Deciphering the link between PTPN22 and Autoimmunity

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A dissertation submitted to Cardiff University in candidature for the degree of Doctor of Philosophy

September 2011

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Acknowledgements

This project would not have been possible without the generous help of many people.

I am truely indebted and thankful to my supervisor, Dr James Matthews for his continued guidance and support throughout the project and for lending me his unlimited enthusiasm which saw me through the project. I would also like to thank my co-supervisor, Dr Awen Gallimore for her valuable input in the project and for her help with the manuscript.

My sincere and earnest thanks to Dr John Bridgeman for encouragement and valuable guidance without which, I am sure this project would not have been successfull.

I would also like to thank Dr Claire Harris for providing valuable assistance with generation of monoclonal antibodies and help with the manuscript.

I am obliged to many of my colleagues both from the Department of Infection, Immunity and Biochemistry and the Department of Pathology who supported me in the lab and during the write-up for past four years and for making the department a pleasant and excellent place to work. I would particularly like to thank Dr Claudia Nunes and Dr Gary Dolton for their help with T cell culture and Flow Cytometry.

I would like to express my gratitude towards Welsh Assembly Government for the financial support without which this project would not have been possible.

I extend a special thanks to my family and friends, particularly Mum, Dad, Gaurav, Ben, Laurence, Anya, Boitelo, Emily, Paul and Kathy for their continual support and encouragement through some of the most challenging part of the project. Lastly, I would like to thank Sara and Jaq for providing the much needed distraction during the writing up process.

Summary

Recent genetic studies have linked a C to T single nucleotide polymorphism (SNP) in the protein tyrosine phosphatase (PTP) non-receptor type 22 (PTPN22) to several autoimmune diseases (ADs). This changes amino acid at position 620 from an Arginine (R) to a Tryptophan (W) in the protein, Lyp. Lyp is thought to be a negatively regulator of TCR signalling by dephosphorylating Src family kinases Lck and Fyn, and Zap70. However, the cellular and molecular mechanisms of predisposition to ADs by the R620W polymorphism are not yet understood. Several studies have reported the R620W polymorphism as a "gain of function" change resulting in an increase in the PTP activity of Lyp. It has been further hypothesised that the W620 isoform suppresses TCR signalling more potently than the R620 isoform, resulting in the survival of auto-reactive cells that would normally be deleted by negative selection in the thymus. Alternatively, the impact of Lyp W620 on TCR signalling may have an effect on the development and functioning of T regulatory cells.

To investigate the effect of the R620W polymorphism in T cells, lentivirus plasmids expressing the R and W isoforms of Lyp were generated and used to introduce the ^{R}Lyp and ^{W}Lyp isoforms in leukaemic T cells thereby generating H9 and E6.1 cell lines over-expressing the ^{R}Lyp and ^{W}Lyp isoforms. Investigation of activation marker expression and cytokine production by these T cell lines post activation showed no differences in CD69 activation marker expression between the ^{R}Lyp and ^{W}Lyp expressing T cells or between the $^{R/W}$Lyp expressing and control cells (not expressing exogenous Lyp).

However, there was a trend towards a reduction in IL-2 production observed by $^{R/W}$Lyp expressing H9 T cells compared to control cells. In addition, a significant reduction in IL-10 production by $^{R/W}$Lyp expressing H9 T cells compared to control cells was observed. This effect of Lyp on IL-10 production suggests a potential mechanism by which ^{W}Lyp, if indeed a more active PTP, may predispose to ADs

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List of abbreviations

AD	Autoimmune diseases
AICD	Activation induced cell death
AIRE	Autoimmune regulator
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
cDNA	Complementary DNA
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal calf serum
Foxp3	Forkhead box P3
GFP	Green fluroscent protein
Grb2	Growth factor receptor binding protein 2
HLA	Human leukocyte antigen
ICOS	TCR- inducible costimulatory receptor
IFN	Interferon
IL	Interleukins
ITAM	Immunoreceptor Tyrosine based Activation Motif
MHC	Major Histocompatability Complex
MFI	Mean fluorescence intensity
MS	Multiple sclerosis
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κB

NKT	Natural killer T cells
NP40	Nonidet-P40
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PE	Phycoerythrin
PTEN	Phosphatase and Tensin homolog deleted on chromosome 10
PTP	Protein tyrosine phosphatase
PTPN22	Protein tyrosine phosphatase non receptor type 22
RA	Rheumatoid arthiritis
SDS	Sodium Dodecyl Sulphate
SHIP	SH2 domain-containing inositol polyphosphate 5'-phosphatase
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
T1D	Type 1 Diabetes
TCR	T cell receptor
Tfh	T follicular helper
TGF-β	Transforming growth factor-beta
T_H	T helper
TNF-α	Tumour necrosis factor-alpha
T reg	T regulatory
ZAP70	Zeta-Associated Protein of 70 kDa

Chapter 1

Introduction

1.1 Autoimmune diseases

Paul Ehrlich first described the concept of autoimmunity at the beginning of 20th century as a "horror autotoxicus". Autoimmune disease (AD) is a complex chronic disease caused by the activation of T cells or B cells or both by self-antigens, in the absence of an ongoing infection or other apparent cause (Davidson and Diamond 2001; Kivity et al. 2009). There are currently over 80 diseases classified as autoimmune. ADs, with the exception of Rheumatoid arthritis (RA) and Autoimmune Thyroiditis are individually rare, but together they affect approximately 5% of individuals in western countries (Jacobson et al. 1997; Sinha et al. 1990). After heart diseases and cancer, ADs are the third leading cause of morbidity and mortality in the industrialised world (Kivity et al. 2009). ADs can affect almost every site in the body although most ADs are not fatal. ADs develop through a combination of genetic, environmental, immunological and hormonal factors (Figure 1.1) (Cojocaru 2008; Kivity et al. 2009).

Although the aetiology of AD is yet to be fully deciphered, it is well documented that T cells are the key mediators of many ADs, such as Type 1 diabetes (T1D), Autoimmune Thyroiditis, and RA (Murphy et al. 2007a). Furthermore, there is mounting evidence that normal healthy individuals have potentially pathogenic self-reactive T cells. Therefore, T cells and their role in preventing and mediating autoimmunity will be the focus of this Introduction.

Each autoimmune disorder has its own aetiology, however, failure of immunological tolerance in one or more lymphocyte subsets appears to be a common observation in all AD (Hoyne 2011; Murphy et al. 2007a). The random gene arrangement mechanisms that occur during the development of T and B cells result in the generation of diverse T and B cell receptors capable of recognising a wide variety of foreign antigens. However, this also results in the generation of auto-reactive receptors that can potentially react to self-antigens (Appleman and Boussiotis 2003; Bluestone 2011; de Souza et al. 2010; Hogquist et al. 2005).

Hence, distinct tolerance mechanisms, central and peripheral have to be in place to prevent ADs in normal individuals.

1.2 Mechanisms of generation of T cell receptor diversity

Huge receptor diversity is encoded in the mammalian genome so that mammals can potentially recognise a huge range of chemical structures in order to fight off possible microorganism induced infections. Autoimmunity is a consequence of this deliberately random receptor generating process (Figure 1.2) (Racanelli et al. 2011) . The vast receptor diversity possessed by lymphocyte populations is achieved by the processes of two somatic genome modifications. First is the mechanism of V(D)J recombination where the Variable (V), Diversity (D) and Joining (J) gene segments are assembled together during T cell differentiation to generate diverse and unique T cell receptors (TCRs) (reviewed in Goldrath and Bevan 1999; reviewed in Kronenberg et al. 1986). There are multiple different copies of V, D and J gene segments encoded in the germline DNA and these can be arranged together in various combinations to give rise to a different V(D)J sequence each time. This combinatorial diversity is the main source of the diversity of the variable antigen binding region (Goldrath and Bevan 1999; Murphy et al. 2007a). In addition, junctional diversity at the joints between the different gene segments due to the addition and subtraction of nucleotides by the recombination process introduces another source of diversity in the V(D)J sequence (Goldrath and Bevan 1999; Murphy et al. 2007a). Finally, the many possible different combinations of alpha and beta chain V region that can pair to form a TCR gives rise to further receptor diversity (reviewed in Goldrath and Bevan 1999; Kronenberg et al. 1986; Wilson et al. 1988). Once these recombination events have succeeded in producing a functional receptor, further rearrangement is prohibited, therefore increasing the likelihood that each lymphocyte expresses only one receptor specificity (Goldrath and Bevan 1999). B cells further diversify their BCR by the process of somatic hypermutation. Somatic hypermutation in activated B cells introduces point mutations into the rearranged V-region genes creating further diversity that can be selected for enhanced binding to antigen (Murphy et al. 2007a). T cells with a functional TCR are not known to diversify their V region after rearrangement through somatic hypermutation (Goldrath and Bevan 1999).

