infections such as tuberculosis (TB) (Gomez et al. 2005). Furthermore, there has been no thorough investigation of the location of Lyp in unstimulated and stimulated T cells and also little study of how this location affects the affinity of Lyp for different substrates. These are

two important questions which must be answered to aid further understanding of the transition from health to autoimmunity in R620W variant expressing individuals.

6.5 PTPN22 R620W and inflammatory T cells

In addition to changes in Lyp phosphatase activity, the R620W variant has also been shown previously to cause significant changes in TCR signalling and consequently T cell effector functions (Burn et al. 2011). The work presented in this study has shown that expression of R620W promotes the occurrence of inflammatory CD4+ T cells, as characterised by production of the pro-inflammatory cytokines TNF-α and IFN-γ (Figure 4.9/10 G+H). An increase in IFN-γ production has also been observed in healthy individuals homozygous (AA) for R620W along with a decrease in IL-17 production. Further to this, it was also found that regulatory T cells isolated from AA individuals were unable to suppress Th1-mediated IFN-γ production (Vang et al. 2013). Taken together these findings suggest a molecular mechanism by which R620W could promote autoimmunity, by increasing production of pro-inflammatory cytokines by effector T cells and also a lack of function of regulatory T cells. The role of PTPN22/Lyp in regulatory T cell development has also been investigated in a mouse model, which showed that lack of PTPN22 increased the numbers of pre-T regulatory cells in the thymus as well as T regulatory cells in the periphery (Maine et al. 2012). In addition to this, a decrease in the function of T regulatory cells has also been observed in human disease states including RA (Ehrenstein et al. 2004), highlighting a potential mechanism by which R620W could contribute to disease development.

6.6 PTPN22 R620W and neutrophil activation

This work has highlighted that study of PTPN22 R620W should not be restricted to lymphocytes and that expression of this genetic variant also causes significant alterations in the function of other types of leukocytes. It was shown in this study that PTPN22 R620W increased neutrophil signalling and activation in health and RA, which was characterised by

increased migration, increased Ca^{2+} release and increased production of ROS following TNF-α priming and stimulation.

The function of Lyp in T cells is to act as a negative regulator of signalling through the TCR (Hermiston et al. 2009) however the function of Lyp in neutrophils is not known at present. Similarly to T cells, neutrophils express a number of PTPs including CD45, CD148 and Lyp (Hoffmeyer et al. 1995, Chien et al. 2003, Hermiston et al. 2009). CD45 and CD148 have been reported to be regulators of neutrophil migration (Zhu et al. 2011) whereas a role of Lyp has not yet been identified. Given that CD148 has been shown to be involved in both positive and negative regulation (Zhu et al. 2011), it is possible that Lyp could have a similar dual role or it could be a solely negative regulator as in T cells. This lack of knowledge makes the effects of R620W in neutrophils difficult to predict and although the mutation induces a structural alteration to the Lyp protein (Bottini et al. 2004), it is unclear as to whether this confers a phosphatase which has a higher or lower activity.

The increase in migration and ROS production observed in neutrophils with R620W only occurred after priming of neutrophils or EC with TNF-α. The signalling pathways mediating the priming effects of TNF-α on neutrophils are not well known, however it could be hypothesised that Lyp is a key regulator of these events (Figure 6.1). For example, TNF-α has been shown to increase intracellular Ca^{2+} in neutrophils which is associated with an increase in the co-localisation of $gp91^{PHOX}$, a component of the NADPH oxidase enzyme necessary for ROS production, and also β2 integrins, important mediators of neutrophil adhesion and subsequent migration (Reumaux et al. 2006). It is possible that Lyp is a direct regulator of Ca^{2+} dependent functions in neutrophils, given that the two effector functions measured in this study (ROS production and cell migration) were enhanced by changes to Lyp function induced by R620W expression.

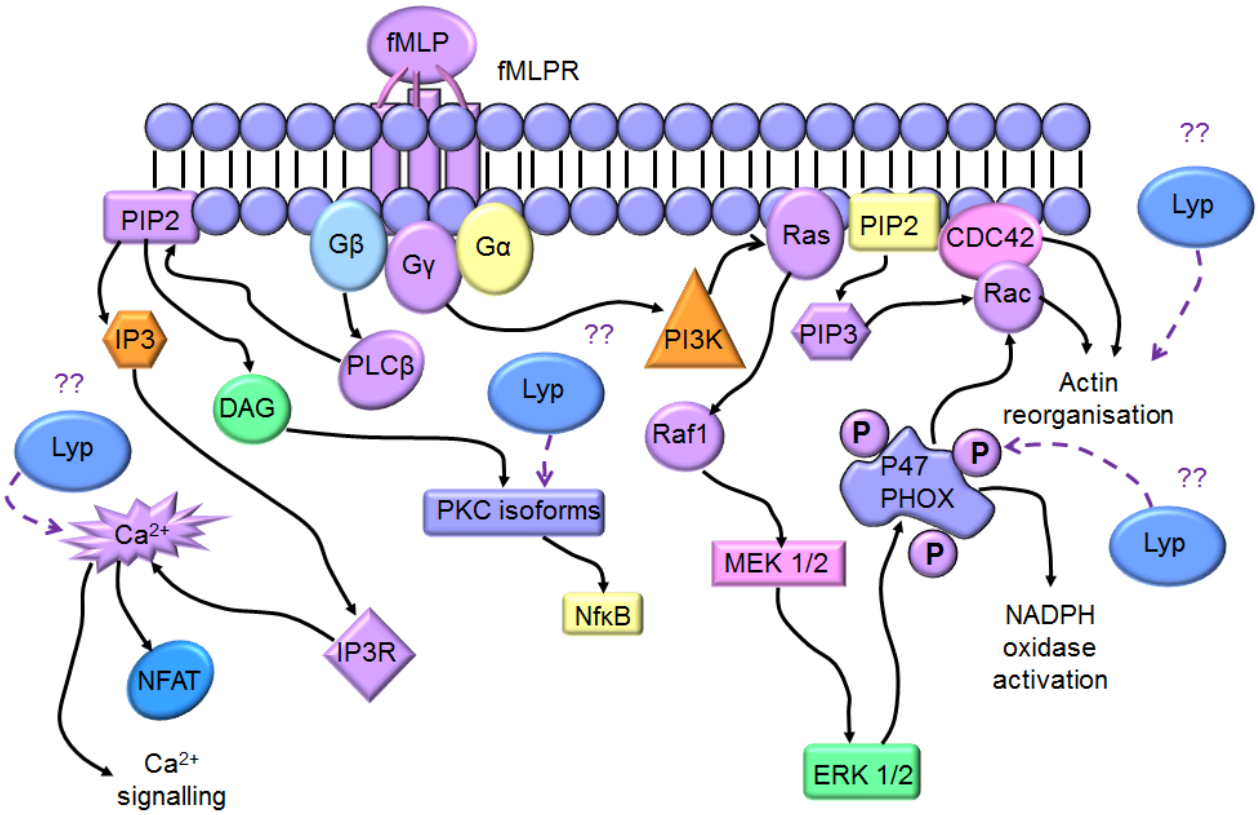


Figure 6.1 Schematic representation of possible roles of Lyp in the regulation of neutrophil signalling and activation mediated by fMLP receptor engagement. Solid black lines represent known protein interactions and dashed purple lines represent possible roles of Lyp. Not all known interactions are depicted. Gβ, Gγ and Gα represent the different G-protein subunits of the fMLP receptor. Ca^{2+}, calcium; DAG, diacylglycerol; ERK, Extracellular Signal-Regulated Kinase; fMLP, N-Formyl-Met-Leu-Phe; fMLPR, N-Formyl-Met-Leu-Phe receptor; IP3, inositol triphosphate; IP3R, inositol triphosphate receptor; MEK, Mitogen activated/ Extracellular Signal-Regulated Kinase; NFAT, Nuclear Factor of Activated T-cells; NFκB, Nuclear Factor Kappa-B; P, phosphotyrosine; PIP2, Phosphatidylinositol 4,5-bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphoinositide 3-kinase; PKC, Protein Kinase C; PLCβ, phospholipase C beta.

With regards to ROS production, it has been proposed that TNF-α prepares the NADPH oxidase for activation by promoting movement of its three cytosolic sub units, ($p40^{PHOX}$ $p47^{PHOX}$, and $p67^{PHOX}$) closer to the cell membrane prior to cell stimulation (Sheppard et al. 2005). There are a number of phosphorylation sites present in the structure of these subunits which regulate their cellular location and activation status (Babior 1999, Dang et al. 1999, Chowdhury et al. 2005). To date only serine and threonine phosphorylation sites have been documented in the neutrophils, but a study in EC has shown that $p47^{PHOX}$ can be subject to tyrosine phosphorylation by Src (Chowdhury et al. 2005). Therefore it is possible that Lyp could be involved in dephosphorylating $p47^{PHOX}$ at this site and a change in Lyp phosphatase activity or localisation induced by R620W could alter the activation state of the NADPH oxidase. Ca^{2+} signalling in neutrophils is regulated by a number of proteins including Vav1, a guanine exchange factor (GEF) which acts as an adaptor molecule during signalling (Moores et al. 2000). Substrate trapping experiments using T cells found that Vav1 is a substrate of Lyp (Wu et al. 2006) which may also be the case in neutrophils. Vav1 is also a mediator of β2 integrin-dependent functions including adhesion and migration (Gakidis et al. 2004), suggesting changes to Vav1 phosphorylation could mediate the changes in neutrophil function induced by R620W.

A higher Ca^{2+} release by R620W neutrophils could result in increased activation of the Ca^{2+} dependent protein kinase C (PKC) isoforms α, β1 and β2 which have also been shown to phosphorylate the $p47^{PHOX}$ sub unit of the NADPH oxidase ready for activation (Dang et al. 1999). Moreover, PKC has been shown *in vivo* to phosphorylate Lyp at Serine-35, which reduces its ability to dephosphorylate target substrates (Yu et al. 2007). This direct association between Lyp and PKC could be could be altered by R620W and have downstream effects on the NADPH oxidase during TNF-α priming. Overall it is unclear as to the exact role of Lyp in the fMLP receptor signalling pathway in neutrophils, however its potential roles are summarised in Figure 6.1.

6.6.1 Potential pathways regulated by Lyp in neutrophils

The work presented in this thesis has shown that expression of PTPN22 R620W has significant effects on neutrophil signalling and activation (Chapter 4). In particular, this work highlights an important mechanism by which R620W could contribute to the pathogenesis and initiation of RA, by altering the function of neutrophils. Indeed, in some mouse models mice fail to develop arthritis if neutrophils are not present, highlighting the importance of neutrophils in the pathogenesis of arthritis (Wipke et al. 2001).

It has long been suggested that RA can be triggered in susceptible individuals by an infection (Edwards et al. 2006). One of the key events which occurs during the immune response to an infection is the activation of neutrophils. Neutrophils use a variety of mechanisms to combat infection, including the production of ROS and neutrophil extracellular traps (NETs). NETs are of particular relevance to RA and are released during NETosis, a novel form of cell death used by neutrophils to aid bacterial killing (Brinkmann et al. 2004). A key part of NETosis is histone citrullination, which is carried out by the enzyme PAD4 and ultimately results in citrullinated proteins being released by neutrophils going through the process of NETosis (Li et al. 2010). The most common autoantibodies specific to RA are those directed against citrullinated proteins (Deane 2014), which could potentially be produced by overactive neutrophils and trigger autoimmunity. Therefore, if PTPN22 R620W results in increased neutrophil effector function upon activation, it could be proposed that hyperactive neutrophils as a result of this genetic variant could be essential to the initiation of RA following an infection. Although in this present study production of NETs in R620W expressing individuals was not measured, it would be reasonable to suggest that this would also be increased given that Ca^{2+} is required for PAD4 activation (Vossenaar et al. 2004) and this was found to be enhanced in neutrophils with R620W.

Given this work has identified a role for Lyp in fMLP signalling in neutrophils, it is also likely that Lyp could be a regulator of other types of signalling in this cell type. Many receptor

signalling pathways in neutrophils involve the recruitment and activation of PTKs following receptor engagement (Majeed et al. 2001). These particular pathways represent good targets for further investigation of the role of Lyp as PTPs including Lyp are known to co-operate with PTKs to fine tune signalling responses. However, one of the biggest challenges for studying the role of Lyp in neutrophils is their short lifespan. The use of gene knock down is not possible as isolated neutrophils do not survive more than 24 hours in culture. Thus a Lyp inhibitor or use of a blocking antibody represent more realistic strategies that could be used to further study the role of Lyp in neutrophils.

An important line of investigation would be to study the role of Lyp when neutrophils are activated via different surface receptors aside from fMLP. For example, the role of Lyp in signalling via Fc receptors (FcR) should be studied. FcR bind the Fc portion of immunoglobulin and are widely expressed by leukocytes of the innate immune system, including neutrophils (Rosales 2013). There are a number of classes of FcR and human neutrophils express only the FcγR for IgG, specifically CD32 (FcγRIIA/B), CD64 (FcγRI) and CD16b (FcγRIIIB) (Fleit et al. 1982, Rosales 2013). When FcγR is activated via the binding of antibody to the receptor, a cascade of signalling events occurs including the phosphorylation of tyrosines within ITAMs present in the receptor structure (Figure 6.2) (Rosales 2013). These tyrosine phosphorylation processes involve activation and recruitment of Src-family kinases and Syk. Further signalling occurs as Syk phosphorylates substrates including the adaptor molecules SLP76 and LAT, PI3K and PLCγ (Bezman et al. 2007). Downstream of these signalling events, second messengers are produced which induce release of Ca^{2+} from the ER, activating Ca^{2+} dependent PKC and subsequently ERK. Lastly, GTPases of the Rac and Rho family are activated by Vav which allows changes to occur within the actin cytoskeleton of the neutrophil. Vav represents a particularly promising target as its absence in mice has shown to protect them from developing arthritis by halting signalling downstream of integrin receptors in neutrophils (Cremasco et al. 2008). If the Lyp

protein alters neutrophil signalling through interactions with Vav, the R620W variant could alter these interactions and lead to hyperactive neutrophils.

The FcR signalling pathway in neutrophils shares many similarities with the T cell receptor signalling pathway, for example a large number of phosphorylation events, release of Ca^{2+} and activation of Vav (Hermiston et al. 2009). Given that Lyp is a key regulator of these events in T cells, it is possible that Lyp has a similar role in the FcR signalling pathway in neutrophils. Furthermore, the regulation of the FcR pathway by PTPs is not well characterised and requires further study. PTPs in general are known to be important regulators of FcR signalling as when neutrophils are treated with PTP inhibitors, FcR mediated increases in tyrosine phosphorylation are augmented (Durden et al. 1994), however the role of specific PTPs are not known. In summary, in order to further characterise the exact role of Lyp in neutrophils, the FcR signalling pathway would be a good starting point.

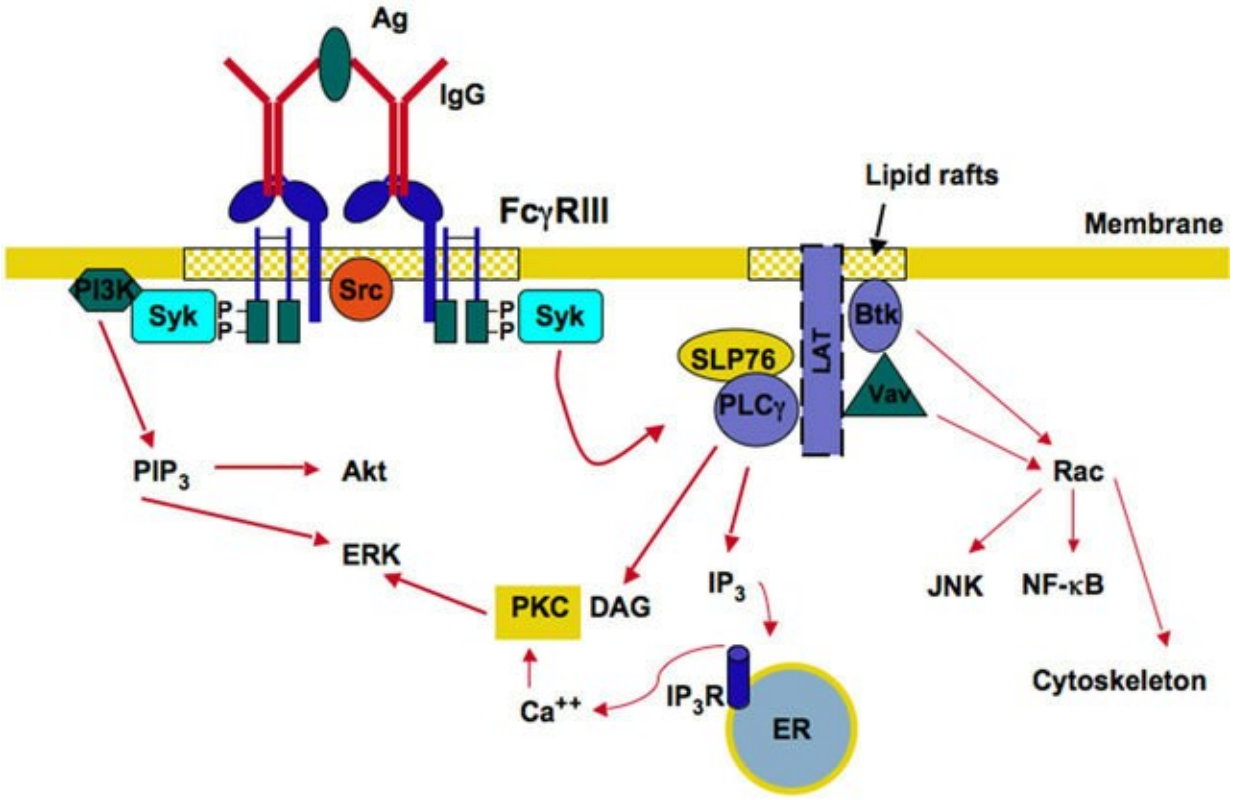


Figure 6.2 Activating Fcγ receptor signaling. FcγR cross-linking by immunoglobulin (IgG) bound to antigen (Ag), induces acti-vation of Src family kinases and Syk family kinases in lipid rafts. These enzymes associate with phosphorylated tyrosines in the ITAM sequences. Syk then phosphorylates enzymes such as phosphatidylinositol 3-kinase (PI 3-K), phospholipase Cγ (PLCγ), and the adaptor molecules SLP76 (SH2-domain-con- taining leukocyte protein of 76 kDa) and LAT (linker for acti-vation of T cells). PI 3-K produces phosphatidylinositol-3,4,5- triphosphate (PIP3), which leads to activation of Akt and ex- tracellular signal-regulated kinase (ERK). PLCγ produces inosi-toltrisphosphate (IP3), and diacylglycerol (DAG). These second messengers cause calcium release from the endoplasmic re- ticulum (ER), and activation of protein kinase C (PKC), respectively. PKC leads to activation of ERK. Vav activates GTPases of the Rho and Rac family, which are involved in the regulation of the actin cytoskeleton. Other enzymes such as Bruton's tyrosine kinase (Btk) also activate the GTPase Rac to induce activation of nuclear factors such as JNK and NF-κB. P represents a phosphate group. IP3R, receptor for IP3. Figure taken from (Rosales 2013).

6.7 Cigarette smoke extract and T cell receptor signalling

It was observed that exposure of T cells to CSE decreases TCR-dependent signalling, as characterised by a loss of function of PTPs, increased phosphorylation of Lck and a depression of Ca^{2+} release upon TCR-stimulation (Chapter 5). It was also observed that CSE exposure had no effect on T cell proliferation, but decreased the production of a number of cytokines. These observations suggest that CSE depresses overall signalling through the TCR which could be due to a decrease in the activity of the CD45 phosphatase, important for early TCR triggering.

In support of this, a previous study investigating T cell signalling in response to CS components using a rat model showed that chronic exposure to nicotine impaired T cell proliferation and reduced Ca^{2+} release following stimulation (Geng et al. 1995), suggesting that CS is a potent immunosuppressant as we have also shown.

T cell dysfunction induced by CS could be one explanation for hyporesponsive T cells observed in human diseases such as RA (Rider et al. 2013). In diseases associated with smoking such as cancer, RA and chronic obstructive pulmonary disease (COPD), profound alterations in T cell function are observed (Stampfli et al. 2009) and could be the end result of long term exposure to the components of CS. To further probe the contribution of T cells to disease processes, a study was conducted in mice to determine if CS exposure in a mouse model could directly promote pathogenic T cells (Motz et al. 2010). In this study mice were exposed to CS, CD3+ T cells taken from the lungs and transferred to mice lacking both B and T cells. It was found that these transferred T cells themselves were pathogenic and induced changes to the pulmonary system similar to those seen in human lung disease. Significant features of these changes included increased accumulation of neutrophils and monocytes in the lungs, apoptosis of alveolar epithelial cells and airspace enlargement. The possibility that specific components of CS drive clonal expansion of these pathogenic T cells

in an antigen-dependent manner was also investigated by exposing T cell recipients to CS for a few weeks following T cell transfer (Motz et al. 2010). CS exposure of T cell recipients had no significant effects on lung inflammation, suggesting that changes to T cell function were stable and did not require further exposure to possible antigens contained within the CS for T cell stimulation and activation. Given the stable nature of these alterations, it could be proposed that CS exposure induces epigenetic changes in T cells contributing to their long term altered function. Smoking has already been shown to induce epigenetic changes in other cell types in the lungs such as epithelial cells (Buro-Auriemma et al. 2013) and this could also be occurring in T cells.

The study described above suggests that inappropriate over-activation of T cells results in the generation of pathogenic T cells, which can then recruit other types of immune cells to a site of inflammation. Our work has shown that in fact exposure of human T cells to CSE decreases their activation and cytokine production, which is the opposite of this study. These conflicting results could be explained by a number of potential reasons. The populations of T cells used in our experiments were total CD4+ T cells, which would contain a mixture of naïve, memory and regulatory T cell subsets. It is possible that CSE may have differential effects on different T cell subsets, for example if CSE severely depressed the signalling and function of T regulatory cells but had less of an effect on memory T cells this would result in unregulated effector T cell function. Indeed, a decrease in the function of regulatory T cells has been reported in disease states such as RA (Ehrenstein et al. 2004). Another reason could be that in the mouse model total CD3+ T cells were transferred to the mice, which would contain both CD4+ and CD8+ T cells. It has been reported that CD8+ T cell numbers correlate with alveolar epithelial cell apoptosis in patients with emphysema (Majo et al. 2001). Furthermore, increased expression of perforin by CD8+ T cells isolated from smokers with COPD has also been observed (Chrysofakis et al. 2004). These observations suggest that increased cytotoxic function of CD8+ T cells could be of importance in mediating lung

damage in smokers and that CD4+ T cells may play a different role. Thus the effects of CSE on CD8+ T cells may be different to the effects we have seen in CD4+ T cells.

Overall studies suggest that T cells are directly involved in the pathogenesis of smoking-related diseases and an important mechanism by which this occurs in through their ability to recruit other immune cells to inflammatory sites. Therefore, it is important to consider the effects of cigarette smoking on the overall immune system and how smoking may alter the way the human immune system responds to pathogens.

6.8 Cigarette smoking and immune responses

The effects of smoking on the responses of the human immune system are complex, which is not surprising given the large number of compounds found within CS (Smith et al. 2000). CS has been shown to activate immune cells and induce pro-inflammatory responses but also have some immunosuppressive effects as well. For example, CSE has been shown to directly activate macrophages, which *in vivo* would prompt the activation of other inflammatory pathways (Yang et al. 2006). Macrophage activation by CSE was characterised by increased IL-8 and TNF-α production, activation of NFκB, depletion of the antioxidant GSH and increased release of ROS. This study highlights that CSE has effects on immune cells at multiple levels, from gene transcription to protein production, and also illustrates the importance of changes in redox balance. In contrast, nicotine and other aromatic compounds present in CS have been shown to alter transcriptional activity in lymphocytes with upstream effects of reduced cell proliferation and effector function (Lambert et al. 2005). Other types of molecules found within CS include carcinogens, toxins, and reactive solids. Oxidants such as nitrogen oxides and superoxide are found in large quantities in fresh smoke and have the ability to directly damage cellular DNA and proteins, thought to be of importance in the pathogenesis of human diseases including cancer (Borgerding et al. 2005). Additionally,

oxidants can directly modify the function of immune cells such as T cells, neutrophils and macrophages in a number of ways (Stampfli et al. 2009).

Our studies have concentrated on the effect of CSE on T cell signalling and we have shown that overall exposure of T cells to CSE decreases T cell activation and function. However, it is important to consider how this immunosuppressive effect of CSE on T cells could impact on overall immune responses in smokers. CS first enters the human body via the lungs, which results in activation of alveolar macrophages. Smokers display increased numbers of alveolar macrophages (Sopori 2002) which produce pro-inflammatory mediators, proteolytic enzymes and ROS (de Boer et al. 2000, Russell et al. 2002). This could provide some explanation for the tissue damage and inflammation found within smoker's lungs. In addition to direct activation of alveolar macrophages there is also some evidence of simply priming these cells to exhibit an inflammatory profile upon subsequent activation (Gaschler et al. 2009).

In contrast to the pro-inflammatory effects of smoking on macrophages, a decrease in the function and numbers of NK cells are seen in smokers (Tollerud et al. 1989). This is believed to be of importance in the pathogenesis of smoking-related diseases such as cancer, given the important role of NK cells in anti-tumour immune surveillance. Another prominent cell type in the lung are DCs and evidence from both human and animal studies indicate that smoking could affect both DC numbers as well as their maturity state (Jahnsen et al. 2006, Tsoumakidou et al. 2008). These changes could have upstream effects on antigen presentation to T cells and consequently T cell-mediated immunity. Given that smoking is associated with an increased risk of autoimmunity it is not surprising that CS also has significant effects on B cells. These cells are capable of producing useful antibodies to fight off infections but also pathogenic autoantibodies to self-antigens. It has been shown that smoking decreases overall serum immunoglobulin levels (Holt 1987) as well as causing the formation of B cell follicles in long term smokers (Hogg et al. 2004). These follicles could play

an important role in generating responses to self-antigens observed in autoimmunity, through actions such as complement fixation and subsequent tissue damage.

In summary, smoking appears to increase the function of innate immune cells in the lungs to promote inflammation and also decrease the ability of adaptive immune cells including B cells and T cells to respond to infectious challenge. Overall it is difficult to study the effects of smoking *in vitro*, as results obtained from short term CS exposure are unlikely to mimic what is really happening *in vivo* in the lungs and bloodstream of long term smokers. Furthermore, a multi-step approach should be taken to study the effects of smoking on gene transcription through to protein production in a range of immune cells, to establish what the predominant effects governing overall immune responses in smokers are.

6.9 How could PTPN22 R620W and cigarette smoking promote autoimmunity?

The main aim of this study was to investigate how R620W and cigarette smoking could interact to promote the development of autoimmunity, in particular RA. Evidence from epidemiological studies clearly show interactions between these genetic and environmental risk factors and their influence on disease susceptibility (Kallberg et al. 2007), but the mechanisms by which these interactions occur are not known. Based on this study, it could be hypothesised that in smokers expressing the R620W variant a number of events could be occurring in a multi stage process which ultimately results in the onset of RA.

We have observed that expression of R620W results in hyperactive neutrophils and a tendency towards pro-inflammatory CD4+ T cells. These moderate functional alterations could be of benefit and contribute to a more rapid and aggressive immune response to invading pathogens in healthy individuals. This supports the idea that the R620W variant is undergoing positive selection in some populations, to provide better immunity against bacterial infections (Gomez et al. 2005). However, this increased immune cell activation could be a disadvantage if other changes to the immune system are occurring such as those

associated with ageing or environmental exposures such as smoking. This work and other studies have shown that cigarette smoke components induce changes to the structure of proteins such as oxidation, citrullination and carbamylation (Trouw et al. 2013). These proteins could be part of immune cells themselves or other self-proteins which form part of the body's tissues. Changes to immune cell proteins would result in dysregulated signalling by the immune system as we have observed in T cells and alterations to self-proteins could increase their immunogenicity allowing recognition by the immune system. Given the hyperactivity of neutrophils and CD4+ T cells induced by R620W expression, this could also be the case for B cells which are important for antibody production.

PTPN22 R620W and smoking are specifically associated with an increased risk of developing ACPA-positive RA (Linn-Rasker et al. 2006, Kallberg et al. 2011) suggesting these factors have a key role in influencing the production of ACPA. It would be reasonable to suggest that R620W has similar effects on both B and T cells and thus you would expect R620W individuals to display alterations in B cell function. Indeed, this has been studied and it has been observed even in healthy individuals with R620W that the variant is associated with an accumulation of autoreactive B cells in the periphery (Menard et al. 2011). In summary, PTPN22 R620W could promote the development of RA by altering B and T cell selection and increasing the activity of T cells in the periphery such that self-peptides modified by cigarette smoking will be recognised by the immune system. Failure of removal of autoreactive B cells would then provide a situation in which autoantibodies such as ACPA are produced. At some stage, neutrophils would also be present during responses of the innate immune system and display enhanced function which would contribute to the initiation or maintenance of inflammation.