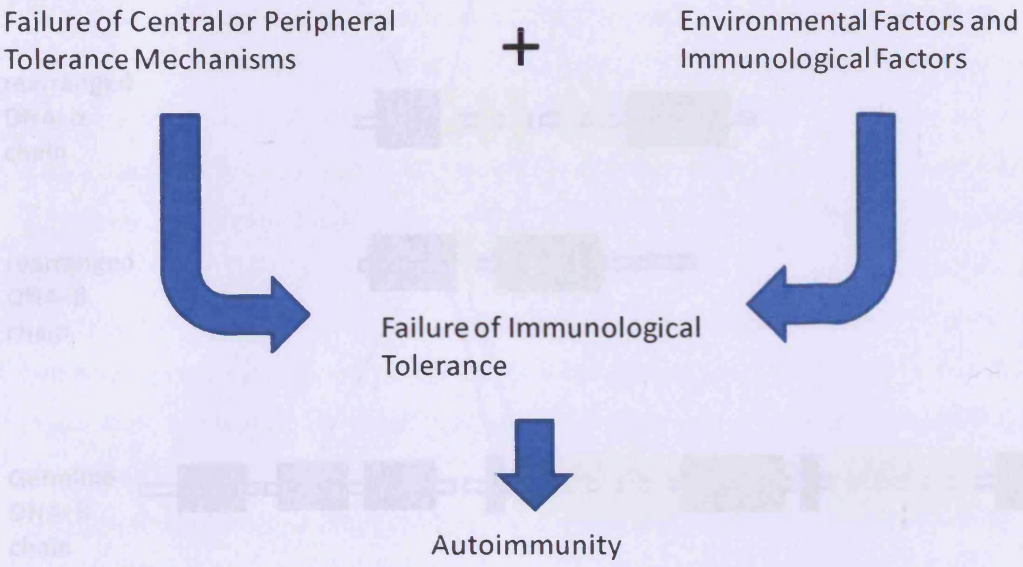


Figure 1.1 Factors involved in development of autoimmunity

Autoimmune diseases result due to a genetic predisposition, which plays a role in the failure of central and peripheral tolerance mechanisms in place to prevent autoimmunity and due to environmental and immunological triggers such as infection, inflammation, molecular mimicry and hormones. This leads to a failure of immunological tolerance, which in turn leads to autoimmunity.

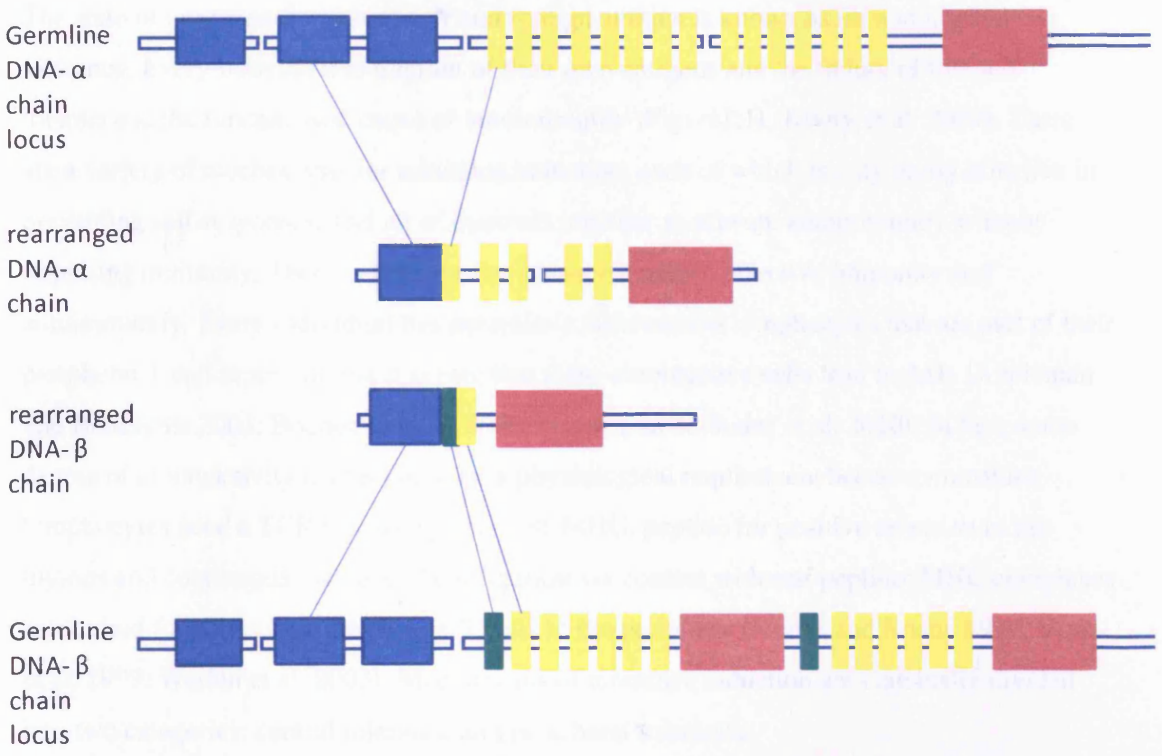


Figure 1.2 The germline organisation and rearrangement of T cell receptor alpha and beta chain gene

The TCRα and β chain genes are composed of discrete variable (V shown as blue rectangle), diversity (D, green rectangle) and joining (J, yellow rectangle) gene segments. The TCR α locus (top) consists of 70-80 Vα gene segments and 61 Jα gene segments followed by a single Constant (C, red rectangle) gene segment. Any one of the functional 70-80 Vα gene segments can rearrange with any one of the 61 Jα gene segments to form a TCR α chain. The TCR β locus (bottom) has a different organisation to TCR α chain locus with a cluster of 52 functional Vβ gene segments located distantly from two separate clusters each containing a single D gene segment together with 6 or 7 J gene segments and a single C gene. Any of the Vβ gene segments can rearrange with either Dβ gene segments and any of the Jβ gene segments to form a rearranged TCR β chain. This is called combinatory diversity and is the main source of diversity of the variable antigen binding region. Adapted from (Murphy et al. 2007b)

1.3 Immunological tolerance

The state of unresponsiveness to self and foreign antigen is known as immunological tolerance. Every individual is tolerant of their own antigens and the failure of this self-tolerance is the fundamental cause of autoimmunity (Figure1.1) (Kivity et al. 2009). There are a variety of mechanisms for tolerance induction, each of which is only partly effective in preventing self-responses, and all of them act together to prevent autoimmunity without impairing immunity. There is hence a fine balance between effective immunity and autoimmunity. Every individual has potentially autoreactive lymphocytes that are part of their peripheral T cell repertoire but it is rare that these autoreactive cells lead to ADs (Appleman and Boussiotis 2003; Bouneaud et al. 2000; reviwed in de Souza et al. 2010). In fact, some degree of autoreactivity is thought to be a physiological requirement because immature lymphocytes need a TCR that recognises self-MHC: peptide for positive selection in the thymus and continuous low level TCR ligation via contact with self peptide: MHC complexes is required for survival of the mature T cells in the periphery (Freitas and Rocha 1999; Viret et al. 1999; Werlen et al. 2003). Mechanisms of tolerance induction are classically divided into two categories: central tolerance and peripheral tolerance.

1.3.1 Central tolerance mechanisms

Central tolerance is the process of deleting self-reactive cells in the thymus and bone marrow (Bluestone, J.A. 2011; Metzger and Anderson 2011; Starr et al. 2003). Central tolerance mechanisms are the first and the most important tolerance mechanisms that take place in the central lymphoid organs during the generation and development of naïve lymphocytes by which the newly developing lymphocytes are ensured to be non reactive to self (reviewed in Bluestone, J.A. 2011; von Boehmer and Melchers 2010). The hallmark mechanism used during central tolerance of both B and T lymphocytes is clonal deletion via negative selection (Bluestone, J.A 2011; von Boehmer and Melchers 2009). Clonal anergy and receptor editing are also thought to play roles in tolerance induction of T cells in the thymus, however these are thought to be of much lower significance compared to clonal deletion (de Souza et al. 2010; Goodnow et al. 2005; Hammerling et al. 1991; Hogquist et al. 2005; Roberts et al. 1990; Wang et al. 1998) .

Most of the double negative (DN, do not express CD4 or CD8 receptors) developing thymocytes in the thymus express a TCR that does not recognise a self peptide: MHC complex and therefore die by neglect (Figure 1.3) (Benoist and Mathis 1989; reviewed in de Souza et al. 2010). Some of these DN thymocytes express TCR that recognises self-peptide: MHC complex but responds only weakly to this TCR ligation thereby receiving survival signals to continue their development. This is called positive selection (Figure 1.3) (de Souza et al. 2010; Metzger and Anderson 2011). These cells are non-responsive to self-antigens in the periphery. A small number of developing thymocytes (about 5%) respond with high affinity to the TCR ligation by self peptide: MHC complex (Laufer et al. 1999; reviewed in Metzger and Anderson 2011; van Meerwijk et al. 1997; reviewed in de Souza et al. 2010). These thymocytes are potentially autoimmune and therefore need to be deleted (clonal deletion) from the peripheral T cell repertoire to avoid autoimmunity (Kappler et al. 1987; Kisielow et al. 1988; MacDonald et al. 1988; Metzger and Anderson 2011; Venanzi et al. 2004; Woodland et al. 1990; Zal et al. 1994; reviewed in de Souza et al. 2010). Negative selection is the mechanism of deleting these self-reactive and potentially autoimmune thymocytes, thereby generating a repertoire of T cells in the periphery that are self-tolerant (Figure 1.3).