The studies conducted here have increased knowledge regarding how PTPN22 R620W and smoking may interact to promote the development of RA, however they have also raised a number of questions. For example, it was observed that expression of R620W resulted in

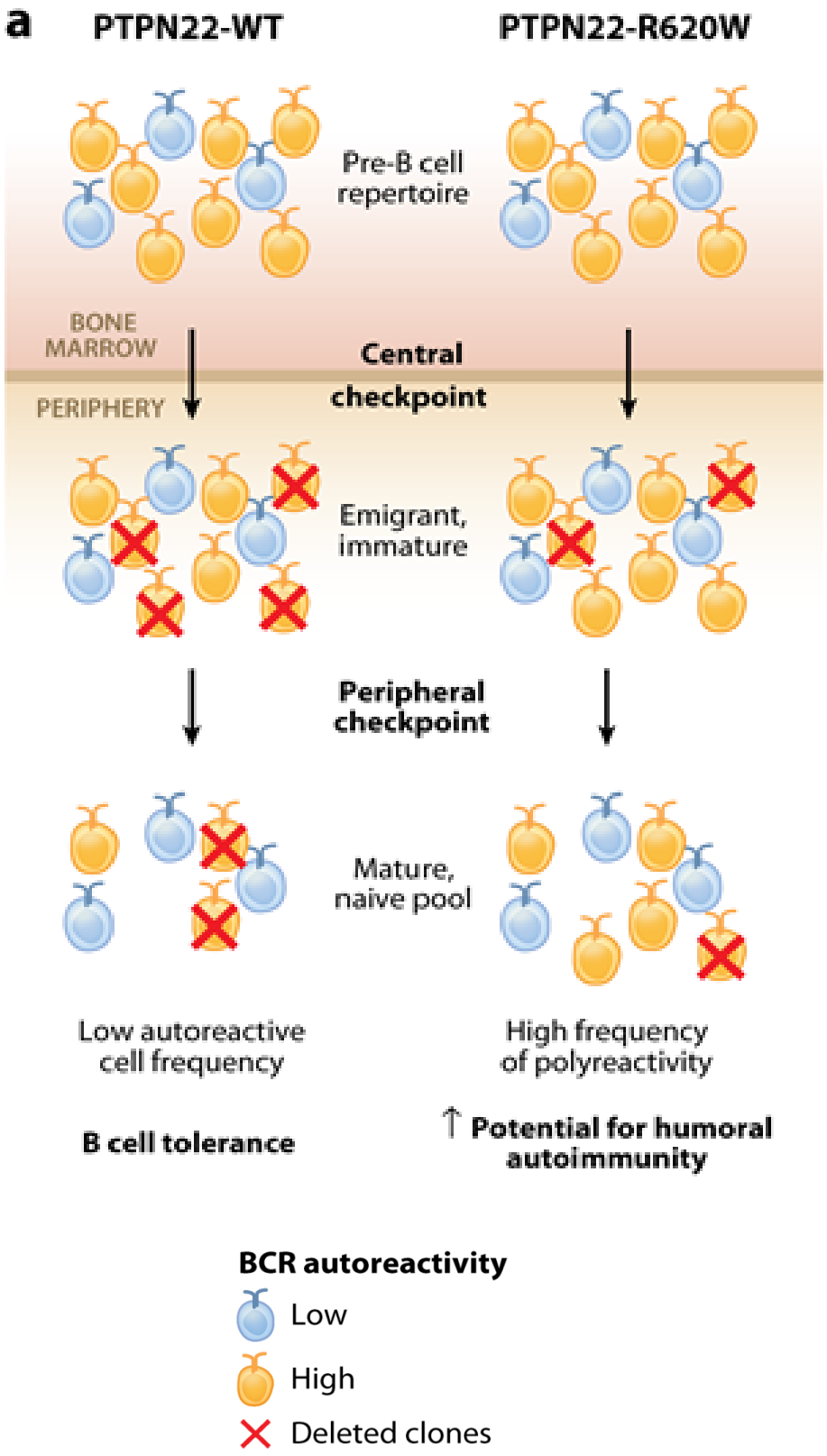
increased production of the pro-inflammatory cytokines TNF-α and IFN-γ by CD4+ T cells (Chapter 4). In contrast, exposure of CD4+ T cells to cigarette smoke resulted in a reduction in the production of these same cytokines (Chapter 5). This raises the question as to why PTPN22 R620W and smoking are both risk factors for RA, but appear to have opposite effects on pro-inflammatory cytokine production. It is possible that because these studies were conducted on whole populations of CD4+ T cells and not on specific subsets that the effects could be different depending on which T cell subset is being studied. Also, given the large number of donors required for this study there may have been large differences in the balance of different T cell subsets between donors, such as an increase in the percentage of memory T cells in older donors. Lastly, it is also important to consider that these *in vitro* studies of isolated T cells are not representative of what may be happening *in vivo*. For example, *in vivo* there will be additional effects on T cell signalling that were not measured here, such as T cell-mediated immune responses to antigens which may be contained within cigarette smoke and also the role of other immune cell types and their influence on T cell activation. Indeed, a study in mice has shown that long term exposure to cigarette smoke caused an expansion of T cell populations in the lungs (Motz et al. 2008), indicating an increase in T cell activation. This is the opposite to what we have observed *in vitro* using isolated human T cells and highlights the complexity of studying responses of the immune system to cigarette smoke.

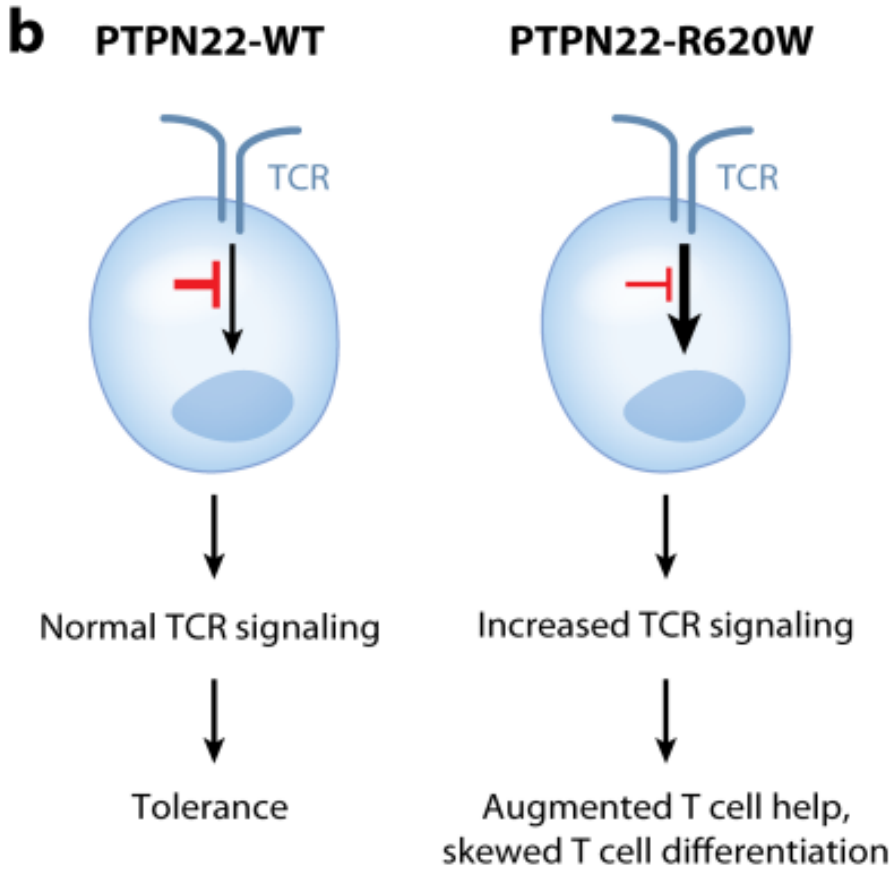
6.10 How could PTPN22 R620W promote rheumatoid arthritis?

This work has largely focussed on identifying mechanisms by which PTPN22 R620W and smoking could interact to promote the development of RA. PTPN22 R620W is an important genetic risk factor in its own right, yet the mechanisms by which it operates in autoimmunity and specifically RA are not yet known. It is clear by the widespread expression profile of PTPN22/Lyp among all immune cells (Chien et al. 2003) that multiple cell types and signalling pathways are likely to be involved. Studies to date have suggested that expression

of R620W alters host defence, immune cell signalling and immune cell development processes, as reviewed in (Bottini et al. 2014).

The characteristic feature of RA is an inflammation of the synovium which is driven by infiltration of leukocytes and an increased production of pro-inflammatory cytokines (Lee et al. 2001). One model suggests that RA stems from the ability of R620W to prevent autoreactive B lymphocyte clones with affinity for citrullinated self-peptides from being deleted during BCR-driven selection (Mahdi et al. 2009) (Figure 6.3). These B cell clones are then permitted into the circulation where an unknown trigger can activate an autoimmune response via these cells. At this point R620W expression may alter T cell help during this response, given that R620W is known to alter the thresholds for TCR signalling as described in this thesis and other studies (Bottini et al. 2014). These immune responses may also stimulate the recruitment of neutrophils to the synovium, which are known to be hyperactive in individuals expressing R620W (Bayley et al. 2014). Later on in the disease process, R620W expression in myeloid cells could reduce their ability to secrete the anti-inflammatory type I IFN (Wang et al. 2013), which downstream would fail to suppress the pro-inflammatory effects of IL-1β and TNF-α (Yarilina et al. 2007). Overall, these R620W-mediated events could increase inflammation and further promote leukocyte infiltration into the synovial joints. In support of this model, work described in this thesis has shown that *in vitro* R620W expressing neutrophils migrate much faster across inflamed endothelium and also display enhanced effector functions such as increased ROS production (Chapter 4). Overall, this model of how R620W contributes to the development of RA involves multiple cell types and can account for why R620W expression may manifest as an organ specific disease.


a
PTPN22-WT
PTPN22-R620W
Pre-B cell repertoire
BONE MARROW
PERIPHERY
Central checkpoint
Emigrant, immature
Peripheral checkpoint
Mature, naive pool
Low autoreactive cell frequency
High frequency of polyreactivity
B cell tolerance
↑ Potential for humoral autoimmunity
BCR autoreactivity
Low
High
Deleted clones


b
PTPN22-WT
PTPN22-R620W
TCR
TCR
Normal TCR signaling
Increased TCR signaling
Tolerance
Augmented T cell help,
skewed T cell differentiation

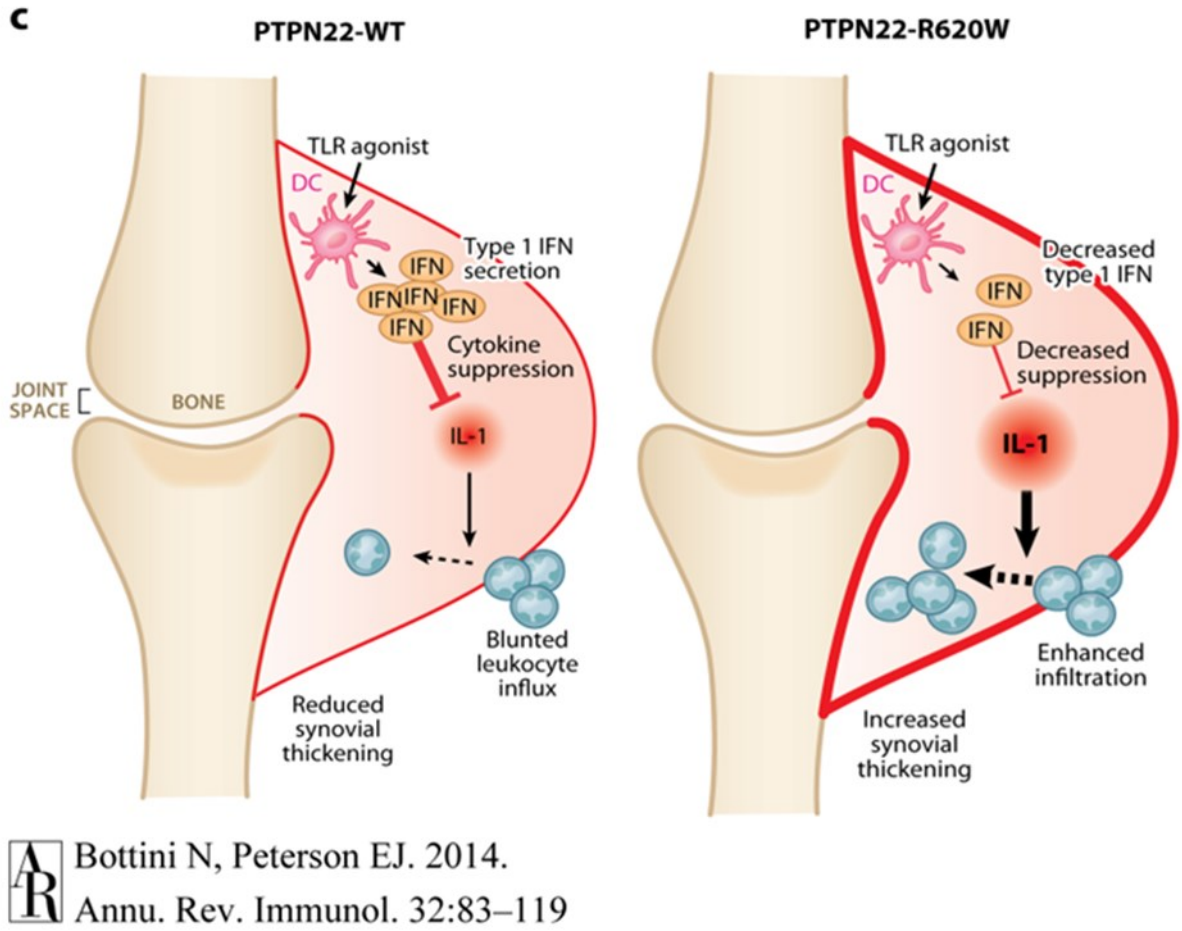


Figure 6.3 The autoimmunity-associated variant PTPN22-R620W may contribute to disease predisposition through differential regulation of B cell repertoire development, T cell signaling, and TLR-driven type 1 IFN production. **(*a*)** Selective removal of B lymphocytes bearing high-affinity self-reactive BCRs occurs at distinct developmental checkpoints in the bone marrow and in the periphery. In PTPN22-R620W carriers, increased numbers of polyreactive B cells escape BCR-mediated deletion as cells progress through central and peripheral checkpoints. Thus, the PTPN22-R620W-carrier naive B cell repertoire harbors a greater tendency toward autoantibody production. **(*b*)** Diminished negative regulation of proximal TCR signaling in the presence of PTPN22-R620W results in augmented second messenger activity and enhanced capacity for cellular activation after TCR stimulation. **(*c*)** TLR signals drive type 1 IFN, which can suppress the activity of proinflammatory cytokines such as IL-1 in inflammatory arthritis. Abbreviations: BCR, B cell receptor; DC, dendritic cell; IFN, interferon; MAPK, mitogen-activated protein kinase; TCR, T cell receptor; TLR, Toll-like receptor. Figure taken from (Bottini et al. 2014).

6.11 Further study

The investigations carried out during this study examined the effects of the genetic variant PTPN22 R620W on the function of neutrophils and CD4+ T cells in healthy individuals and patients with RA. The effects of CS on CD4+ T cell signalling and function were also studied to try and determine possible mechanisms by which these genetic and environmental factors may interact to promote the development of RA. Throughout these investigations, findings have been considered with reference to current literature regarding R620W and present knowledge of T cell and neutrophil dysfunction in autoimmune inflammatory diseases. This work has shown that R620W promotes inflammatory T cells, perhaps with a decline in the function of T regulatory cells, which adds to previous findings (Minton 2012, Vang et al. 2013). In addition this work details the effect of R620W in neutrophils, which has not been documented before and suggests that R620W enhances neutrophil activation in health and RA. However, findings from this work have also raised additional questions regarding how R620W and CS interact to promote the development of autoimmunity.

6.11.1 Patient study groups

The experiments carried out using leukocytes isolated from RA patients who had been genotyped for PTPN22 R620W could be improved in a number of ways. Importantly, experiments should be carried out using leukocytes isolated from 'disease control' subjects who were recruited in the study cohort. This group included patients with other musculoskeletal diseases excluding RA, for example osteoarthritis and psoriatic arthritis. By comparing measurements from healthy controls, disease controls and RA patients it could be determined if any changes seen in R620W RA patient cells are specific to RA or are a more general effect observed in all inflammatory joint diseases. Another improvement could be to increase the numbers of patients recruited, in order to increase the numbers of individuals homozygous (AA) for R620W. This present study largely focussed on the effects of

heterozygosity (AG) for R620W on leukocyte function, as a reasonable number of heterozygotes were recruited in both the healthy control and RA patient populations. A total of 9 homozygotes were recruited to the cohort of RA patients and it would be advantageous to have a larger population to isolate leukocytes from. Another reason for increasing the size of the cohort would be to investigate the effects of the Lyp variant in anti-cyclic citrullinated peptide (CCP) negative RA patients. Given that R620W has only been previously associated with anti-CCP positive patients (Kokkonen et al. 2007) it would be interesting to identify if leukocytes with the Lyp variant behave differently in anti-CCP negative disease.

A further improvement to this work would be to measure the specific activity of the Lyp phosphatase in leukocytes isolated from RA patients with R620W. These experiments were carried out in healthy individuals and showed that R620W CD4+ T cells expressed decreased amounts of Lyp protein which had a higher specific phosphatase activity. Given the complexity of the inflammatory processes occurring in RA, this may not be the case in patient leukocytes with R620W. For example, the processes controlling Lyp degradation could be altered in RA and by measuring the Lyp protein amount using the Lyp phosphatase assay this could give an indirect indication of these possible changes to Lyp stability. It would also be useful to obtain CD4+ T cells and neutrophils from the synovial fluid (SF) of RA patients to determine any differences in Lyp phosphatase activity between peripheral blood (PB) cells and those infiltrating the SF.

6.11.2 Mechanisms of enhanced TNF-α and IFN-γ production by Lyp variant T cells

It was observed that R620W T cells produced more of the pro-inflammatory cytokines IFN-γ and TNF-α. Further work would include investigation of the mechanisms behind this observation. It would be important to investigate if this still occurs in sorted populations of naïve and memory CD4+ T cells as this would determine if R620W T cells are more likely to differentiate into Th1 cells, if T regulatory cell function is impaired, or if perhaps R620W

expressing individuals have a larger population of memory T cells. Experiments could also be carried out to determine the effect of signal strength on cytokine production by R620W T cells, as this could enhance or diminish the effects on cytokine production observed here. This could be done *in vitro* using different amounts of anti-CD3 and anti-CD28 to mimic changes to the level of antigenic stimulation present *in vivo.* The effects of changes to Lyp phosphatase activity could further be investigated using an inhibitor specific to Lyp. These results could then be compared to Lyp phosphatase activity in R620W expressing cells, to further characterise whether the variant is a gain or loss of function.

The effect of R620W on Lck and Zap-70 phosphorylation status has been investigated in this study which could be extended to other known substrates of Lyp in T cells. An example is Vav1, which is known to be a regulator of Ca^{2+} signalling in T cells. Previous work has shown that R620W T cells display decreased Ca^{2+} mobilisation (Rieck et al. 2007) which could potentially be mediated by a change in Vav1 activation.

The production of cytokines by T cells is regulated at many levels, one of which is expression of transcription factors required for protein production. Transcriptional regulation of the TNF gene could be investigated to determine if this is altered in R620W T cells. The pathways downstream of TNF gene activation could also be measured including activation of other signalling proteins such as ERK and p38 MAP kinase. Lastly other T cell effector functions could also be measured, for example the ability of T cells to adhere and migrate across endothelium. This is important for T cells to exit the bloodstream and enter sites of inflammation. It is anticipated that R620W will affect these T cell functions as loss of Lyp in T cells has been shown to enhance migratory responses (Brownlie et al. 2012).

6.11.3 Mechanisms of enhanced activation of Lyp variant neutrophils

A major part of this study was to determine the effects of R620W on neutrophil function in health and RA. It was found that R620W enhanced neutrophil function in healthy individuals

and RA patients, in particular when neutrophils were primed with the pro-inflammatory cytokine TNF-α. The mechanisms behind this enhanced neutrophil function induced by TNF-α was not determined. Further work on these investigations would include measuring the specific activity of the Lyp phosphatase in neutrophils expressing R620W to determine whether similar effects on Lyp protein amount and activity are also true of R620W neutrophils. As ROS production by R620W neutrophils was significantly enhanced following TNF-α priming and fMLP stimulation, signalling downstream of the TNF receptor should be investigated. This would involve measuring the activation of ERK and p38 MAP kinase, to try and determine exactly where Lyp is involved in these pathways. Little is known about how TNF-α primes neutrophils in terms of ROS production, but it has been hypothesised that it activates processes which mediate recruitment of components of the NADPH oxidase to the plasma membrane (Sheppard et al. 2005). In order to produce ROS, all components of the NADPH oxidase must be present at the membrane and some of these components contain phosphorylation sites (Babior 1999, Dang et al. 1999, Chowdhury et al. 2005). Lyp could be responsible for controlling the phosphorylation status of some of these components, which could be differentially regulated in R620W neutrophils.

Overall, this thesis has contributed to the existing knowledge regarding how changes to Lyp phosphatase function may promote the development of autoimmune inflammatory diseases in humans. This work has highlighted the complexity of genetic and environmental interactions which act together to drive the pathogenesis of human disease. Further questions have been raised as to exactly how the genetic variant PTPN22 R620W contributes to the development of autoimmunity, and this work has indicated that there is a need for study of this variant in other immune cell types aside from T cells. With the further work suggested here, these studies could increase our understanding of the complex mechanisms involved in RA pathogenesis and hopefully provide novel therapeutic targets of the future.

REFERENCES

References

Aarnisalo, J., A. Treszl, P. Svec, et al. (2008). "Reduced CD4+T cell activation in children with type 1 diabetes carrying the PTPN22/Lyp 620Trp variant." Journal Of Autoimmunity **31**(1): 13-21.

Abi Abdallah, D. S., C. E. Egan, B. A. Butcher, et al. (2011). "Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation." International Immunology **23**(5): 317-326.

Adrianto, I., F. Wen, A. Templeton, et al. (2011). "Association of a functional variant downstream of TNFAIP3 with systemic lupus erythematosus." Nature Genetics **43**(3): 253-258.

Ahlmen, M., B. Svensson, K. Albertsson, et al. (2010). "Influence of gender on assessments of disease activity and function in early rheumatoid arthritis in relation to radiographic joint damage." Annals of the Rheumatic Diseases **69**(1): 230-233.

Akimaru, K., T. Utsumi, E. F. Sato, et al. (1992). "Role of tyrosyl phosphorylation in neutrophil priming by tumor necrosis factor-alpha and granulocyte colony stimulating factor." Archives of Biochemistry and Biophysics **298**(2): 703-709.

Alberg, A. (2002). "The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients." Toxicology **180**(2): 121-137.

Aletaha, D., T. Neogi, A. J. Silman, et al. (2010). "2010 Rheumatoid Arthritis Classification Criteria An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative." Arthritis and Rheumatism **62**(9): 2569-2581.

Amadori, A., R. Zamarchi, G. De Silvestro, et al. (1995). "Genetic control of the CD4/CD8 T-cell ratio in humans." Nature Medicine **1**(12): 1279-1283.

Amos, C. I., W. V. Chen, A. Lee, et al. (2006). "High-density SNP analysis of 642 Caucasian families with rheumatoid arthritis identifies two new linkage regions on 11p12 and 2q33." Genes and Immunity **7**(4): 277-286.

Arechiga, A. F., T. Habib, Y. He, et al. (2009). "Cutting edge: the PTPN22 allelic variant associated with autoimmunity impairs B cell signaling." Journal of Immunology **182**(6): 3343-3347.

Asami, S., H. Manabe, J. Miyake, et al. (1997). "Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung." Carcinogenesis **18**(9): 1763-1766.

Avraham, H., S. Avraham and Y. Taniguchi (2000). "Receptor protein tyrosine phosphatases in hematopoietic cells." Journal of Hematotherapy & Stem Cell Research **9**(4): 425-432.

Babior, B. M. (1999). "NADPH oxidase: an update." Blood **93**(5): 1464-1476.

Balandraud, N., J. Roudier and C. Roudier (2004). "Epstein-Barr virus and rheumatoid arthritis." Autoimmunity Reviews **3**(5): 362-367.

Bartok, B. and G. S. Firestein (2010). "Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis." Immunological Reviews **233**(1): 233-255.

Bayley, R., K. A. Kite, H. M. McGettrick, et al. (2014). "The autoimmune-associated genetic variant PTPN22 R620W enhances neutrophil activation and function in patients with rheumatoid arthritis and healthy individuals." Annals of the Rheumatic Diseases.

Bayley, R., P. Yang, C. D. Buckley, et al. (2013). "Measuring the specific activity of the protein tyrosine phosphatase Lyp." Journal of Immunological Methods **388**(1-2): 33-39.

Beckman, J. S., R. L. Minor, Jr., C. W. White, et al. (1988). "Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance." The Journal of Biological Chemistry **263**(14): 6884-6892.

Begovich, A. B., V. E. Carlton, L. A. Honigberg, et al. (2004). "A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis." American Journal of Human Genetics **75**(2): 330-337.

Berton, G. and C. A. Lowell (1999). "Integrin signalling in neutrophils and macrophages." Cellular signalling **11**(9): 621-635.

Bezman, N. and G. A. Koretzky (2007). "Compartmentalization of ITAM and integrin signaling by adapter molecules." Immunological Reviews **218**: 9-28.

Blair, H. C., S. L. Teitelbaum, R. Ghiselli, et al. (1989). "Osteoclastic bone resorption by a polarized vacuolar proton pump." Science **245**(4920): 855-857.

Blanco, P., A. K. Palucka, V. Pascual, et al. (2008). "Dendritic cells and cytokines in human inflammatory and autoimmune diseases." Cytokine Growth Factor Reviews **19**(1): 41-52.

Boivin, B., S. Zhang, J. L. Arbiser, et al. (2008). "A modified cysteinyl-labeling assay reveals reversible oxidation of protein tyrosine phosphatases in angiomyolipoma cells." Proceedings of the National Academy of Sciences of the United States of America **105**(29): 9959-9964.

Borgerding, M. and H. Klus (2005). "Analysis of complex mixtures--cigarette smoke." Experimental and Toxicologic Pathology **57 Suppl 1**: 43-73.

Borretzen, M., C. Chapman, J. B. Natvig, et al. (1997). "Differences in mutational patterns between rheumatoid factors in health and disease are related to variable heavy chain family and germ-line gene usage." European Journal of Immunology **27**(3): 735-741.

Bottini, N., L. Musumeci, A. Alonso, et al. (2004). "A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes." Nature Genetics **36**(4): 337-338.

Bottini, N. and E. J. Peterson (2014). "Tyrosine phosphatase PTPN22: multifunctional regulator of immune signaling, development, and disease." Annual Reviews Immunology **32**: 83-119.

Brennan, F. M., R. N. Maini and M. Feldmann (1992). "TNF alpha--a pivotal role in rheumatoid arthritis?" British Journal of Rheumatology **31**(5): 293-298.

Brinkmann, V., U. Reichard, C. Goosmann, et al. (2004). "Neutrophil extracellular traps kill bacteria." Science **303**(5663): 1532-1535.

Bromley, M. and D. E. Woolley (1984). "Chondroclasts and osteoclasts at subchondral sites of erosion in the rheumatoid joint." Arthritis and Rheumatism **27**(9): 968-975.

Brown, P. B., F. A. Nardella and M. Mannik (1982). "Human complement activation by self-associated IgG rheumatoid factors." Arthritis and Rheumatism **25**(9): 1101-1107.

Brownlie, R. J., L. A. Miosge, D. Vassilakos, et al. (2012). "Lack of the phosphatase PTPN22 increases adhesion of murine regulatory T cells to improve their immunosuppressive function." Science Signalling **5**(252): ra87.

Brumell, J. H., C. K. Chan, J. Butler, et al. (1997). "Regulation of Src homology 2-containing tyrosine phosphatase 1 during activation of human neutrophils. Role of protein kinase C." Journal of Biological Chemistry **272**(2): 875-882.

Burkle, A., G. Caselli, C. Franceschi, et al. (2007). "Pathophysiology of ageing, longevity and age related diseases." Immunity & Ageing **4**: 4.

Burn, G. L., L. Svensson, C. Sanchez-Blanco, et al. (2011). "Why is PTPN22 a good candidate susceptibility gene for autoimmune disease?" FEBS Letters **585**(23): 3689-3698.

Buro-Auriemma, L. J., J. Salit, N. R. Hackett, et al. (2013). "Cigarette smoking induces small airway epithelial epigenetic changes with corresponding modulation of gene expression." Human Molecular Genetics.

Cao, Y., J. Yang, K. Colby, et al. (2012). "High basal activity of the PTPN22 gain-of-function variant blunts leukocyte responsiveness negatively affecting IL-10 production in ANCA vasculitis." Plos One **7**(8): e42783.

Carlton, V. E., X. Hu, A. P. Chokkalingam, et al. (2005). "PTPN22 genetic variation: evidence for multiple variants associated with rheumatoid arthritis." American Journal of Human Genetics **77**(4): 567-581.

Carpentier, I. and R. Beyaert (1999). "TRAF1 is a TNF inducible regulator of NF-kappaB activation." FEBS Letters **460**(2): 246-250.

Carruthers, D. M., H. P. Arrol, P. A. Bacon, et al. (2000). "Dysregulated intracellular Ca2+ stores and Ca2+ signaling in synovial fluid T lymphocytes from patients with chronic inflammatory arthritis." Arthritis and Rheumatism **43**(6): 1257-1265.

Cassatella, M. A. (1995). "The production of cytokines by polymorphonuclear neutrophils." Immunology Today **16**(1): 21-26.

Castagnetta, L. A., G. Carruba, O. M. Granata, et al. (2003). "Increased estrogen formation and estrogen to androgen ratio in the synovial fluid of patients with rheumatoid arthritis." Journal of Rheumatology **30**(12): 2597-2605.

Cemerski, S., J. P. van Meerwijk and P. Romagnoli (2003). "Oxidative-stress-induced T lymphocyte hyporesponsiveness is caused by structural modification rather than proteasomal degradation of crucial TCR signaling molecules." European Journal of Immunology **33**(8): 2178-2185.

Chang, H. H., T. S. Tai, B. Lu, et al. (2012). "PTPN22.6, a dominant negative isoform of PTPN22 and potential biomarker of rheumatoid arthritis." PLoS One **7**(3): e33067.

Chien, W., N. Tidow, E. A. Williamson, et al. (2003). "Characterization of a myeloid tyrosine phosphatase, Lyp, and its role in the Bcr-Abl signal transduction pathway." Journal of Biological Chemistry **278**(30): 27413-27420.

Chowdhury, A. K., T. Watkins, N. L. Parinandi, et al. (2005). "Src-mediated tyrosine phosphorylation of p47phox in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells." Journal of Biological Chemistry **280**(21): 20700-20711.

Chrysofakis, G., N. Tzanakis, D. Kyriakoy, et al. (2004). "Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD." Chest **125**(1): 71-76.

Cloutier, J. F. and A. Veillette (1996). "Association of inhibitory tyrosine protein kinase p50csk with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells." The EMBO Journal **15**(18): 4909-4918.

Cohen, S., H. Dadi, E. Shaoul, et al. (1999). "Cloning and characterization of a lymphoid-specific, inducible human protein tyrosine phosphatase, Lyp." Blood **93**(6): 2013-2024.

Cook, N. J., A. J. Silman, J. Propert, et al. (1993). "Features of systemic sclerosis (scleroderma) in an identical twin pair." British Journal of Rheumatology **32**(10): 926-928.

Cooke, M. P. and R. M. Perlmutter (1989). "Expression of a novel form of the fyn proto-oncogene in hematopoietic cells." The New Biologist **1**(1): 66-74.

Coornaert, B., I. Carpentier and R. Beyaert (2009). "A20: central gatekeeper in inflammation and immunity." Journal of Biological Chemistry **284**(13): 8217-8221.

Cope, A. P., H. Schulze-Koops and M. Aringer (2007). "The central role of T cells in rheumatoid arthritis." Clinical and Experimental Rheumatology **25**(5 Suppl 46): S4-11.

Corrigall, V. M. and G. S. Panayi (2002). "Autoantigens and immune pathways in rheumatoid arthritis." Critical Reviews in Immunology **22**(4): 281-293.