The thymic cortex is the site for positive selection and cortical thymic epithelial cells (TEC) are the main players in mediating positive selection of the developing thymocytes. However, the developmental stage and therefore the site at which negative selection occurs has been a source of some controversy in the past as the stage of development at which negative selection occurs differs depending on the particular experimental system and the particular antigen used (reviewed in Goodnow et al. 2005). Some experiments indicate that clonal deletion (negative selection) occurs in the thymic cortex and others indicate that it occurs in the thymic medulla. Initially, it was also thought that thymic negative selection was a sequential process taking place after positive selection. However, it is now accepted that negative selection of thymocytes takes place throughout thymocyte development both in the thymic cortex and the thymic medulla but the thymic medulla is thought to be the main site of negative selection (Baldwin et al. 1999; reviewed in Goodnow et al. 2005; Metzger and Anderson 2011; Ohashi et al. 1990; Spain and Berg 1992).

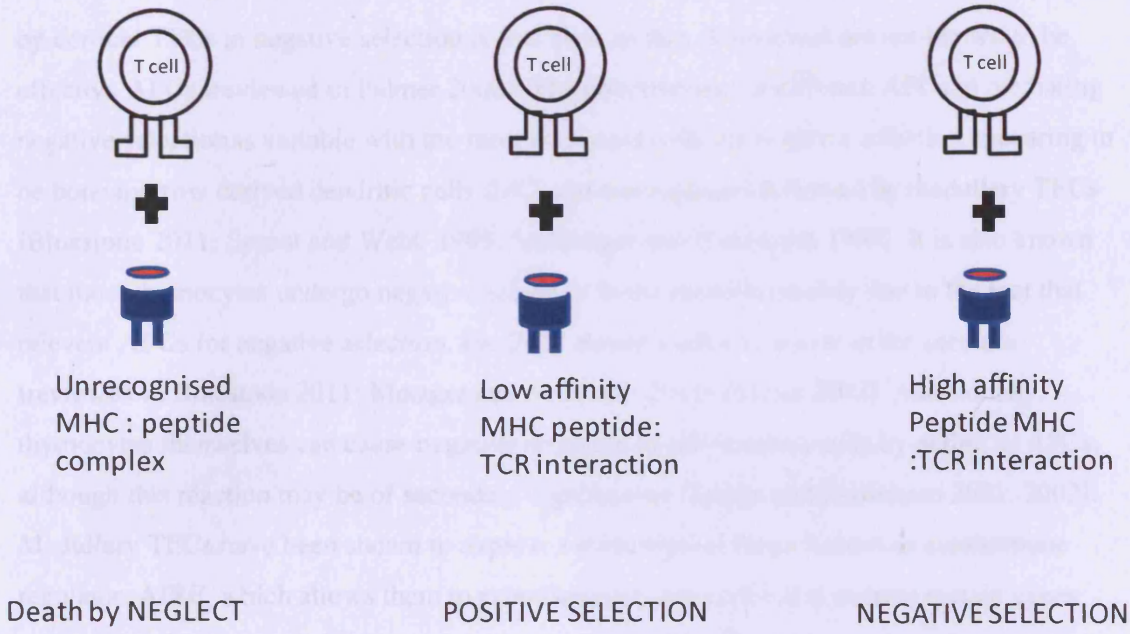


Figure 1.3 Checkpoints in the development of T cells in the thymus

The Immature thymocytes in the thymus need to be able to generate a TCR that recognises self MHC: peptide complexes to undergo positive selection. Failure to recognise a self MHC: peptide complex leads to apoptotic death of the cell, a phenomenon known as "death by neglect". A Low affinity TCR: MHC-self peptide interaction provides signal for positive selection, which allows continuation down the developmental pathways. However, high affinity TCR: MHC-self peptide interactions lead to clonal deletion via negative selection.

There is a general concensus in literature that cortical TECs are the mediators of positive selection. However, the importance of the role played by cortical TECs in negative selection has been controversial (Lorenz and Allen 1989a, b; Palmer 2003). Medullary TECs have been shown by several studies to significantly contribute to negative selection and therefore tolerance induction (reviewed in Metzger and Anderson 2011; Palmer 2003). The role played by cortical TECs in negative selection is less clear as they themselves are not known to be effective APCs (reviewed in Palmer 2003). The effectiveness of different APCs in mediating negative selection is variable with the most important cells for negative selection appearing to be bone marrow derived dendritic cells (DC) and macrophages followed by medullary TECs (Bluestone 2011; Sprent and Webb 1995; Stockinger and Hausmann 1994). It is also known that most thymocytes undergo negative selection in the medulla, mainly due to the fact that relevant APCs for negative selection, the DCs, almost exclusive reside in the medulla (reviewed in Bluestone 2011; Metzger and Anderson 2011; Palmer 2003). Additionally, thymocytes themselves can cause negative selection of self-reactive cells by acting as APCs, although this reaction may be of secondary significance (Sprent and Kishimoto 2001, 2002). Medullary TECs have been shown to express a transcription factor known as autoimmune regulator, AIRE, which allows them to promiscuously transcribe and express certain genes such as insulin and myelin basic protein that are normally only expressed in peripheral tissue (reviewed in Anderson et al. 2000; Bluestone 2011; Hanahan 1998; Heino et al. 1999; Klein et al. 2000; Metzger and Anderson 2011). People who express a defective form of AIRE suffer from autoimmune polyendocrine syndrome type 1, a multi organ form of autoimmunity (Consortium. 1997). Mice deficient in AIRE expression develop a pattern of ADs similar to the human syndrome (Ramsey et al. 2002). The absence of AIRE expression only from the medulla is sufficient to cause disease (Villasenor et al. 2005).

The role of costimulatory molecules in negative selection has been a source of some controversy. Some studies have shown that costimulatory molecules are important for negative selection (Buhlmann et al. 2003; Gao et al. 2002; Kishimoto and Sprent 1999; Page 1999; Page et al. 1993; Palmer 2003; Punt et al. 1994; Sprent and Kishimoto 2001, 2002). However, other studies with mice genetically deficient in expression of costimulatory molecules have failed to show a defect in negative selection (Dautigny et al. 1999; Li and Page 2001; Palmer 2003; Walunas et al. 1996; Williams et al. 2002). The lack of a defect seen in mice genetically deficient for individual costimulation genes is that many costimulatory molecules may have the redundant same function. Therefore, any single gene

knockout will fail to show any defect. Alternatively it maybe that costimulatory molecules are not required for the deletion of high affinity thymocytes but only for the deletion of low affinity thymocytes. If the deletion of high affinity thymocytes is possible in the absence of costimulatory signal then it may be possible for the TCR to deliver signals for negative selection (Palmer 2003; Sprent and Kishimoto 2002; Sprent and Kosaka 1993).

It is also not clear how a developing thymocyte can discriminate between a positive selection and a negative selection signal and initiate the positive or negative selection pathway respectively. Although it is generally agreed that low affinity peptide: MHC ligand leads to positive selection and high affinity peptide: MHC ligand leads to negative selection, it is not entirely understood how a TCR can discriminate between a low affinity and a high affinity signal (reviewed in Gascoigne and Palmer 2011; Palmer 2003; Starr et al. 2003). There are currently two proposed mechanisms of how a developing thymocyte might make the distinction between a low affinity and a high affinity signal. First, is the serial triggering model whereby a single peptide: MHC complex can engage and activate multiple TCRs over time (reviewed in Palmer and Naeher 2009; Valitutti et al. 1995). An increase in number of TCR interactions by a peptide: MHC complex repeatedly engaging multiple TCRs on the T cell surface (serial triggering) would lead to a multiplication of the peptide: MHC induced signals resulting in negative selection (Rachmilewitz and Lanzavecchia 2002). In this way one or two peptide: MHC complexes can lead to apoptosis via negative selection. Second, is the kinetic proofreading model of T cell activation whereby a T cell detects ligand affinity by measuring how long a peptide: MHC complex remains bound to the TCR. In this model, a high affinity TCR: peptide: MHC ligands would occupy the TCR for a longer period of time (slower off-rate) and have a longer half life providing sufficient time for the formation of fully activated TCR: CD3 complexes (Kalergis et al. 2001; Kersh et al. 1998; reviewed in Palmer and Naeher 2009; Savage and Davis 2001; Teague et al. 2008). A low affinity TCR: peptide: MHC complex in contrast would have a faster off-rate and a shorter half life resulting in incomplete activation of the TCR: CD3 complexes (reviewed in Palmer and Naeher 2009; Rabinowitz et al. 1996). In terms of signal transduction, TCR ligation by high affinity ligand for a longer period of time would induce a set of early and late signals whereas TCR ligation by a low affinity ligand would only induce a set of early signals (Alam et al. 1996; Gascoigne and Palmer 2011; Palmer 2003; Starr et al. 2003). Therefore, a single receptor complex might determine the affinity of the ligand molecule by determining the time

it remains bound to the TCR and thus the signal transduced (Gascoigne and Palmer 2011; McKeithan 1995).