Costenbader, K. H., S. C. Chang, I. De Vivo, et al. (2008). "Genetic polymorphisms in PTPN22, PADI-4, and CTLA-4 and risk for rheumatoid arthritis in two longitudinal cohort studies: evidence of gene-environment interactions with heavy cigarette smoking." Arthritis Research & Therapy **10**(3): R52.

Cremasco, V., D. B. Graham, D. V. Novack, et al. (2008). "Vav/Phospholipase Cgamma2-mediated control of a neutrophil-dependent murine model of rheumatoid arthritis." Arthritis and Rheumatism **58**(9): 2712-2722.

Cutolo, M., A. Sulli, S. Capellino, et al. (2004). "Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity." Lupus **13**(9): 635-638.

Cutolo, M., A. Sulli, C. Pizzorni, et al. (2001). "Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis." Annals of the Rheumatic Diseases **60**(8): 729-735.

Dai, X., R. G. James, T. Habib, et al. (2013). "A disease-associated PTPN22 variant promotes systemic autoimmunity in murine models." Journal of Clinical Investigation **123**(5): 2024-2036.

Dang, P. M., C. Dewas, M. Gaudry, et al. (1999). "Priming of human neutrophil respiratory burst by granulocyte/macrophage colony-stimulating factor (GM-CSF) involves partial phosphorylation of p47(phox)." Journal of Biological Chemistry **274**(29): 20704-20708.

Davidson, D., B. Schraven and A. Veillette (2007). "PAG-associated FynT regulates calcium signaling and promotes anergy in T lymphocytes." Molecular and Cellular Biology **27**(5): 1960-1973.

Davies, E. V., B. D. Williams, R. J. Whiston, et al. (1994). "Altered Ca2+ signalling in human neutrophils from inflammatory sites." Annals of the Rheumatic Diseases **53**(7): 446-449.

de Boer, W. I., J. K. Sont, A. van Schadewijk, et al. (2000). "Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD." The Journal of Pathology **190**(5): 619-626.

Deane, K. D. (2014). "Preclinical rheumatoid arthritis (autoantibodies): an updated review." Current Rheumatology Reports **16**(5): 419.

Deane, K. D., C. I. O'Donnell, W. Hueber, et al. (2010). "The number of elevated cytokines and chemokines in preclinical seropositive rheumatoid arthritis predicts time to diagnosis in an age-dependent manner." Arthritis and Rheumatism **62**(11): 3161-3172.

Douroudis, K., A. Shcherbakova, H. Everaus, et al. (2010). "PTPN22 gene regulates natural killer cell proliferation during in vitro expansion." Tissue Antigens **76**(4): 315-318.

Droge, W., K. Schulze-Osthoff, S. Mihm, et al. (1994). "Functions of glutathione and glutathione disulfide in immunology and immunopathology." FASEB Journal **8**(14): 1131-1138.

Drost, E. M., C. Selby, M. M. Bridgeman, et al. (1993). "Decreased leukocyte deformability after acute cigarette smoking in humans." The American Review of Respiratory Disease **148**(5): 1277-1283.

Durden, D. L., H. Rosen, B. R. Michel, et al. (1994). "Protein tyrosine phosphatase inhibitors block myeloid signal transduction through the Fc gamma RI receptor." Experimental Cell Research **211**(1): 150-162.

Edmiston, J. S., J. W. Flora, M. J. Scian, et al. (2009). "Cigarette smoke extract induced protein phosphorylation changes in human microvascular endothelial cells in vitro." Analytical and Bioanalytical Chemistry **394**(6): 1609-1620.

Edwards, C. J. and C. Cooper (2006). "Early environmental factors and rheumatoid arthritis." Clinical and Experimental Immunology **143**(1): 1-5.

Ehrenstein, M. R., J. G. Evans, A. Singh, et al. (2004). "Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy." Journal of Experimental Medicine **200**(3): 277-285.

Emery, P. (2006). "Treatment of rheumatoid arthritis." British Medical Journal **332**(7534): 152-155.

Eyre, S., J. Bowes, D. Diogo, et al. (2012). "High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis." Nature Genetics **44**(12): 1336-1340.

Fearon, D. T. and R. M. Locksley (1996). "The instructive role of innate immunity in the acquired immune response." Science **272**(5258): 50-53.

Feldmann, M., F. M. Brennan and R. N. Maini (1996). "Role of cytokines in rheumatoid arthritis." Annual Review of Immunology **14**: 397-440.

Fialkow, L., C. K. Chan and G. P. Downey (1997). "Inhibition of CD45 during neutrophil activation." Journal of Immunology **158**(11): 5409-5417.

Filby, A., B. Seddon, J. Kleczkowska, et al. (2007). "Fyn regulates the duration of TCR engagement needed for commitment to effector function." Journal of Immunology **179**(7): 4635-4644.

Fiorillo, E., V. Orru, S. M. Stanford, et al. (2010). "Autoimmune-associated PTPN22 R620W variation reduces phosphorylation of lymphoid phosphatase on an inhibitory tyrosine residue." The Journal of Biological Chemistry **285**(34): 26506-26518.

Firestein, G. S. (2003). "Evolving concepts of rheumatoid arthritis." Nature **423**(6937): 356-361.

Fleit, H. B., S. D. Wright and J. C. Unkeless (1982). "Human neutrophil Fc gamma receptor distribution and structure." Proceedings of the National Academy of Sciences of the U S A **79**(10): 3275-3279.

Foyouzi-Youssefi, R., F. Petersson, D. P. Lew, et al. (1997). "Chemoattractant-induced respiratory burst: increases in cytosolic Ca2+ concentrations are essential and synergize with a kinetically distinct second signal." The Biochemical Journal **322 (Pt 3)**: 709-718.

Franceschi, C., M. Capri, D. Monti, et al. (2007). "Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans." Mechanisms of Ageing and Development **128**(1): 92-105.

Frank, C., H. Keilhack, F. Opitz, et al. (1999). "Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation." Biochemistry **38**(37): 11993-12002.

Fukao, T., D. M. Frucht, G. Yap, et al. (2001). "Inducible expression of Stat4 in dendritic cells and macrophages and its critical role in innate and adaptive immune responses." Journal of Immunology **166**(7): 4446-4455.

Gakidis, M. A., X. Cullere, T. Olson, et al. (2004). "Vav GEFs are required for beta2 integrin-dependent functions of neutrophils." The Journal of Cell Biology **166**(2): 273-282.

Gaschler, G. J., M. Skrtic, C. C. Zavitz, et al. (2009). "Bacteria challenge in smoke-exposed mice exacerbates inflammation and skews the inflammatory profile." American Journal of Respiratory and Critical Care Medicine **179**(8): 666-675.

Gaudry, M., A. C. Caon, C. Gilbert, et al. (1992). "Evidence for the involvement of tyrosine kinases in the locomotory responses of human neutrophils." Journal of Leukocyte Biology **51**(2): 103-108.

Geng, Y., S. M. Savage, L. J. Johnson, et al. (1995). "Effects of nicotine on the immune response. I. Chronic exposure to nicotine impairs antigen receptor-mediated signal transduction in lymphocytes." Toxicology and Applied Pharmacology **135**(2): 268-278.

Ghio, A. J., E. D. Hilborn, J. G. Stonehuerner, et al. (2008). "Particulate matter in cigarette smoke alters iron homeostasis to produce a biological effect." American Journal of Respiratory and Critical Care Medicine **178**(11): 1130-1138.

Gillis, S. and J. Watson (1980). "Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2-producing human leukemia T cell line." The Journal of Experimental Medicine **152**(6): 1709-1719.

Gjorloff-Wingren, A., M. Saxena, S. Williams, et al. (1999). "Characterization of TCR-induced receptor-proximal signaling events negatively regulated by the protein tyrosine phosphatase PEP." European Journal of Immunology **29**(12): 3845-3854.

Glader, P., S. Moller, J. Lilja, et al. (2006). "Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells." Respiratory Medicine **100**(5): 818-827.

Gomez-Cambronero, J., C. K. Huang, V. A. Bonak, et al. (1989). "Tyrosine phosphorylation in human neutrophil." Biochemical and Biophysical Research Communications **162**(3): 1478-1485.

Gomez, L. M., J. M. Anaya and J. Martin (2005). "Genetic influence of PTPN22 R620W polymorphism in tuberculosis." Human Immunology **66**(12): 1242-1247.

Goncalves, R. B., R. D. Coletta, K. G. Silverio, et al. (2011). "Impact of smoking on inflammation: overview of molecular mechanisms." Inflammation Research **60**(5): 409-424.

Gosselin, E. J., K. Wardwell, W. F. Rigby, et al. (1993). "Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3." Journal of Immunology **151**(3): 1482-1490.

Gregersen, P. K. and F. Batliwalla (2005). "PTPN22 and rheumatoid arthritis: Gratifying replication." Arthritis and Rheumatism **52**(7): 1952-1955.

Gregersen, P. K., J. Silver and R. J. Winchester (1987). "The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis." Arthritis and Rheumatism **30**(11): 1205-1213.

Groen, A., S. Lemeer, T. van der Wijk, et al. (2005). "Differential oxidation of protein-tyrosine phosphatases." The Journal of Biological Chemistry **280**(11): 10298-10304.

Guatura, S. B., J. A. Martinez, P. C. Santos Bueno, et al. (2000). "Increased exhalation of hydrogen peroxide in healthy subjects following cigarette consumption." Sao Paulo Medical Journal **118**(4): 93-98.

Gubner, R., S. August and V. Ginsberg (1951). "Therapeutic suppression of tissue reactivity. II. Effect of aminopterin in rheumatoid arthritis and psoriasis." The American Journal of the Medical Sciences **221**(2): 176-182.

Guo, R. F. and P. A. Ward (2005). "Role of C5a in inflammatory responses." Annual Reviews Immunology **23**: 821-852.

Hammer, J., F. Gallazzi, E. Bono, et al. (1995). "Peptide binding specificity of HLA-DR4 molecules: correlation with rheumatoid arthritis association." Journal of Experimental Medicine **181**(5): 1847-1855.

Han, M. K., J. S. Kim, B. H. Park, et al. (2003). "NF-kappaB-dependent lymphocyte hyperadhesiveness to synovial fibroblasts by hypoxia and reoxygenation: potential role in rheumatoid arthritis." Journal of Leukocyte Biology **73**(4): 525-529.

Harvath, L., J. A. Balke, N. P. Christiansen, et al. (1991). "Selected antibodies to leukocyte common antigen (CD45) inhibit human neutrophil chemotaxis." Journal of Immunology **146**(3): 949-957.

Hasegawa, K., F. Martin, G. Huang, et al. (2004). "PEST domain-enriched tyrosine phosphatase (PEP) regulation of effector/memory T cells." Science **303**(5658): 685-689.

Hermiston, M. L., J. Zikherman and J. W. Zhu (2009). "CD45, CD148, and Lyp/Pep: critical phosphatases regulating Src family kinase signaling networks in immune cells." Immunological Reviews **228**(1): 288-311.

Hill, J. A., S. Southwood, A. Sette, 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." Journal of Immunology **171**(2): 538-541.

Hill, R. J., S. Zozulya, Y. L. Lu, et al. (2002). "The lymphoid protein tyrosine phosphatase Lyp interacts with the adaptor molecule Grb2 and functions as a negative regulator of T-cell activation." Experimental Hematology **30**(3): 237-244.

Hitchon, C. A. and H. S. El-Gabalawy (2004). "Oxidation in rheumatoid arthritis." Arthritis Research and Therapy **6**(6): 265-278.

Hoffmeyer, F., K. Witte, U. Gebhardt, et al. (1995). "The low affinity Fc gamma RIIa and Fc gamma RIIIb on polymorphonuclear neutrophils are differentially regulated by CD45 phosphatase." Journal of Immunology **155**(8): 4016-4023.

Hogg, J. C., F. Chu, S. Utokaparch, et al. (2004). "The nature of small-airway obstruction in chronic obstructive pulmonary disease." The New England Journal of Medicine **350**(26): 2645-2653.

Holmes, N. (2006). "CD45: all is not yet crystal clear." Immunology **117**(2): 145-155.

Holt, P. G. (1987). "Immune and inflammatory function in cigarette smokers." Thorax **42**(4): 241-249.

Huang, Q., Y. Ma, A. Adebayo, et al. (2007). "Increased macrophage activation mediated through toll-like receptors in rheumatoid arthritis." Arthritis and Rheumatism **56**(7): 2192-2201.

Huang, Z., N. A. Olson, W. You, et al. (1992). "A sensitive competitive ELISA for 2,4-dinitrophenol using 3,6-fluorescein diphosphate as a fluorogenic substrate." Journal of Immunological Methods **149**(2): 261-266.

Isomaki, P., M. Panesar, A. Annenkov, et al. (2001). "Prolonged exposure of T cells to TNF down-regulates TCR zeta and expression of the TCR/CD3 complex at the cell surface." Journal of Immunology **166**(9): 5495-5507.

Jahnsen, F. L., D. H. Strickland, J. A. Thomas, et al. (2006). "Accelerated antigen sampling and transport by airway mucosal dendritic cells following inhalation of a bacterial stimulus." Journal of Immunology **177**(9): 5861-5867.

Jaswal, S., H. C. Mehta, A. K. Sood, et al. (2003). "Antioxidant status in rheumatoid arthritis and role of antioxidant therapy." Clinical Chimica Acta **338**(1-2): 123-129.

Johnson, K. G., S. K. Bromley, M. L. Dustin, et al. (2000). "A supramolecular basis for CD45 tyrosine phosphatase regulation in sustained T cell activation." Proceedings of the National Academy of Sciences of the United States of America **97**(18): 10138-10143.

Jury, E. C., P. S. Kabouridis, A. Abba, et al. (2003). "Increased ubiquitination and reduced expression of LCK in T lymphocytes from patients with systemic lupus erythematosus." Arthritis and Rheumatism **48**(5): 1343-1354.

Kallberg, H., B. Ding, L. Padyukov, et al. (2011). "Smoking is a major preventable risk factor for rheumatoid arthritis: estimations of risks after various exposures to cigarette smoke." Annals of the Rheumatic Diseases **70**(3): 508-511.

Kallberg, H., L. Padyukov, R. M. Plenge, et al. (2007). "Gene-gene and gene-environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis." American Journal of Human Genetics **80**(5): 867-875.

Kay, B. K., M. P. Williamson and M. Sudol (2000). "The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains." The FASEB Journal **14**(2): 231-241.

Kitamura, H., K. Iwakabe, T. Yahata, et al. (1999). "The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells." Journal of Experimental Medicine **189**(7): 1121-1128.

Kokkonen, H., M. Johansson, L. Innala, et al. (2007). "The PTPN22 1858C/T polymorphism is associated with anti-cyclic citrullinated peptide antibody-positive early rheumatoid arthritis in northern Sweden." Arthritis Research and Therapy **9**(3): R56.

Kolaczkowska, E. and P. Kubes (2013). "Neutrophil recruitment and function in health and inflammation." Nature reviews. Immunology **13**(3): 159-175.

Kraaij, M. D., N. D. Savage, S. W. van der Kooij, et al. (2010). "Induction of regulatory T cells by macrophages is dependent on production of reactive oxygen species." Proceedings of the National Academy of Sciences of the United States of America **107**(41): 17686-17691.

Kremer, J. M., R. Westhovens, M. Leon, et al. (2003). "Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig." New England Journal of Medicine **349**(20): 1907-1915.

Kruger, J., J. R. Butler, V. Cherapanov, et al. (2000). "Deficiency of Src homology 2-containing phosphatase 1 results in abnormalities in murine neutrophil function: Studies in motheaten mice." Journal of Immunology **165**(10): 5847-5859.