Central tolerance via negative selection is very effective in getting rid of T cells with high affinity TCRs against self-peptides. However, it does not purge the system of all self-reactive T cells. Self-reactive T cells are present in the periphery of healthy individuals. So how is it that despite having self-reactive T cells present in the periphery only 3 to 6% of individuals go on to develop autoimmunity (Jacobson et al. 1997)?

1.3.2 Peripheral tolerance mechanisms

Negative selection in the thymus does not eliminate all self-reactive lymphocytes (Bluestone 2011; Bouneaud et al. 2000; Metzger and Anderson 2011; Mueller 2009; Peterson et al. 1999; von Boehmer and Melchers 2009). Self-reactive lymphocytes do exist in the natural immune repertoire and they can be activated by immunization with self constituents along with potent adjuvant or by repeated stimulation with self-antigens in vitro (Weigle 1980; Wekerle et al. 1996). Deleting every weakly self reactive lymphocyte would compromise the immune system because every TCR, self reactive or not, is potentially capable of generating an effective immune response against foreign antigens. The immature DN progenitor cells in the thymus can only successfully undergo positive selection if they recognise self-peptide: MHC complexes and signal via their TCR. These cells are therefore capable of reacting against self-peptide (de Souza et al. 2010). However, in the periphery these same cells effectively ignore the self-peptide that triggered their positive selection in the thymus (Davey et al. 1998; Wong et al. 2001). Such normally "self-ignorant" cells may still be capable of being activated in the periphery if the stimulus is strong enough (Sandberg et al. 2000). However, these cells are highly unlikely to be activated and lead to autoimmunity because the strength of TCR signal produced by the self-peptide that lead to positive selection is not enough to activate the cell in the periphery. There are intrinsic difference between a developing thymocyte and a mature T cell. Immature thymocytes are undergoing a developmental programme in the thymus, genes are being switched on/off, epigenetic changes taking place and thresholds being set. It may be the case that certain signalling regulators are also developmentally regulated and therefore all the regulatory mechanisms that is functional in a mature T cell may not be present in a developing thymocyte. Thus upon encounter with the same antigen an

immature thymocyte that recognises this antigen may signal differently (higher thereshold) at this stage than when it has differntiated into a mature T cell. Therefore, a signal that is enough to activate a thymocyte in the thymus and lead to positive selection is not normally enough to activate the same T cell in the periphery. A more significant risk to autoimmunity is posed by those autoreactive cells that have medium to high affinity for self-antigens which escape tolerance mechanisms in the central lymphoid organs (Van Kaer 2010). T cell with lower avidity for self-peptide: MHC complexes than that required for induction of negative selection will fail to be deleted in the thymus and will escape to the periphery (Jiang and Chess 2004). An autoreactive T cell might escape negative selection because the particular self-antigen that it recognises is not expressed in the thymus or is not expressed in sufficient quantity to induce negative selection or is only mildly immunogenic (reviewed in Walker and Abbas 2002). In these cases peripheral tolerance mechanisms play an important role in preventing autoimmunity and AD (Figure 1.4).

Peripheral tolerance mechanisms act on mature self-reactive lymphocytes to prevent them from causing autoimmune disorders. Indeed, autoreactive cells that have broken tolerance can still be regulated so that they do not cause ADs (de Souza et al. 2010; von Boehmer and Melchers 2009). This regulation takes two forms, intrinsic regulation and extrinsic regulation (Figure 1.4)

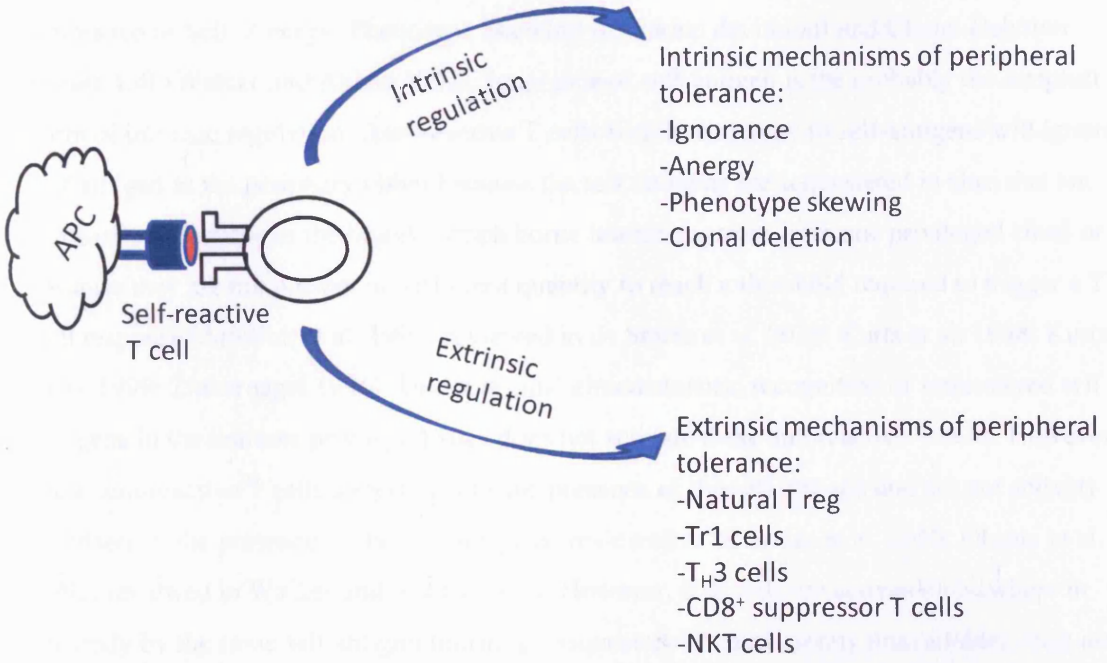


Figure 1.4 Pathways of peripheral tolerance

Not all self-reactive cells are deleted in the thymus. Cells that escape negative selection can still be regulated in the periphery by mechanisms of peripheral tolerance acting either directly on the self-reactive T cell (T cell intrinsic) or indirectly via additional cells (T cell extrinsic).

1.3.2.1 Intrinsic regulation

Intrinsic regulation involves built in mechanisms in the cell itself to control autoreactivity. The mechanisms used for intrinsic regulation of autoreactive cells in the periphery are Ignorance of Self, Anergy, Phenotype Skewing (Cytokine deviation) and Clonal Deletion Figure 1.4) (Walker and Abbas 2002). Ignorance of self-antigen is the probably the simplest form of intrinsic regulation. Autoreactive T cells with low affinity to self-antigens will ignore self antigen in the periphery either because the self antigens are sequestered in sites that are not easily accessible to the blood/ lymph borne immune system (immune privileged sites) or because they are not present in sufficient quantity to reach a threshold required to trigger a T cell response (Alferink et al. 1999; reviewed in de Souza et al. 2010; Kurts et al. 1998; Kurts et al. 1999; Zinkernagel 1996). Under normal circumstances, recognition of sequestered self antigens in the immune privileged sites does not activate these autoreactive T cells. However, these autoreactive T cells simply ignore the presence of the self-antigen and are not actively tolerised by the presence of the self antigens (reviewed in de Souza et al. 2010; Ohashi et al. 1991; reviewed in Walker and Abbas 2002). However, if T cells are activated elsewhere in the body by the same self antigen (normally sequestered or sufficiently unavailable), they are capable of mounting an immune response towards these autoantigens, even in immune privileged sites (Goverman et al. 1993; Lafaille et al. 1994; Oehen et al. 1992; Ohashi et al. 1991; Streilein et al. 1997; Walker and Abbas 2002).

Phenotype skewing or cytokine deviation can help maintain tolerance by avoiding a pathogenic response even when the T cells are fully activated (reviewed in Nurieva et al. 2011; Walker and Abbas 2002). During the course of a normal immune response, $CD4^+$ T cells can differentiate into various types of effector cells, namely T helper type 1 (T_H1), T helper type 2 (T_H2) and T helper type 17 (T_H17) (de Souza et al. 2010). This differentiation can help maintain tolerance because only certain cytokines and chemokines and particular T_H responses are linked with pathogenicity (Bradley et al. 1999; de Souza et al. 2010; Young et al. 2000). Therefore, differentiation into phenotypes that produce immunosuppressive cytokines may help in controlling autoreactivity. However, the involvement of different phenotypes in disease pathogenicity is not always straightforward. For example, T_H1 cells and cytokines have been found to be responsible for the pathogenicity of T1D (Hancock et al. 1995; Lyons et al. 2000; Tian et al. 1998) and Multiple Scelerosis (MS) whereas Systemic Lupus Erthromatous (SLE) pathogenicity involves both T_H1 and T_H2 cells. In general, T_H2

responses are associated with downregulation of autoimmunity (Bradley et al. 1999; Young et al. 2000), although this is not always the case (Pakala et al. 1997). IL-17 producing T_H17 cells have also been implicated in having a central role in the pathogenesis of experimental models of AD including RA, SLE and MS (reviewed in de Souza et al. 2010). There is also a close association between cytokine differentiation and chemokine receptor expression (Charles et al. 1999; Chensue et al. 2001). Chemokine receptor expression can play an important role in recruitment of T cells into sites of inflammation and infection by acting as chemo-attractant to autoreactive T cells (Borges et al. 1997; Hill et al. 2003).

Alternatively, T cell encounters with self-antigens might lead to functional inactivation or anergy (reviewed in de Souza et al. 2010; Lamb et al. 1983; Rocha et al. 1993; Schwartz 1990). The fact that a given peptide can both activate or induce unresponsiveness depending on the presence of APC, indicated the requirement of a second signal, other than that from TCR ligation which influences the T cell fate (activation or anergy) (Appleman and Boussiotis 2003; Lafferty et al. 1978). Later studies showed that TCR ligation in the absence of costimulation leads to anergy (Appleman and Boussiotis 2003; Bour-Jordan et al. 2011; reviewed in de Souza et al. 2010; Quill and Schwartz 1987). However this costimulation is not limited to activation of cell surface receptors that potentiate TCR signalling such as CD28 or inducible costimulator (ICOS) but also includes immuno-inhibitory receptors such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death 1 (PD-1) and the soluble cytokine, IL-2 (reviewed in Appleman and Boussiotis 2003; Mueller 2009; Nurieva et al. 2011). In fact, it was demonstrated that the absence of activatory costimulatory signals (CD28) alone in the absence of inhibitory costimulation (CTLA-4 or PD-1) is not sufficient to induce anergy in vivo (reviewed in Appleman and Boussiotis 2003; Bour-Jordan et al. 2011; Wells et al. 2001). It has been demonstrated that active ligation of immunoinhibitory receptor is required for anergy induction rather than just the absence of costimulation (Greenwald et al. 2001; Perez et al. 1997). CTLA-4 exerts negative regulatory effects on T cell activation and blocking CTLA-4 with antibody has been shown to lead to higher T cell activation (Greenwald et al. 2001; Kearney et al. 1995; reviewed in Mueller 2009; Vanasek et al. 2001). CTLA-4 has been shown to inhibit T cell proliferation, cell cycle progression and IL-2 production(Bour-Jordan et al. 2011). CTLA-4 knockout mice have a lymphoproliferative disorder and die within 3-4 weeks (Tivol et al. 1995; reviewed in Walker and Abbas 2002; Waterhouse et al. 1995). In addition, Programmed Cell Death 1 (PD-1) has been shown to be expressed in high amount by anergic cells and mice lacking PD-1 receptors

or its two ligands PD ligand 1 (PD-L1) and PD-L2 develop ADs (Ansari et al. 2003; Bour-Jordan et al. 2011; Freeman et al. 2000; Keir et al. 2006; reviewed in Mueller 2009; Nishimura et al. 1999; reviewed in Walker and Abbas 2002).

A hallmark of anergic T cells is that they do not produce IL-2 when stimulated with an antigen, even in the presence of co-stimulatory signals (Appleman and Boussiotis 2003; Jenkins et al. 1987; Quill and Schwartz 1987). IL-2 is not only critical for T-cell expansion but also has been shown to have a non-redundant tolerogenic function. Mice and humans deficient for IL-2 or IL-2 receptor develop lymphoproliferation and multi-organ autoimmunity (Sadlack et al. 1995; Suzuki et al. 1995). Polymorphisms in the IL-2 and IL-2 receptor genes have also been associated with autoimmunity (Cavanillas et al. 2010; reviewed in Wang et al. 2009) although this might not be a direct effect but an effect due to the role played by IL-2 in regulatory T cell biology.

Another effective mechanism of intrinsic peripheral tolerance is the clonal deletion of autoreactive cells. There is a limit on survival and proliferation of lymphocytes due to activation induced cell death (AICD) that is in place to limit damage. As self-antigens cannot be easily cleared, repetitive TCR engagement might be a feature of T-cell encounter with self-proteins, which perhaps serves as a trigger for AICD (de Souza et al. 2010). Apoptosis is induced by Fas receptor engagement by FasL and Bim-dependent triggering of a Bcl-2 and Bcl-xL–regulated mitochondrial death pathway (reviewed in Marrack and Kappler 2004; Mueller 2009; Walker and Abbas 2002). Spontaneous autoimmunity and T cell lymphoproliferative disease is observed in mice with a mutant allele of *Fas* that fails to transmit a death-inducing signal (de Souza et al. 2010; Watanabe-Fukunaga et al. 1992). Defects in the Fas pathway in humans are also associated with autoimmune lymphoproliferative syndrome (de Souza et al. 2010; Fisher et al. 1995; Walker and Abbas 2002). Cells in mice deficient for Bim are also resistant to apoptosis, and with age these mice spontaneously develop immune complex–mediated glomerulonephritis (Bouillet et al. 1999). Bim is thought to function as a natural antagonist of the survival protein Bcl-2, and both Bim-deficient and Bcl-2 transgenic OT-I $CD8^+$ T cells fail to undergo peripheral deletion after their adoptive transfer (Davey et al. 2002). Therefore, peripheral deletion of autoreactive T cells is essential for maintaining peripheral tolerance (Mueller 2009; Nagata et al. 2010).

1.3.2.2 Extrinsic regulation

The extrinsic regulation of peripheral autoreactive cells also involves a small subset of T cells with regulator phenotypes such as Natural killer T (NKT) cells, $CD8^+$ suppressor T cells, and T regulatory (T reg) cells that exert their effect on activated T and B cells as well as antigen presenting cells (de Souza et al. 2010; Jiang and Chess 2004) (Figure 1.5).

Natural Killer T cells

Natural Killer T (NKT) cells are a distinctive population of T cells generated in the thymus. They express alpha, beta TCR and have properties of Natural Killer cells (Bendelac 1995; Cerundolo et al. 2009; Godfrey et al. 2004; Jiang and Chess 2004). NKT cells specifically recognize glycolipids related to the glycolipid (alpha)-galactosylceramide that often occurs in pathogenic microorganisms and tumor cells via binding to the CD1 molecule (Cerundolo et al. 2009; Kinjo et al. 2006; Kinjo et al. 2005; Mattner et al. 2005). The CD1-glycolipid complex activates NKT cells and triggers it to lyse targets and secrete cytokines (Bendelac et al. 1995; Bendelac et al. 1997; Cerundolo et al. 2009; Mattner et al. 2005). Originally NKT cells were thought to mediate the innate immune responses that lyse tumor cells and pathogens (Cerundolo et al. 2009; Cui et al. 1997; Godfrey et al. 2004). NKT cells have also been implicated in human ADs where they have been shown to have a protective role (Mattner et al. 2008; Novak et al. 2007; Novak and Lehuen 2011). In monozygotic twins that are discordant for T1D, the twin with diabetes tends to have fewer NKT cells than the twin without diabetes (Wilson et al. 1998). However, the numbers of NKT cells were found to be unaltered in T1D compared with healthy controls (Lee et al. 2002). In the NOD diabetic mouse, injection of cell populations enriched for NKT cells prevents T1D (Beaudoin et al. 2002; Cain et al. 2006; Falcone et al. 1999; Sharif et al. 2001), whereas depletion of NKT cells early in the development of diabetes accelerates the onset of diabetes (Frey and Rao 1999). In mouse models of T1D and MS, depletion of NKT cells accelerates the onset of disease, while activation of NKT cells by treatment with (alpha)-galactosylceramide ameliorates, prevents or reduces severity of the disease (Furlan et al. 2003; Kojo et al. 2005; Wu and Van Kaer 2009). These effects are absent in mice that are deficient in CD1d (Wu and Van Kaer 2009). In mouse models of TID, RA and MS, NKT cells have been shown to have

a suppressive function by secretion of IL-10 and IL-4 that favor T_H2 responses (Baxter et al. 1997; Chiba et al. 2004; Hammond et al. 1998; Miellot et al. 2005; Wu and Van Kaer 2009).

$CD8^+$ suppressor T cells

$CD8^+$ suppressor T cells are thought to regulate autoreactive cells in the periphery by deleting or suppressing potentially pathogenic self-reactive T cells. They selectively suppress self-reactive lymphocytes with intermediate avidity for self and foreign antigens and possibly play an important role during the remission phase of AD (Jiang and Chess 2004; Jiang et al. 2003). Two key findings have highlighted the existence of this suppression pathway. Firstly, it was noticed that $CD8^+$ T cells participate in resistance to the re-induction of experimental autoimmune encephalomyelitis (Jiang and Chess 2004; Jiang et al. 1992; Koh et al. 1992; reviewed in Van Kaer 2010) and secondly, they suppress relapses of the disease (Panoutsakopoulou et al. 2004). The mechanism of this suppression was shown to be via preferential down regulation of autoreactive T cells that have intermediate avidity for peptides derived from myelin basic protein. The regulatory functions of $CD8^+$ T cells were also observed in other models of ADs, including collagen-induced arthritis, autoimmune myocarditis, and herpes simplex virus–induced stromal keratitis (Lu and Cantor 2008). $CD8^+$ T cells with suppressor activities have also been implicated in human ADs, including MS and inflammatory bowel disease (Brimnes et al. 2005; Tennakoon et al. 2006).