Kruger, J. M., T. Fukushima, V. Cherepanov, et al. (2002). "Protein-tyrosine phosphatase MEG2 is expressed by human neutrophils. Localization to the phagosome and activation by polyphosphoinositides." Journal of Biological Chemistry **277**(4): 2620-2628.

Kurts, C., H. Kosaka, F. R. Carbone, et al. (1997). "Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells." Journal of Experimental Medicine **186**(2): 239-245.

Kuschner, W. G., A. D'Alessandro, H. Wong, et al. (1996). "Dose-dependent cigarette smoking-related inflammatory responses in healthy adults." The European Respiratory Journal **9**(10): 1989-1994.

Lambert, C., J. McCue, M. Portas, et al. (2005). "Acrolein in cigarette smoke inhibits T-cell responses." Journal of Allergy and Clinical Immunology **116**(4): 916-922.

Larbre, J. P., A. R. Moore, J. A. Da Silva, et al. (1994). "Direct degradation of articular cartilage by rheumatoid synovial fluid: contribution of proteolytic enzymes." The Journal of Rheumatology **21**(10): 1796-1801.

Lard, L. R., H. Visser, I. Speyer, et al. (2001). "Early versus delayed treatment in patients with recent-onset rheumatoid arthritis: comparison of two cohorts who received different treatment strategies." The American Journal of Medicine **111**(6): 446-451.
Lee, D. M. and M. E. Weinblatt (2001). "Rheumatoid arthritis." Lancet **358**(9285): 903-911.

Lee, E. G., D. L. Boone, S. Chai, et al. (2000). "Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice." Science **289**(5488): 2350-2354.

Lefvert, A. K., Y. Zhao, R. Ramanujam, et al. (2008). "PTPN22 R620W promotes production of anti-AChR autoantibodies and IL-2 in myasthenia gravis." Journal of Neuroimmunology **197**(2): 110-113.

Lemaitre, V., A. J. Dabo and J. D'Armiento (2011). "Cigarette smoke components induce matrix metalloproteinase-1 in aortic endothelial cells through inhibition of mTOR signaling." Toxicological Sciences **123**(2): 542-549.

Ley, K., C. Laudanna, M. I. Cybulsky, et al. (2007). "Getting to the site of inflammation: the leukocyte adhesion cascade updated." Nature reviews. Immunology **7**(9): 678-689.

Li, P., M. Li, M. R. Lindberg, et al. (2010). "PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps." Journal of Experimental Medicine **207**(9): 1853-1862.

Liao, K. P., L. Alfredsson and E. W. Karlson (2009). "Environmental influences on risk for rheumatoid arthritis." Current Opinion in Rheumatology **21**(3): 279-283.

Lichtman, M. A., J. H. Vaughan and C. G. Hames (1967). "The distribution of serum immunoglobulins, anti-gamma-G globulins ("rheumatoid factors") and antinuclear antibodies in White and Negro subjects in Evans County, Georgia." Arthritis and Rheumatism **10**(3): 204-215.

Linn-Rasker, S. P., A. H. van der Helm-van Mil, F. A. van Gaalen, et al. (2006). "Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles." Annals of the Rheumatic Diseases **65**(3): 366-371.

Liu, Y., S. K. Shaw, S. Ma, et al. (2004). "Regulation of leukocyte transmigration: cell surface interactions and signaling events." Journal of Immunology **172**(1): 7-13.

Lorenz, U. (2011). "Protein tyrosine phosphatase assays." Current Protocols in Immunology **Chapter 11**: Unit 11 17.

Lutzky, V., S. Hannawi and R. Thomas (2007). "Cells of the synovium in rheumatoid arthritis. Dendritic cells." Arthritis Research and Therapy **9**(4): 219.

MacGregor, A. J., H. Snieder, A. S. Rigby, et al. (2000). "Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins." Arthritis and Rheumatism **43**(1): 30-37.

Mahdi, H., B. A. Fisher, H. Kallberg, et al. (2009). "Specific interaction between genotype, smoking and autoimmunity to citrullinated alpha-enolase in the etiology of rheumatoid arthritis." Nature Genetics **41**(12): 1319-1324.

Maine, C. J., E. E. Hamilton-Williams, J. Cheung, et al. (2012). "PTPN22 alters the development of regulatory T cells in the thymus." Journal of Immunology **188**(11): 5267-5275.

Majeed, M., E. Caveggion, C. A. Lowell, et al. (2001). "Role of Src kinases and Syk in Fcgamma receptor-mediated phagocytosis and phagosome-lysosome fusion." Journal of Leukocyte Biology **70**(5): 801-811.

Majo, J., H. Ghezzo and M. G. Cosio (2001). "Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema." The European Respiratory Journal **17**(5): 946-953.

Manning, G., D. B. Whyte, R. Martinez, et al. (2002). "The protein kinase complement of the human genome." Science **298**(5600): 1912-1934.

Mantovani, A., M. A. Cassatella, C. Costantini, et al. (2011). "Neutrophils in the activation and regulation of innate and adaptive immunity." Nature reviews. Immunology **11**(8): 519-531.

Martinez, F. O., L. Helming and S. Gordon (2009). "Alternative activation of macrophages: an immunologic functional perspective." Annual Review of Immunology **27**: 451-483.

Maurice, M. M., A. C. Lankester, A. C. Bezemer, et al. (1997). "Defective TCR-mediated signaling in synovial T cells in rheumatoid arthritis." Journal of Immunology **159**(6): 2973-2978.

McGettrick, H. M., L. M. Butler and G. B. Nash (2007). "Analysis of leukocyte migration through monolayers of cultured endothelial cells." Methods in Molecular Biology **370**: 37-54.

McGettrick, H. M., K. Hunter, P. A. Moss, et al. (2009). "Direct observations of the kinetics of migrating T cells suggest active retention by endothelial cells with continual bidirectional migration." Journal of Leukocyte Biology **85**(1): 98-107.

McNeill, L., R. J. Salmond, J. C. Cooper, et al. (2007). "The differential regulation of Lck kinase phosphorylation sites by CD45 is critical for T cell receptor signaling responses." Immunity **27**(3): 425-437.

Megiovanni, A. M., F. Sanchez, M. Robledo-Sarmiento, et al. (2006). "Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes." Journal of Leukocyte Biology **79**(5): 977-988.

Menard, L., D. Saadoun, I. Isnardi, et al. (2011). "The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans." Journal of Clinical Investigation **121**(9): 3635-3644.

Meng, T. C., T. Fukada and N. K. Tonks (2002). "Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo." Molecular Cell **9**(2): 387-399.

Miller, C. L., R. Longnecker and E. Kieff (1993). "Epstein-Barr virus latent membrane protein 2A blocks calcium mobilization in B lymphocytes." Journal of Virology **67**(6): 3087-3094.

Minton, K. (2012). "Regulatory T cells: Stabilizing immune regulation in the gut." Nature Reviews. Immunology **12**(6): 397.

Mocsai, A., M. J. Zhou, F. Y. Meng, et al. (2002). "Syk is required for integrin signaling in neutrophils." Immunity **16**(4): 547-558.

Mohr, W., H. Westerhellweg and D. Wessinghage (1981). "Polymorphonuclear granulocytes in rheumatic tissue destruction. III. an electron microscopic study of PMNs at the pannus-cartilage junction in rheumatoid arthritis." Annals of the Rheumatic Diseases **40**(4): 396-399.

Montalibet, J., K. I. Skorey and B. P. Kennedy (2005). "Protein tyrosine phosphatase: enzymatic assays." Methods **35**(1): 2-8.

Moores, S. L., L. M. Selfors, J. Fredericks, et al. (2000). "Vav family proteins couple to diverse cell surface receptors." Molecular and Cell Biology **20**(17): 6364-6373.

Mortaz, E., A. D. Kraneveld, J. J. Smit, et al. (2009). "Effect of cigarette smoke extract on dendritic cells and their impact on T-cell proliferation." PLoS One **4**(3): e4946.

Motz, G. T., B. L. Eppert, G. Sun, et al. (2008). "Persistence of lung CD8 T cell oligoclonal expansions upon smoking cessation in a mouse model of cigarette smoke-induced emphysema." Journal of Immunology **181**(11): 8036-8043.

Motz, G. T., B. L. Eppert, S. C. Wesselkamper, et al. (2010). "Chronic cigarette smoke exposure generates pathogenic T cells capable of driving COPD-like disease in Rag2-/- mice." American Journal of Respiratory and Critical Care Medicine **181**(11): 1223-1233.

Mulherin, D., O. Fitzgerald and B. Bresnihan (1996). "Synovial tissue macrophage populations and articular damage in rheumatoid arthritis." Arthritis and Rheumatism **39**(1): 115-124.

Mustelin, T. and A. Altman (1990). "Dephosphorylation and activation of the T cell tyrosine kinase pp56lck by the leukocyte common antigen (CD45)." Oncogene **5**(6): 809-813.

Mydel, P., Z. Wang, M. Brisslert, et al. (2010). "Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis." Journal of Immunology **184**(12): 6882-6890.

Nathan, C. (2006). "Neutrophils and immunity: challenges and opportunities." Nature Reviews. Immunology **6**(3): 173-182.

Nell, V. P. K., K. P. Machold, T. A. Stamm, et al. (2005). "Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis." Annals of the Rheumatic Diseases **64**(12): 1731-1736.

Nielen, M. M., D. van Schaardenburg, H. W. Reesink, et al. (2004). "Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors." Arthritis and Rheumatism **50**(2): 380-386.

Niggli, V. (2003). "Signaling to migration in neutrophils: importance of localized pathways." The International Journal of Biochemistry & Cell Biology **35**(12): 1619-1638.

Noss, E. H. and M. B. Brenner (2008). "The role and therapeutic implications of fibroblast-like synoviocytes in inflammation and cartilage erosion in rheumatoid arthritis." Immunological Reviews **223**: 252-270.

Okada, Y., D. Wu, G. Trynka, et al. (2014). "Genetics of rheumatoid arthritis contributes to biology and drug discovery." Nature **506**(7488): 376-381.

Orru, V., S. J. Tsai, B. Rueda, et al. (2009). "A loss-of-function variant of PTPN22 is associated with reduced risk of systemic lupus erythematosus." Human Molecular Genetics **18**(3): 569-579.

Ouyang, Y., N. Virasch, P. Hao, et al. (2000). "Suppression of human IL-1beta, IL-2, IFN-gamma, and TNF-alpha production by cigarette smoke extracts." Journal of Allergy and Clinical Immunology **106**(2): 280-287.

Pahl, H. L. (1999). "Activators and target genes of Rel/NF-kappa B transcription factors." Oncogene **18**(49): 6853-6866.

Pelletier, M., L. Maggi, A. Micheletti, et al. (2010). "Evidence for a cross-talk between human neutrophils and Th17 cells." Blood **115**(2): 335-343.

Pereira, S. and C. Lowell (2003). "The Lyn tyrosine kinase negatively regulates neutrophil integrin signaling." Journal of Immunology **171**(3): 1319-1327.

Peters, G. H., T. M. Frimurer and O. H. Olsen (1998). "Electrostatic evaluation of the signature motif (H/V)CX5R(S/T) in protein-tyrosine phosphatases." Biochemistry **37**(16): 5383-5393.

Plenge, R. M., C. Cotsapas, L. Davies, et al. (2007). "Two independent alleles at 6q23 associated with risk of rheumatoid arthritis." Nature Genetics **39**(12): 1477-1482.

Pozzan, T., D. P. Lew, C. B. Wollheim, et al. (1983). "Is Cytosolic Ionized Calcium Regulating Neutrophil Activation." Science **221**(4618): 1413-1415.

Pronai, L., Y. Ichikawa, K. Ichimori, et al. (1991). "Association of enhanced superoxide generation by neutrophils with low superoxide scavenging activity of the peripheral blood, joint fluid, and their leukocyte components in rheumatoid arthritis: effects of slow-acting anti-rheumatic drugs and disease activity." Clinical and Experimental Rheumatology **9**(2): 149-155.

Raychaudhuri, S., C. Sandor, E. A. Stahl, et al. (2012). "Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis." Nature Genetics **44**(3): 291-296.

Raza, K., M. Breese, P. Nightingale, et al. (2005). "Predictive value of antibodies to cyclic citrullinated peptide in patients with very early inflammatory arthritis." The Journal of Rheumatology **32**(2): 231-238.

Raza, K., C. E. Buckley, M. Salmon, et al. (2006). "Treating very early rheumatoid arthritis." Best Practice & Research in Clinical Rheumatology **20**(5): 849-863.

Remmers, E. F., R. M. Plenge, A. T. Lee, et al. (2007). "STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus." New England Journal of Medicine **357**(10): 977-986.

Reumaux, D., P. L. Hordijk, P. Duthilleul, et al. (2006). "Priming by tumor necrosis factor-alpha of human neutrophil NADPH-oxidase activity induced by anti-proteinase-3 or anti-myeloperoxidase antibodies." Journal of Leukocyte Biology **80**(6): 1424-1433.

Rhee, S. G. (1999). "Redox signaling: hydrogen peroxide as intracellular messenger." Experimental & Molecular Medicine **31**(2): 53-59.

Rider, D. A., R. Bayley, E. Clay, et al. (2013). "Does oxidative inactivation of CD45 phosphatase in rheumatoid arthritis underlie immune hyporesponsiveness?" Antioxidants & Redox Signaling.

Rider, D. A., A. J. Sinclair and S. P. Young (2003). "Oxidative inactivation of CD45 protein tyrosine phosphatase may contribute to T lymphocyte dysfunction in the elderly." Mechanisms of Ageing and Development **124**(2): 191-198.

Rieck, M., A. Arechiga, S. Onengut-Gumuscu, et al. (2007). "Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes." Journal of Immunology **179**(7): 4704-4710.

Robbins, C. S., F. Franco, M. Mouded, et al. (2008). "Cigarette smoke exposure impairs dendritic cell maturation and T cell proliferation in thoracic lymph nodes of mice." Journal of Immunology **180**(10): 6623-6628.

Rosales, C., ; Uribe-Querol, E. (2013). "Fc receptors: Cell activators of antibody functions." Advances in Bioscience and Biotechnology **4**(4A): 21-33.

Russell, R. E., A. Thorley, S. V. Culpitt, et al. (2002). "Alveolar macrophage-mediated elastolysis: roles of matrix metalloproteinases, cysteine, and serine proteases." American Journal of Physiology **283**(4): L867-873.

Sallusto, F. and A. Lanzavecchia (1999). "Mobilizing dendritic cells for tolerance, priming, and chronic inflammation." Journal of Experimental Medicine **189**(4): 611-614.

Salvarani, C., P. L. Macchioni, W. Mantovani, et al. (1998). "HLA-DRB1 alleles associated with rheumatoid arthritis in Northern Italy: correlation with disease severity." British Journal of Rheumatology **37**(2): 165-169.

Sato, K., A. Suematsu, K. Okamoto, et al. (2006). "Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction." Journal of Experimental Medicine **203**(12): 2673-2682.

Scally, S. W., J. Petersen, S. C. Law, et al. (2013). "A molecular basis for the association of the HLA-DRB1 locus, citrullination, and rheumatoid arthritis." Journal of Experimental Medicine **210**(12): 2569-2582.

Schaff, U. Y., I. Yamayoshi, T. Tse, et al. (2008). "Calcium flux in neutrophils synchronizes beta2 integrin adhesive and signaling events that guide inflammatory recruitment." Annals of Biomedical Engineering **36**(4): 632-646.