Some activated T cells express self-peptides bound to MHC class Ib molecule (Qa1 in mice and HLA-E in humans) (reviewed in Jiang and Chess 2000; Jiang et al. 1995; Li et al. 2001; Rodgers and Cook 2005; reviewed in Van Kaer 2010; Ware et al. 1995). The presentation of Qa1-self peptide complexes to the $CD8^+$ suppressor T cells is thought to trigger their differentiation into effector cells, which suppress any activated T cells expressing the same Qa1-self peptide complexes during secondary immune response (reviewed in Jiang and Chess 2004; Jiang et al. 1992). Activated $CD4^+$ T cells with intermediate avidity for their cognate antigen express Qa1 (HLA-E) predominantly bound by Heat shock protein 60 species (HSP60sp). These cells are effectively suppressed by HSP60sp specific $CD8^+$ suppressor T cells. In contrast to intermediate avidity CD4 T cells, CD4 T cells with high or low avididty for their cognate antigen: MHC complexes express Qa1 (HLA-E) molecules that are predominantly occupied by Qdm (B7 species) peptides and are therefore out of the control of

HSP60sp specific $CD8^+$ suppressor T cells. In addition, $CD4^+$ T cells with specificity for myelin basic protein-derived peptides can be used as a vaccine to induce $CD8^+$ T cells that protect against experimental autoimmune encephalomyelitis (Jiang et al. 2001; Jiang et al. 1998a; reviewed in Van Kaer 2010). Antibodies directed against the Qa1 molecule block this protection. Mice deficient in Qa1 or CD8 develop severe experimental autoimmune encephalitis when exposed to myelin-associated peptides (Hu et al. 2004; reviewed in Van Kaer 2010).

Furthermore, another distinct population of non Qa1-restricted $CD8^+$ suppressor T cells have also been shown to suppress immune responses by directly interacting with dendritic cells (DCs) and rendering them tolerogenic in vitro (Chang et al. 2002; Jiang et al. 2010; Jiang et al. 1998b). Up-regulation of inhibitory Ig-like transcript 3 (ILT3) and ILT4 receptors expressed on the DCs is thought to be involved in the suppression (reviewed in Jiang et al. 2010; Suzuki et al. 2008). APCs tolerised by $CD8^+$ T cells induce antigen specific unresponsiveness in $CD4^+$ T cells and have reduced expression of costimulatory molecules. The function of these cells in vivo and the mechanisms of suppression are still not clear (Jiang et al. 2010; Van Kaer 2010). In humans, in vitro studies have shown that Qa1 restricted $CD8^+$ T cells can be induced to differentiate into suppressor cells whose phenotype depends on HLA-E expression (Jiang et al. 2010; Li et al. 2001).

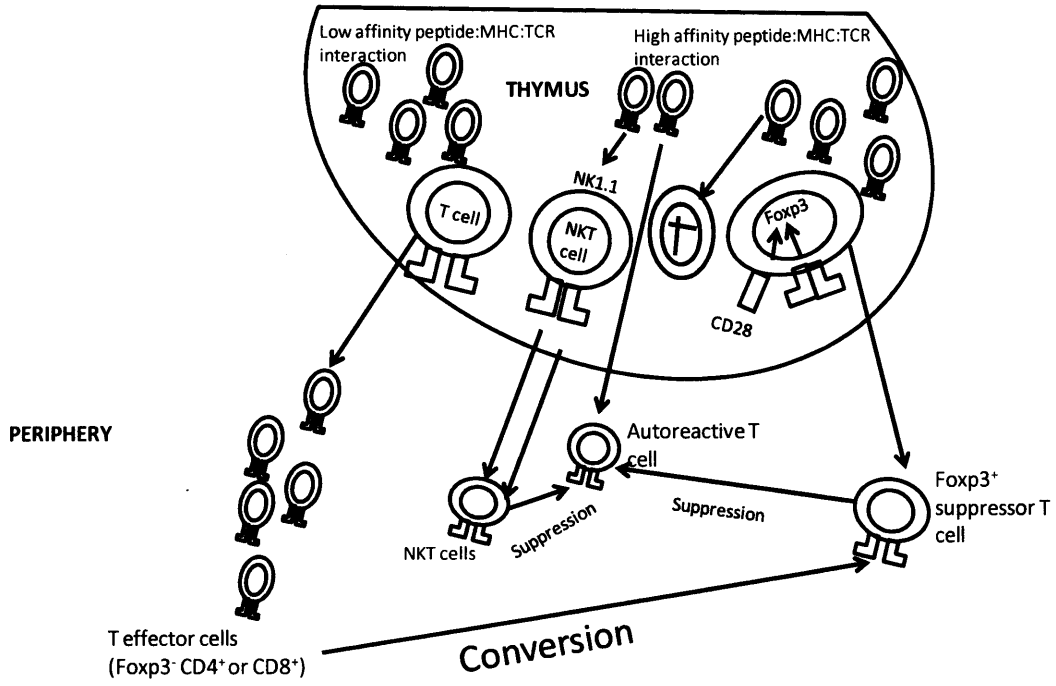


Figure 1.5 Generation of T suppressor cells in the thymus and periphery

Inductive model of T suppressor cell lineage commitment. High affinity TCR: peptide: MHC interactions in augmented by CD28 signalling and additional yet unknown signals converge to induce Foxp3 expression and thus T Suppressor lineage commitment. Foxp3 expressing T reg cells are highly enriched in but not restricted to $CD4^+$ T cell population. Certain conditions and cytokine environment in the periphery can induce conversion of Foxp3- effector T cells to a $Foxp3^+$ or $Foxp3^-$ regulatory T cell phenotype. Adapted from (Fontenot and Rudensky 2005)

$CD4^+$ regulatory T cells

One of the major cell types with a regulatory phenotype is the specialised subset of $CD4^+$ T cells that are able to control destructive immune responses to pathogens and to prevent immune responses against inappropriate targets, such as self antigens or non harmful external antigens. These cells are known as T regulatory (T reg) cells. They are generated in the thymus and their function is to maintain peripheral tolerance (Figure 1.5) (reviewed in Germain 2008; Itoh et al. 1999; reviewed by Sakaguchi 2000; reviewed in Vignali et al. 2008).

The idea of T reg cells dates back to early 1970s when immunologists noticed the phenomena of induced $CD4^+$ T cell non-responsiveness by a suppression mechanism via thymically derived "suppressor" T cells (Baker et al. 1970; Droege 1971; Gershon and Kondo 1971; Okumura and Tada 1971). This idea was further developed by the observation that patients with ADs have a greater percentage of MHC class II^+ $CD4^+$ cells in peripheral blood than healthy patients. RA patients and SLE patients were observed to have a higher than normal percentage of MHC class II^+ $CD4^+$ cells in peripheral blood (reviewed in Costantino et al. 2008; Yu et al. 1980). This was further supported by the observation that $MHCII^+$ $CD4^+$ T cell clones induce anergy when they present antigen to other $CD4^+$ T cells (Costantino et al. 2008; Lanzavecchia et al. 1988).

Evidence from these studies was used to argue for the existence of anti-idiotypic T cell networks and suppressor T cells that were thought to recognise the antigen receptor of autologous $CD4^+$ cells and prohibit activation with specificity directed against the suppressed cell (Germain 2008). Immunologists had a clear grasp of how these T cells were activated and what interleukins were necessary for their activation and expansion. However, whilst there was detailed information about the cytotoxic and helper T cells, whereby their specificity and restriction element of their antigen-binding receptor were known, there was little or no knowledge about these parameters for suppressor T cells. It was unclear whether or not suppressor T cells were antigen specific, or if they were MHC restricted (Germain 2008). No information was available about how they exhibited their suppressive function; whether they directly inhibited helper T cells or if they also influenced B cells (Germain 2008; Moller 1988).

In fact, the existence of suppressor T cells was highly debated (Costantino et al. 2008). There was a lack of unique markers to identify these suppressor T cells. In addition, the inability to confirm the existence of the so called "I-J gene", described by several papers as a governor of functions of suppressor T cells and suppressive factors and mapped to within the I complex of MHC region and the constantly changing soluble suppressor molecules (they have at different times contained Ia antigen and I-J antigen, been VH restricted and I-J restricted or both been antigen specific or non-specific) which were never clearly identified and characterised posed questions about their real existence (Reviewed in Costantino et al. 2008; Germain 2008; Moller 1988). In the years that followed, the biochemical and molecular mechanisms behind these suppression mechanisms could not be determined. MHC II was relegated to be a marker of late $CD4^+$ T cell activation, many early studies of suppressor T cells were discredited or disapproved and therefore the idea of T suppressor cells fell out of fashion (reviewed in Costantino et al. 2008; Germain 2008; Rouse 2007).

However, T suppressor cells came back due to the persistence of a few scientists who were able to demonstrate their presence or existence by using animal models (Fukuma et al. 1988; Penhale et al. 1975). The existence of T reg cells was demonstrated not as suppression of an immune response to non-self-antigen, but rather as a spontaneous development of AD after the depletion of a particular T cell subpopulation (Penhale et al. 1975; Sakaguchi et al. 1985; Sugihara et al. 1988). The field of T reg cells experienced a renaissance with the observation from Shimon Sakaguchi's group in 1995, which identified a subset of $CD4^+$ T cells that co-expressed the IL-2α chain receptor, CD25 (Sakaguchi et al. 1995). Further studies by Thornton and Shevach (1998) supported the existence of these suppressor cells by showing that they could limit expansion of $CD4^+$ and $CD8^+$ T cells in vitro (Thornton and Shevach 1998). These suppressor T cells were henceforth referred to as natural T reg cells.

Since the 1990s, research into these T reg cells has really gathered momentum and contributed greatly to our knowledge of their character, generation and function (Rudensky 2011; Sakaguchi 2008). T reg cells found in the periphery are a heterogeneous population of cells with different origins. Upon activation, T reg cells suppress proliferation and IL-2 production by responder CD4 or CD8 T cells (reviewed in Rouse 2007). T reg cells themselves have a reduced capacity to proliferate and produce IL-2 or pro-inflammatory cytokines under these conditions (Rouse 2007; Sakaguchi 2008). The main subset of T reg cells, known as natural T reg cells, are generated in the thymus by intermediate affinity interaction with agonist ligands and express CD25 and CD62L (Baecher-Allan et al. 2001;

Jordan et al. 2001; Suto et al. 2002). Instead of deletion by negative selection, the intermediate affinity self-peptide: MHC interaction in these T cells leads to the generation of T reg cells. Unlike conventional $CD4^+$ $CD25^-$ T cells, with natural T reg cells, it is thought that the same agonist ligand that leads to the generation of T reg phenotype in the thymus also activates the same T reg cell in the periphery. This means that the organ specific peptide ligands that are ectopically expressed in the thymus (Gotter and Kyewski 2004) can be involved both in the intrathymic generation of natural T reg cells and their activation in the periphery (Apostolou et al. 2002). In addition to high constitutive expression of the high affinity IL-2 receptor alpha chain (CD25) they are positive for CTLA-4 (Sakaguchi et al. 1995; Takahashi et al. 2000), glucocorticoids induced tumour necrosis factor receptor (GITR) and HLA-DR (Shimizu et al. 2002). They also display low expression of the IL-7 receptor alpha chain (CD127) (Bolacchi et al. 2006).

However, to date, the most reliable marker for the naturally occurring T reg is the transcription factor forkheadbox 3 (Foxp3), a member of the forkhead box winged helix family (Rudensky 2011). The Foxp3 transcription factor was identified from the X linked recessive mutant mouse strain, Scurfy, which shows hemizygous lethality in males a month after birth due to hyperactivation of $CD4^+$ T cells and overproduction of proinflammatory cytokines (Godfrey et al. 1991). The gene defective in Scurfy mice was identified and designated Foxp3 (Brunkow et al. 2001). The human homologue for the same gene Foxp3 was also found to be the cause of IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome in humans (Bennett et al. 2001; Chatila et al. 2000; Wildin et al. 2001). IPEX is an X-linked immunodeficiency syndrome associated with multi-organ autoimmunity, atopic dermatitis and fatal infections (Bennett and Ochs 2001). Foxp3 mainly acts as a repressor of transcription and acts as a master regulator in the development and function of T reg cells (Rudensky 2011). The full protein transcribed by the Foxp3 gene is encoded by 11 exons and contains a forkhead DNA binding domain at the C-terminus, which directly or by forming a repressor complex with nuclear factor of activated T cells (NFAT) can bind to the IL-2 promoter and repress IL-2 mRNA transcription (Bettelli et al. 2005; Mantel et al. 2006). In addition to a DNA binding domain, the protein encodes zinc finger and leucine zipper domains that permit homodimerization or heterodimerization with other forkhead family members or other DNA binding co-factor. In addition, the N terminus of protein has essential sites for repressor activity (Bettelli et al. 2005; Mantel et al. 2006). In bone marrow chimera with a mixture of cells from wild-type and Foxp3-deficient mice,

Foxp3-deficient bone marrow cells fail to give rise to $CD25^+CD4^+$ T reg cells, whereas Foxp3-intact bone marrow cells generate T reg cells that suppress disease development (Fontenot et al. 2003; Fontenot et al. 2005b). In transgenic mice that overexpress Foxp3, the number of $CD25^+CD4^+$ T cells is enhanced; $CD25^-CD4^+$ T cells and $CD8^+$ T cells expressed high levels of Foxp3 and exerted suppression in vitro (Khattri et al. 2003). Additionally, forced expression of Foxp3 in naïve T cells can convert them to cells with T reg like phenotype (Fontenot et al. 2003; Hori et al. 2003) However, it is now recognized that Foxp3 expression is necessary for suppressive function but its expression alone is not sufficient for lineage commitment (Lin et al. 2007).

IL-2 is also a critical factor required for T reg cell development (Sakaguchi 2008). This explains the puzzling observation that IL-2 or IL-2 receptor deficiency lead to autoimmunity instead of defects in T cell activation as IL-2 is thought to be an inflammatory cytokine required for proliferation and differentiation of activated T cells (reviewed in Akdis et al. 2011). IL-2 is also required for the sustained expression of Foxp3 and CD25 in natural T reg cells and enhances their suppressive function, at least in vitro (Fontenot et al. 2005b; Shevach et al. 2006)

The induction of suppression by natural T reg cells requires antigen stimulation but the exerted suppression is antigen non specific (Thornton and Shevach 2000). Activated T regs can inhibit a wide range of immune suppression through bystander suppression (Karim et al. 2005). Natural T reg cells exert suppressive function mainly via cell to cell contact (Takahashi et al. 1998; Thornton and Shevach 1998) however, in vivo, secreted cytokines are found to play a role in induction of suppression (Figure 1.6) (Annacker et al. 2001; McGeachy et al. 2005; Uhlig et al. 2006). Cytotoxicity is another mechanism by which natural T reg cells can exert their suppressive function (Figure 1.6). There is evidence that natural T reg cells can kill by mediating granzyme B dependent cytotoxicity against target cells (Grossman et al. 2004) which might be perforin dependent (Zhao et al. 2006) or independent (Gondek et al. 2005). T reg cells are also known to induce suppression of effector T cells by metabolic disruption. T reg cells have also been found to induce cytokine deprivation mediated apoptosis (Pandiyan et al. 2007). The expression of CD39 and CD73 by T reg cells has been shown to generate pericellular adenosine, which suppresses effector T cell function through activation of adenosine 2A receptor (Borsellino et al. 2007; Deaglio et al. 2007; Kobie et al. 2006). T reg cells have also been shown to inhibit effector T cells directly by transferring cyclic adenosine monophosphate (cAMP) into effector T cells

through membrane gap junctions (Bopp et al. 2007). In addition, T reg cells have been shown to suppress T cell function by inhibiting APC maturation and function, which are required for the activation of effector T cells (Tadokoro et al. 2006; Tang et al. 2006). T reg cells have been shown to condition DCs to express indoleamine 2,3-dioxygenase (IDO), a potent regulatory molecule known to induce the production of pro-apoptotic metabolites from the catabolism of tryptophan (Fallarino et al. 2003).

Adaptive T reg cells, like all T cells are thought to initially originate in the thymus but are derived from either classical T cell subsets in the periphery (Curotto de Lafaille and Lafaille 2009). These cells may be generated when the TCR of these naive T cells is stimulated by agonist peptide in low concentration or/ and with lack of stimulation (tolerogenic conditions) (Apostolou and von Boehmer 2004; Mahnke et al. 2003; Sakaguchi 2000; Thorstenson and Khoruts 2001). Several types of adaptive T reg cells have also been described; IL-10 producing type 1 regulatory T (Tr1) cells and IL-10, IL-4 and TGF-β producing T_H3 cells. Tr1 cells can be cultured in vitro in the presence of a large amount of IL-10 and their development is helped by the presence of IFN-α (Bacchetta et al. 1994; Groux et al. 1997; O'Garra and Vieira 2004). They secrete high amounts of the immunosuppressive cytokine IL-10 and low to moderate amounts of TGF-β when activated but no IL-4. Once activated, Tr1 cells suppress APCs and T cells in an antigen non-specific manner (Cavani et al. 2000; Kitani et al. 2000). This suppression by Tr1 cells may be mediated by both cell-to-cell contact (Vieira et al. 2004) and by production of cytokines (reviewed in Roncarolo et al. 2001; Vieira et al. 2004).

T_H3 are found to play a role in tolerance induction in the mucosal immune system (Coombes et al. 2007; Sun et al. 2007). They were first identified in mice after oral tolerance induction using myelin basic protein (MBP) (Chen et al. 1994). In vivo, after treatment with MBP, the majority of the MBP specific $CD4^+$ T cells secrete TGF-β and suppress the induction of MBP specific experimental autoimmune encephalitis (Fukaura et al. 1996). They function to control immune activation in the mucosa and lack of these cells is associated with autoimmunity in the gut and is linked to inflammatory bowel disease (Weiner 2001). T_H3 produce IL-10, IL-4 and a large amount of TGF-β and hence the mechanism of suppression is thought to be cytokine mediated.