Schett, G. (2007). "Cells of the synovium in rheumatoid arthritis. Osteoclasts." Arthritis Research Therapy **9**(1): 203.

Scott, D. L., F. Wolfe and T. W. Huizinga (2010). "Rheumatoid arthritis." The Lancet **376**(9746): 1094-1108.

Scott, I. C., S. Steer, C. M. Lewis, et al. (2011). "Precipitating and perpetuating factors of rheumatoid arthritis immunopathology: linking the triad of genetic predisposition, environmental risk factors and autoimmunity to disease pathogenesis." Best Practice & Research. Clinical Rheumatology **25**(4): 447-468.

Shadidi, K. R., K. M. Thompson, J. E. Henriksen, et al. (2002). "Association of antigen specificity and migratory capacity of memory T cells in rheumatoid arthritis." Scandinavian Journal of Immunology **55**(3): 274-283.

Sheppard, F. R., M. R. Kelher, E. E. Moore, et al. (2005). "Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation." Journal of Leukocyte Biology **78**(5): 1025-1042.

Shi, J., R. Knevel, P. Suwannalai, et al. (2011). "Autoantibodies recognizing 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 **108**(42): 17372-17377.

Shigeyama, Y., T. Pap, P. Kunzler, et al. (2000). "Expression of osteoclast differentiation factor in rheumatoid arthritis." Arthritis and Rheumatism **43**(11): 2523-2530.

Silverman, G. J. and D. A. Carson (2003). "Roles of B cells in rheumatoid arthritis." Arthritis Research and Therapy **5 Suppl 4**: S1-6.

Sirpal, S. (2009). "Myeloperoxidase-mediated lipoprotein carbamylation as a mechanistic pathway for atherosclerotic vascular disease." Clinical Science (London; England: 1979) **116**(9): 681-695.

Smith, C. J. and C. Hansch (2000). "The relative toxicity of compounds in mainstream cigarette smoke condensate." Food and Chemical Toxicology **38**(7): 637-646.

Sopori, M. (2002). "Effects of cigarette smoke on the immune system." Nature Reviews. Immunology **2**(5): 372-377.

Spalinger, M. R., S. Lang, A. Weber, et al. (2013). "Loss of Protein Tyrosine Phosphatase Nonreceptor Type 22 Regulates Interferon-gamma-Induced Signaling in Human Monocytes." Gastroenterology.

Stampfli, M. R. and G. P. Anderson (2009). "How cigarette smoke skews immune responses to promote infection, lung disease and cancer." Nature Reviews. Immunology **9**(5): 377-384.

Stecher, R. M. (1958). "Ankylosis of the finger joints in rheumatoid arthritis." Annals of the Rheumatic Diseases **17**(4): 365-375.

Steptoe, R. J., J. M. Ritchie, L. K. Jones, et al. (2005). "Autoimmune diabetes is suppressed by transfer of proinsulin-encoding Gr-1+ myeloid progenitor cells that differentiate in vivo into resting dendritic cells." Diabetes **54**(2): 434-442.

Storey, G. O., M. Comer and D. L. Scott (1994). "Chronic Arthritis before 1876 - Early British Cases Suggesting Rheumatoid-Arthritis." Annals of the Rheumatic Diseases **53**(9): 557-560.

Straus, D. B. and A. Weiss (1992). "Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor." Cell **70**(4): 585-593.

Sugiyama, D., K. Nishimura, K. Tamaki, et al. (2010). "Impact of smoking as a risk factor for developing rheumatoid arthritis: a meta-analysis of observational studies." Annals of the Rheumatic Diseases **69**(1): 70-81.

Suzuki, A., R. Yamada, X. Chang, et al. (2003). "Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis." Nature Genetics **34**(4): 395-402.

Svensson, L., G. Burn, C. Sanchez-Blanco, et al. (2011). "Lyp/Ptpn22 Is a Negative Regulator of Integrin Mediated T Cell Adhesion and Migration; the Disease Associated Ptpn22 Allelic Variant Is a Loss of Function Mutant That Perturbs T Cell Migration." Annals of the Rheumatic Diseases **70**: A7-A7.

Swat, W. and K. Fujikawa (2005). "The Vav family: at the crossroads of signaling pathways." Immunologic research **32**(1-3): 259-265.

Symmons, D., G. Turner, R. Webb, et al. (2002). "The prevalence of rheumatoid arthritis in the United Kingdom: new estimates for a new century." Rheumatology **41**(7): 793-800.

Tak, P. P., T. J. Smeets, M. R. Daha, et al. (1997). "Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity." Arthritis and Rheumatism **40**(2): 217-225.

Takakura, K., J. S. Beckman, L. A. MacMillan-Crow, et al. (1999). "Rapid and irreversible inactivation of protein tyrosine phosphatases PTP1B, CD45, and LAR by peroxynitrite." Archives of Biochemistry and Biophysics **369**(2): 197-207.

Takemura, S., P. A. Klimiuk, A. Braun, et al. (2001). "T cell activation in rheumatoid synovium is B cell dependent." Journal of Immunology **167**(8): 4710-4718.

Takesono, A., L. D. Finkelstein and P. L. Schwartzberg (2002). "Beyond calcium: new signaling pathways for Tec family kinases." Journal of Cell Science **115**(Pt 15): 3039-3048.

Tengstrand, B., K. Carlstrom, L. Fellander-Tsai, et al. (2003). "Abnormal levels of serum dehydroepiandrosterone, estrone, and estradiol in men with rheumatoid arthritis: high correlation between serum estradiol and current degree of inflammation." Journal of Rheumatology **30**(11): 2338-2343.

Tetlow, L. C., M. Lees, Y. Ogata, et al. (1993). "Differential expression of gelatinase B (MMP-9) and stromelysin-1 (MMP-3) by rheumatoid synovial cells in vitro and in vivo." Rheumatology International **13**(2): 53-59.

Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, et al. (1996). "Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells." Nature **382**(6587): 171-174.

Thomas, M. L. (1989). "The leukocyte common antigen family." Annual Review of Immunology **7**: 339-369.

Thomas, M. L. and L. Lefrancois (1988). "Differential expression of the leucocyte-common antigen family." Immunology Today **9**(10): 320-326.

Thomas, R., L. S. Davis and P. E. Lipsky (1994). "Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells." Journal of Immunology **152**(5): 2613-2623.

Thomas, S. M. and J. S. Brugge (1997). "Cellular functions regulated by Src family kinases." Annual Review of Cell and Developmental Biology **13**: 513-609.

Thomson, W., A. Barton, X. Ke, et al. (2007). "Rheumatoid arthritis association at 6q23." Nature Genetics **39**(12): 1431-1433.

Tollerud, D. J., J. W. Clark, L. M. Brown, et al. (1989). "Association of cigarette smoking with decreased numbers of circulating natural killer cells." The American Review of Respiratory Disease **139**(1): 194-198.

Toms, T. E., V. F. Panoulas, J. P. Smith, et al. (2011). "Rheumatoid arthritis susceptibility genes associate with lipid levels in patients with rheumatoid arthritis." Annals of the Rheumatic Diseases **70**(6): 1025-1032.

Tonks, N. K. (2006). "Protein tyrosine phosphatases: from genes, to function, to disease." Nature Reviews. Molecular Cell Biology **7**(11): 833-846.

Trouw, L. A., T. W. Huizinga and R. E. Toes (2013). "Autoimmunity in rheumatoid arthritis: different antigens--common principles." Annals of the Rheumatic Diseases **72 Suppl 2**: ii132-136.

Tsai, S. J., U. Sen, L. Zhao, et al. (2009). "Crystal structure of the human lymphoid tyrosine phosphatase catalytic domain: insights into redox regulation." Biochemistry **48**(22): 4838-4845.

Tsoumakidou, M., I. K. Demedts, G. G. Brusselle, et al. (2008). "Dendritic cells in chronic obstructive pulmonary disease: new players in an old game." American Journal of Respiratory and Critical Care Medicine **177**(11): 1180-1186.

Tsuchiya, M., A. Asada, E. Kasahara, et al. (2002). "Smoking a single cigarette rapidly reduces combined concentrations of nitrate and nitrite and concentrations of antioxidants in plasma." Circulation **105**(10): 1155-1157.

Turrens, J. F. (2003). "Mitochondrial formation of reactive oxygen species." The Journal of Physiology **552**(Pt 2): 335-344.

Turunen, S., M. K. Koivula, L. Risteli, et al. (2010). "Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits." Arthritis and Rheumatism **62**(11): 3345-3352.

van der Helm-van Mil, A. H., K. N. Verpoort, S. le Cessie, et al. (2007). "The HLA-DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide." Arthritis and Rheumatism **56**(2): 425-432.

van der Vaart, H., D. S. Postma, W. Timens, et al. (2004). "Acute effects of cigarette smoke on inflammation and oxidative stress: a review." Thorax **59**(8): 713-721.

van Gisbergen, K. P., M. Sanchez-Hernandez, T. B. Geijtenbeek, et al. (2005). "Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN." The Journal of Experimental Medicine **201**(8): 1281-1292.

Van Snick, J. L., E. Van Roost, B. Markowetz, et al. (1978). "Enhancement by IgM rheumatoid factor of in vitro ingestion by macrophages and in vivo clearance of aggregated IgG or antigen-antibody complexes." European Journal of Immunology **8**(4): 279-285.

Van Veldhoven, P. P. and G. P. Mannaerts (1987). "Inorganic and organic phosphate measurements in the nanomolar range." Analytical Biochemistry **161**(1): 45-48.

Vang, T., M. Congia, M. D. Macis, et al. (2005). "Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant." Nature Genetics **37**(12): 1317-1319.

Vang, T., J. Landskron, M. K. Viken, et al. (2013). "The autoimmune-predisposing variant of lymphoid tyrosine phosphatase favors T helper 1 responses." Human Immunology **74**(5): 574-585.

Vang, T., W. H. Liu, L. Delacroix, et al. (2012). "LYP inhibits T-cell activation when dissociated from CSK." Nature Chemical Biology **8**(5): 437-446.

Vassallo, R., K. Tamada, J. S. Lau, et al. (2005). "Cigarette smoke extract suppresses human dendritic cell function leading to preferential induction of Th-2 priming." Journal of Immunology **175**(4): 2684-2691.

Vaughan, J. H. and T. Chihara (1975). "Lymphocyte function in rheumatic disorders." Archives of Internal Medicine **135**(10): 1324-1328.

Vessey, M. P., L. Villard-Mackintosh and D. Yeates (1987). "Oral contraceptives, cigarette smoking and other factors in relation to arthritis." Contraception **35**(5): 457-464.

Viatte, S., D. Plant and S. Raychaudhuri (2013). "Genetics and epigenetics of rheumatoid arthritis." Nature Reviews Rheumatology **9**(3): 141-153.

Vossenaar, E. R., T. R. Radstake, A. van der Heijden, et al. (2004). "Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages." Annals of the Rheumatic Diseases **63**(4): 373-381.

Wang, S., H. Dong, J. Han, et al. (2010). "Identification of a variant form of tyrosine phosphatase LYP." BMC Molecular Biology **11**: 78.

Wang, Y., W. Guo, L. Liang, et al. (1999). "Phosphorylation of CD45 by casein kinase 2. Modulation of activity and mutational analysis." The Journal of Biological Chemistry **274**(11): 7454-7461.

Wang, Y., I. Shaked, S. M. Stanford, et al. (2013). "The autoimmunity-associated gene PTPN22 potentiates toll-like receptor-driven, type 1 interferon-dependent immunity." Immunity **39**(1): 111-122.

Wang, Z., S. J. Nicholls, E. R. Rodriguez, et al. (2007). "Protein carbamylation links inflammation, smoking, uremia and atherogenesis." Nature Medicine **13**(10): 1176-1184.

Watford, W. T., B. D. Hissong, J. H. Bream, et al. (2004). "Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4." Immunological Reviews **202**: 139-156.

Weissmann, G. and H. Korchak (1984). "Rheumatoid arthritis. The role of neutrophil activation." Inflammation **8 Suppl**: S3-14.

Winterbourn, C. C. and M. B. Hampton (2008). "Thiol chemistry and specificity in redox signaling." Free Radical Biology & Medicine **45**(5): 549-561.

Wipke, B. T. and P. M. Allen (2001). "Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis." Journal of Immunology **167**(3): 1601-1608.

Wittmann, S., D. Frohlich and S. Daniels (2002). "Characterization of the human fMLP receptor in neutrophils and in Xenopus oocytes." British Journal of Pharmacology **135**(6): 1375-1382.

Wright, H. L., R. J. Moots, R. C. Bucknall, et al. (2010). "Neutrophil function in inflammation and inflammatory diseases." Rheumatology (Oxford) **49**(9): 1618-1631.

Wu, J., A. Katrekar, L. A. Honigberg, et al. (2006). "Identification of substrates of human protein-tyrosine phosphatase PTPN22." The Journal of Biological Chemistry **281**(16): 11002-11010.

Wu, S., M. Bottini, R. C. Rickert, et al. (2009). "In silico screening for PTPN22 inhibitors: active hits from an inactive phosphatase conformation." ChemMedChem **4**(3): 440-444.

Yamada, R., A. Suzuki, X. Chang, et al. (2005). "Citrullinated proteins in rheumatoid arthritis." Frontiers in Bioscience **10**: 54-64.

Yamaguchi, H. and W. A. Hendrickson (1996). "Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation." Nature **384**(6608): 484-489.

Yang, S. R., A. S. Chida, M. R. Bauter, et al. (2006). "Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational

modifications of histone deacetylase in macrophages." American Journal of Physiology **291**(1): L46-57.

Yanni, G., A. Whelan, C. Feighery, et al. (1994). "Synovial tissue macrophages and joint erosion in rheumatoid arthritis." Annals of the Rheumatic Diseases **53**(1): 39-44.

Yarilina, A., E. DiCarlo and L. B. Ivashkiv (2007). "Suppression of the effector phase of inflammatory arthritis by double-stranded RNA is mediated by type I IFNs." Journal of Immunology **178**(4): 2204-2211.

Yeo, L., K. M. Toellner, M. Salmon, et al. (2011). "Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis." Annals of the Rheumatic Diseases **70**(11): 2022-2028.

Yu, X., J. P. Sun, Y. He, et al. (2007). "Structure, inhibitor, and regulatory mechanism of Lyp, a lymphoid-specific tyrosine phosphatase implicated in autoimmune diseases." Proceedings of the National Academy of Sciences of the United States of America **104**(50): 19767-19772.

Zavitz, C. C., G. J. Gaschler, C. S. Robbins, et al. (2008). "Impact of cigarette smoke on T and B cell responsiveness." Cellular Immunology **253**(1-2): 38-44.

Zhang, J., N. Zahir, Q. Jiang, et al. (2011). "The autoimmune disease-associated PTPN22 variant promotes calpain-mediated Lyp/Pep degradation associated with lymphocyte and dendritic cell hyperresponsiveness." Nature Genetics **43**(9): 902-907.

Zhang, Q., M. Raoof, Y. Chen, et al. (2010). "Circulating mitochondrial DAMPs cause inflammatory responses to injury." Nature **464**(7285): 104-107.

Zhu, J., D. Zhang, F. Wu, et al. (2011). "Single nucleotide polymorphisms at the TRAF1/C5 locus are associated with rheumatoid arthritis in a Han Chinese population." BMC Medical Genetics **12**: 53.

Zhu, J. W., K. Doan, J. Park, et al. (2011). "Receptor-like tyrosine phosphatases CD45 and CD148 have distinct functions in chemoattractant-mediated neutrophil migration and response to S. aureus." Immunity **35**(5): 757-769.

Zikherman, J., M. Hermiston, D. Steiner, et al. (2009). "PTPN22 deficiency cooperates with the CD45 E613R allele to break tolerance on a non-autoimmune background." Journal of Immunology **182**(7): 4093-4106.

Zikherman, J. and A. Weiss (2009). "Antigen receptor signaling in the rheumatic diseases." Arthritis Research and Therapy **11**(1): 202.

PUBLICATIONS

Publications

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

Metabolomics in the analysis of inflammatory diseases. Kapoor S, Fitzpatrick M, Clay E, Bayley R, Wallace GR, Young SP, Textbook chapter, Metabolomics, Chapter 11; p269-288, 2012

Measuring the activity of the protein tyrosine phosphatase Lyp. Bayley R, Yang P, Buckley CD, Young SP, Journal of Immunological Methods, 2012, 388: 1-2, p33-39

Differential Expression of CD148 on leucocyte subsets in inflammatory arthritis. Richa D, Naylor A, Young SP, Bayley R, Hardie D, Haworth O, Rider DA, Cook A, Buckley CD, Kellie S, Arthritis Research and Therapy, 2013, 15:R108

Does Oxidative Inactivation of CD45 Phosphatase in Rheumatoid Arthritis Underlie Immune Hyporesponsiveness? Rider DA, Bayley R, Clay E, Young SP, Antioxidants and Redox Signalling, 2013, 19(18): p2280–p2285

The autoimmune-associated genetic variant PTPN22 R620W enhances neutrophil activation and function in rheumatoid arthritis patients and healthy controls. Bayley R, Kite KA, McGettrick HM, Smith JP, Kitas GD, Buckley CD, Young SP, Annals of the Rheumatic Diseases, March 2014 [Epub ahead of print]

APPENDICES

Appendix 1 – Immunoblotting recipes

10% SDS-PAGE gel

10ml Protogel

12.5ml H_2O

7.5ml 1.5M Tris pH 8.8

300µl 10% SDS

150µl of 10% APS

30µl TEMED

5% SDS-PAGE stacking gel

2.6ml Protogel

12.2ml H_2O

5ml 0.5M Tris pH 6.8

200µl 10% SDS

100µl of 10% APS

20µl TEMED

SDS loading buffer

800µl 10% SDS

200µl 4% β-mercaptoethanol

800µl glycerol

1ml 0.5M Tris pH 6.8

Pinch of bromophenol blue

2ml dH_2O

Appendix 2 – Patient samples details

This appendix contains a summary of all the patient samples used during this study. Copies of the healthy volunteer screening form, consent form and information sheets prepared by Dr Stephen Young which were used throughout the study are also enclosed, along with a letter from the Edgbaston Research Ethics Committee confirming ethical approval for the study.

	Healthy controls (n=17)			Rheumatoid arthritis patients (n=27)		
PTPN22 R620W genotype	GG (n=8)	AG (n=8)	AA (n=1)	GG (n=12)	AG (n=12)	AA (n=3)
Sex, female n (%)	5 (63)	5 (63)	0 (0)	7 (58)	8 (67)	1 (33)
Age (years) median (IQR)	29 (16)	26 (19)	35 (0)	73 (25)	65 (15)	56 (48)
Smoker n (%)	0 (0)	0 (0)	0 (0)	3 (25)	6 (50)	2 (66)
CRP (mg/l) median (IQR)	-	-	-	11 (28)	3 (5)	6 (10)
RF positive n (%)	-	-	-	8 (67)	9 (75)	2 (66)
Disease duration ≥10 year's n (%)	-	-	-	10 (83)	5 (42)	1 (33)
Treatment with Methotrexate n (%)	-	-	-	4 (33)	5 (42)	3 (100)
Treatment with DMARD's n (%)	-	-	-	6 (67)	11 (92)	3 (100)
Treatment with steroids n (%)	-	-	-	4 (33)	3 (25)	3 (100)
Treatment with NSAID's n (%)	-	-	-	8 (100)	7 (88)	3 (100)

Table 9.1 Demographic data of study participants. IQR; interquartile range, CRP; C-reactive protein, RF; Rheumatoid factor, DMARD's; Disease modifying anti-rheumatic drugs, NSAID's; Non-steroidal anti-inflammatory drugs.

Names of Researchers: Dr Stephen P Young, Rachel Bayley

Healthy volunteer screening form

Do you consider yourself to be in good health? Yes ☐ No ☐

Do you currently have an infection? Yes ☐ No ☐

Have you had an infection in the last month? Yes ☐ No ☐

Do you have a chronic inflammatory disease? Yes ☐ No ☐

To include any of the following;

inflammatory arthritis, inflammatory bowel disease, psoriasis, liver disease, inflammatory lung disease or asthma which requires regular steroids, kidney disease, multiple sclerosis.

Have you ever smoked? Yes ☐ No ☐

Are you a current smoker? Yes ☐ No ☐

If “Yes” approximately how many Less than 10 ☐ 10-19 ☐ 20+ ☐

cigarettes do you smoke per day?

Age (Years):

Gender: Male Female

UNIVERSITY OF BIRMINGHAM

School of Immunity & Infection
Rheumatology Research Group
College of Medical and Dental Sciences
Edgbaston
Birmingham B15 2TT
United Kingdom
Tel: +44-(0)121 414 6480

Consent form

Title of Project: Immune cell signalling and metabolism in inflammatory arthritis

Name of Researcher: Rachel Bayley/Dr Stephen Young

Please initial box

1. I confirm that I have read and understand the information sheet dated 3rd Apr-2012 (Version 2.0) for the above study and have had the opportunity to ask questions ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason. ☐

3. I understand that sections of any of my medical notes may be looked at by the clinical research team and responsible individuals from regulatory authorities where it is relevant to my taking part in research. ☐

4. I agree to take part and have the urine and serum from my blood and joint fluid (if appropriate) studied for research. ☐

5. I agree to having the samples used for future, currently unforeseen, medical research projects in the UK and Europe (these projects would have been approved by a properly constituted research ethics committee before being conducted). ☐

Name	Date	Signature
Researcher	Date	Signature

1 ***for donor;*** *1 for* ***researcher;***

Information leaflet for Healthy Volunteers

UNIVERSITY OF BIRMINGHAM

School of Immunity & Infection
Rheumatology Research Group
College of Medical and Dental Sciences
Edgbaston
Birmingham B15 2TT
Tel: +44-(0)121 414 6480

Study title: Immune cell signalling and metabolism in inflammatory arthritis

Invitation to take part

You are being invited to take part in a research study by donating samples of blood and/or urine for research. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read this information carefully. Please discuss it with others if you wish. Ask us if there is anything that is not clear or if you need to know more. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

Rheumatoid arthritis (RA) can run in families, indicating that the factors you inherit have a strong influence on the disease. Other things may also play a role, for example, smoking and a low intake of vitamin C in the diet.

We believe that these factors together promote development of RA and several other diseases. About 1 person in 5 inherits the factors that promote RA but only 1 in 100 develop the disease and so we wish to see how these factors work in people with RA compared with people who do not have this disease. This information will help us understand the disease better and may help in developing new treatments.

Why have I been chosen?

You have been chosen because you are a healthy person, who does not have rheumatoid arthritis. This allows us to make comparisons between samples from people of various backgrounds. Comparing results in this way is essential for making sure that we draw valid conclusions.

What will happen to my samples?

If you agree to help us with our research we will use your blood and other samples in laboratory experiments at the University of Birmingham. We will analyse the blood to find out whether you have inherited some of the factors that promote RA. We will measure some of the natural chemicals present in the fluids which may be influenced by the inherited factors and your diet. If you agree to donate your samples for this project, some of the sample will be used for the immediate research and some will be stored to repeat the tests later. These will become the property of the University of Birmingham and will not be used for commercial gain. Sometimes new questions arise from a research project and new tests might be needed in future on the stored samples. We will not ask your permission to do these extra tests, but we will seek approval from the NHS Research Ethics Committee.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you choose to take part you will be given this information sheet to keep and be asked to sign a consent form which you will be given a copy of. If you choose not to take part you do not have to explain why. We may ask you for more than one blood sample, but you may refuse to give further samples without having to explain the reasons.

What will happen to me if I take part?
We will collect a blood sample, approximately 10 mls (approximately 3 tablespoons collected into one syringe). This blood will be analysed to see if you have the inherited factors that promote RA. On another occasion we will collect another slightly bigger blood sample (30mls – around an eggcup full) which we will use to analyse the natural chemicals in your blood and assess the function of the cells that protect you from infection, which are influenced by the inherited factors. We will also ask you to produce a small sample of urine or provide you with a plastic bottle to collect all the urine you produce over a 24hr period for assessment of the natural chemicals in it and to compare with those in your blood.

We may ask you for another blood and urine sample, so that we can check what we may have found with the first sample.

What do I have to do?
If you agree to take part in this study we will record details about you (your name, age, sex) and collect the samples.

Can I withdraw from the study?
You are free to withdraw from the study at any point and you do not have to give a reason why. If you do decide that you no longer wish to participate, you can request that all samples in storage will be destroyed, and this will be done. If you change your mind after a long period of time the samples may already have been used to generate research data.

What are the side effects of taking part?
You may develop a bruise on the arm where the blood is taken; this will settle within a few days. There are no other side effects you are likely to experience.

What are the possible disadvantages and risks of taking part?
None besides the possible side effects noted above.

What are the possible benefits of taking part?
Whilst there will be no direct benefit to you, the information we get will allow a better understanding of why patients develop arthritis and other diseases and hopefully lead to improved treatments.

What if something goes wrong?
If you are harmed by taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?
All information given by you and all results obtained will be treated in the strictest confidence. If you are a member of laboratory personnel, culture flasks and storage tubes will be labelled with your name. This is due to University Health and Safety regulations.

The samples will be assigned a unique code. This code will be used in all experiments so that research staff cannot identify you. We will keep a record that links the unique code for your sample back to you. This record will be kept confidential within the research group. This means that we can link together different samples taken at different times from the same person. This is scientifically useful.

In general, all information about you and any results will remain confidential within the research group and will be stored in accordance with the UK Data Protection Act 1998. Your General Practitioner will not be informed of your participation. No results will ever be released to an insurance company. We will hope to publish or present the results of this work in medical/scientific journals or conferences but the data will be completely anonymised.

What will happen to the results of the research study?
The results of the research study are likely to be published in a scientific journal. Information from the tests carried out on you will be included but you will not be individually identified.

Can I find out my results?

The experiments that we do with your sample will not help you by telling you something important about your blood. For this reason, we do not routinely report back the results of your own tests to you, your family or your GP. However, we are very happy to explain more generally about what we learn from the experiments. Please use the contact details below.

Who is organising and funding the research?
This study is organised by the University of Birmingham. This study is being funded, in part by Arthritis Research UK and the Wellcome Trust.

Who has reviewed the study?
The study was reviewed by an NHS Research Ethics Committee.

Contact for Further Information
Should you require further information please do not hesitate to contact Dr Stephen Young (on 0121 414 6480). If you have any concerns or complaints you can contact the Patient Advice and Liaison Service (PALS) on 0121 371 3280.

A copy of this information sheet and a signed consent form will be given to you to keep.
Thank you for taking the time reading this information sheet, please complete the attached consent form if you wish to participate.

Information leaflet for Patients

School of Immunity & Infection
Rheumatology Research Group
College of Medical and Dental Sciences
Edgbaston
Birmingham B15 2TT
Tel: +44-(0)121 414 6480

Study title: Immune cell signalling and metabolism in inflammatory arthritis

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If you agree to help us with our research we will use your blood and other samples in laboratory experiments at the University of Birmingham. We will analyse the blood to find out whether you have inherited some of the factors that promote RA. We will measure some of the natural chemicals present in the fluids which may be influenced by the inherited factors and your diet. If you agree to donate your samples for this project, some of the sample will be used for the immediate research and some will be stored to repeat the tests later. These will become the property of the University of Birmingham and will not be used for commercial gain. Sometimes new questions arise from a research project and new tests might be needed in future on the stored samples. We will not ask your permission to do these extra tests, but we will seek approval from the NHS Research Ethics Committee.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you choose to take part you will be given this information sheet to keep and be asked to sign a consent form which you will be given a copy of. If you choose not to take part you do not have to explain why. Your usual treatment will not be affected in any way whatsoever. We may ask you for more than one blood sample, but you may refuse to give further samples without having to explain the reasons.

What will happen to me if I take part?
At the same time as your usual blood samples are being collected we will fill a small extra tube. This blood will be analysed to see if you have the inherited factors that promote RA. On another of your usual appointments at the hospital, we will collect another slightly bigger blood sample (30mls – around an eggcup full) which we will use to analyse the natural chemicals in your blood and assess the function of the cells that protect you from infection, which are influenced by the inherited factors. We will also ask you to produce a small sample of urine for assessment of the natural chemicals in it and to compare with those in your blood. If you have swollen joints your doctor may remove fluid from these as part of your normal treatment. Instead of throwing this away we will collect it for analysis of the natural chemicals and to isolate cells for study. During another of your usual appointments at the hospital we may ask you for another blood and urine sample, so that we can check what we may have found with the first sample.

What do I have to do?
If you agree to take part in this study, at your next clinic visit, we will collect the extra blood sample through the same needle as used for your routine samples and ask you to collect a small urine sample.

Can I withdraw from the study?
You are free to withdraw from the study at any point and you do not have to give a reason why. If you do decide that you no longer wish to participate, you can request that all samples in storage will be destroyed, and this will be done. If you change your mind after a long period of time the samples may already have been used to generate research data.

What are the side effects of taking part?
You may develop a bruise on the arm where the blood is taken; this will settle within a few days. There are no other side effects you are likely to experience.

What are the possible disadvantages and risks of taking part?
None besides the possible side effects noted above.

What are the possible benefits of taking part?
Whilst there will be no direct benefit to you, the information we get will allow a better understanding of why patients develop arthritis and other diseases and hopefully lead to improved treatments.

What if something goes wrong?
If you are harmed by taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?
All information given by you and all results obtained will be treated in the strictest confidence.
The samples will be assigned a unique code. This code will be used in all experiments so that research staff cannot identify you. We will keep a record that links the unique code for your sample back to you. This record will be kept confidential within the research group. This means that we can link together different samples taken at different times from the same person. This is scientifically useful.
In general, all information about you and any results will remain confidential within the research group and will be stored in accordance with the UK Data Protection Act 1998. Your General Practitioner will not be informed of your participation. No results will ever be released to an insurance company. We will hope to publish or present the results of this work in medical/scientific journals or conferences but the data will be completely anonymised.

What will happen to the results of the research study?
The results of the research study are likely to be published in a scientific journal. Information from the tests carried out on you will be included but you will not be individually identified.

Can I find out my results?
The experiments that we do with your sample will not help you by telling you something important about your blood. For this reason, we do not routinely report back the results of your own tests to you, your family or your GP. However, we are very happy to explain more generally about what we learn from the experiments. Please use the contact details below.

Who is organising and funding the research?
This study is organised by the University of Birmingham. This study is being funded, in part by Arthritis Research UK and the Wellcome Trust.

Who has reviewed the study?
The study was reviewed by an NHS Research Ethics Committee.

Contact for Further Information
Should you require further information please do not hesitate to contact Dr Stephen Young (on 0121 414 6480). If you have any concerns or complaints you can contact the Patient Advice and Liaison Service (PALS) on 0121 371 3280.

Thank you for taking the time reading this information sheet. If you wish to participate, bring this sheet with you to the clinic where your doctor will give you a consent form to complete.