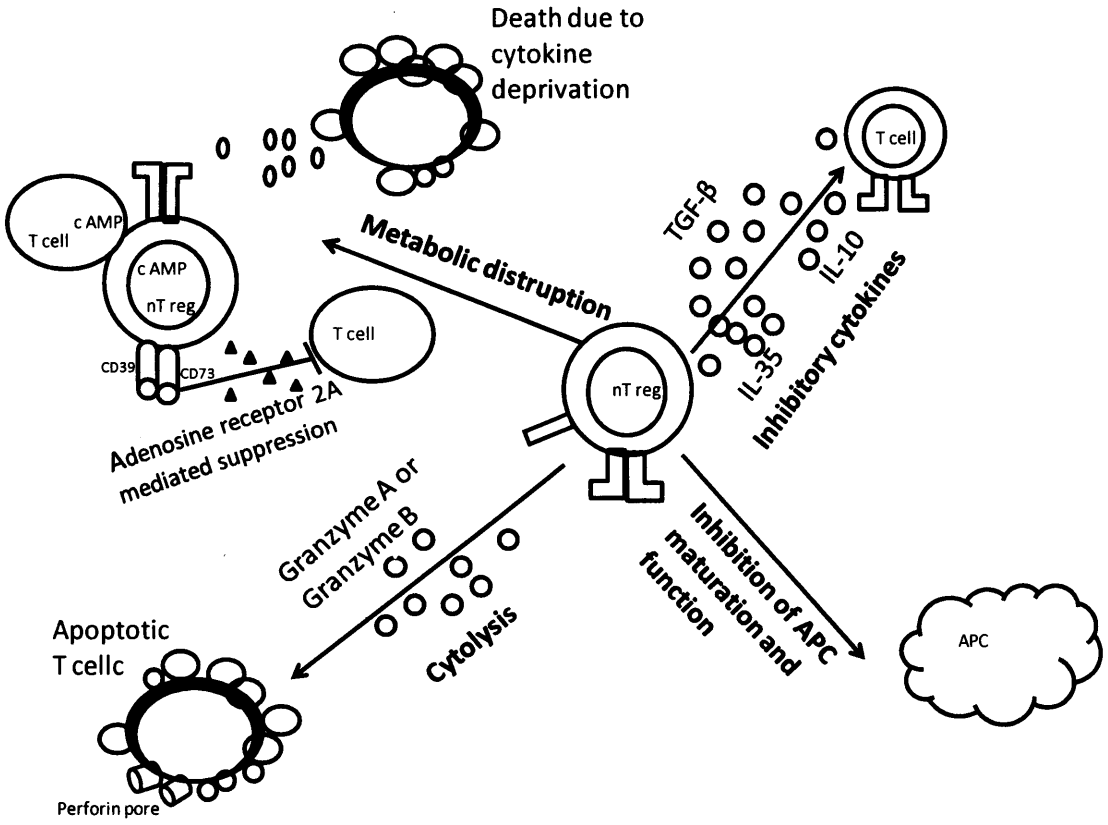


Figure 1.6 Mechanisms of suppression used by natural T reg cells

Inhibitory cytokines produced by natural T reg (nTreg) cells inhibit effector T cell activation. Metabolic disruption by production of adenosine, cyclic adenosine monophosphate (cAMP) mediated inhibition and cytokine deprivation are also mechanisms of suppression used by natural T reg cells. Cytolysis by Granzyme A and B dependent perforin mediated killing and suppression of APCs by targeting mechanisms that modulate APC maturation and function are yet other mechanisms used by natural Treg to suppress T effector cells. Adapted from (Vignali et al. 2008)

The role of cytokines in immune suppression

TGF-β, IL-35 and IL-10 are the key cytokines associated with T reg cell mediated immune suppression. IL-10 and TGF-β have generated a lot of interest for their role in the extrathymic generation of T_R1 and T_H3 cells, respectively. However, whilst the general immunosuppressive role of IL-10 and TGF- β is appreciated, the role of these cytokines in T reg cells biology in vivo is not yet clearly understood. Whereas in vitro data suggests a limited role for soluble IL-10 and TGF- β in T reg cell mediated immune suppression, studies involving in vivo models suggest otherwise.

TGF-β

The role of TGF-β in mediating suppression of T cell proliferation in vitro is controversial (Nakamura et al. 2001), however, there is some consensus that, in vivo, TGF-β plays an important role in mediating suppression. Fahlen et al (2005) using a mouse model of colitis showed that T cells unable to respond to TGF-β escaped immune suppression by T reg cells (Fahlen et al. 2005). In addition, TGF- β deficient natural T reg cells were unable to prevent the development of colitis (Li et al. 2007). There is also increasing evidence for the role of membrane bound TGF-β in T reg cell mediated immune suppression (Chen et al. 2006; Green et al. 2003). $CD4^+CD25^+$ cells have been reported to mediate suppression of $CD8^+$ T cells involved in autoimmunity or tumour rejection. This suppression requires intact TGF-β II receptor on the $CD8^+$ T cells (Chen et al. 2006; Green et al. 2003). It is however not apparent whether this inhibition requires TGF-β production by the $CD4^+CD25^+$ T reg cells or by other cells such as APCs. Some studies have reported that $CD4^+CD25^+$ T reg cells stain with antibodies to TGF-β whereas increased expression of TGF-β RNA has not been noted in $CD4^+CD25^+$ Treg cells (Green et al. 2003).

IL-35

IL-35 is a newly discovered cytokine thought to play a very important role in T reg cells mediated suppression of effector cells both in vitro and in vivo. IL-35 is the member of the IL-12 family alongside IL-12, IL-23 and IL-27 (reviewed by Akdis et al. 2011). The IL-12 family members are all heterodimeric proteins composed of two chains, one of which is either p19, p28, or p35 and the second chain is either p40 or Epstein-Barr virus induced gene 3 (EBI3) (reviewed by Akdis et al. 2011; Devergne et al. 1997). IL-35 is formed by heterodimeric pairing of an EBI3 and the p35 subunit of IL-12 (IL-12α) (Devergne et al.

1997; reviewed by Zhao Ning-Wei 2010). EBI3 is preferentially upregulated in mouse $Foxp3^+$ T reg cells (Gavin et al. 2007) and the EBI3/IL-12α heterodimer was found to be constitutively secreted by these cells (Collison et al. 2007; Gavin et al. 2007). Increased expression of the EBI3/IL-12 α heterodimer in mouse $Foxp3^+$ T reg cells and further transcriptional analysis indicated that expression of EBI3 is regulated by Foxp3 (Collison et al. 2007). Assessment of the suppressive ability of EBI3 knockout or IL-12α knockout T reg cells *in vitro* has showed that in the absence of EBI3 or IL-12α, the suppressive capacity of the T reg cells is reduced (Collison et al. 2007).

Stimulation of $CD4^+CD25^+$ mouse T reg cells with IL-35 has been shown to induce proliferation and IL-10 production by these cells without affecting Foxp3 expression (Ning-Wei 2010). However, stimulation of $CD4^+CD25^-$ effector T cells with IL-35 and anti CD3/CD28 antibodies increases IFN-γ production (Niedbala et al. 2007). In addition, $CD4^+CD25^+$ T cells expanded in the presence of IL-35 were shown to suppress $CD4^+CD25^-$ effector T cells (Niedbala et al. 2007). However, in human the role of IL-35 in T reg cell mediated immune suppression is questionable. A study by Allan et al (2008) demonstrated that unlike mouse T reg cells, ex vivo human T reg cells did not express significant EBI3 mRNA. The level of p35 mRNA in T reg cells was also found to be similar to the levels in T effector cells. Therefore neither EBI3 nor p35 mRNA was affected by over-expression of Foxp3 in human T reg cells, suggesting that IL-35 might not contribute to the suppressive mechanism of human T reg cells (Allan et al. 2008; reviewed by Zhao Ning-Wei 2010).

IL-10

IL-10 is considered to be one of the key molecules involved in immunosuppression in vivo. Its immunosuppressive effect protects the host from exaggerated immune responses to microbial infection as well as ADs. Several studies have reported the importance of IL-10 in inducing unresponsiveness in vivo (Bacchetta et al. 1994; Sundstedt et al. 2003; Van Parijs et al. 1997). T_R1 cells are generated by and mediate their suppressive function via IL-10 (Roncarolo et al. 2006). $CD4^+CD25^+$ natural T reg cells have also been reported to produce IL-10 in vivo (Annacker et al. 2001; Klein et al. 2003). In fact, certain diseases e.g. colitis, are suppressed by $CD4^+CD25^+$ natural Treg cells and this suppression requires the secretion of IL-10 by these cells (Asseman et al. 1999; Suri-Payer and Cantor 2001).

IL-10 exhibits multiple modulatory effects on the immune system. The IL-10 gene maps to a cytokine cluster that includes IL-19, IL-20, IL-24, IL-26 on chromosome 1q31-32 (reviewed by Akdis et al. 2011 2011; Kim et al. 1992). As an anti-inflammatory and immunosuppressive cytokine, IL-10 strongly inhibits the activation of myeloid derived cells, macrophages, dendritic cells and monocytes resulting in a reduced production of pro-inflammatory mediators and results in diminished T cell stimulation (de Waal Malefyt et al. 1991a; Peguet-Navarro et al. 1994). IL-10 directly affects APC functions by downregulating the expression of MHC class II molecules (Akdis and Akdis 2009). IL-10 is known to inhibit the expression of many cytokines including IL-1a, IL-1b, IL-6, IL-12, IL-18 and TNF-α; chemokines including monocyte chemoattractant protein (MCP)–1, MCP5, macrophage inflammatory protein (MIP)–1α (CCL3), MIP1β (CCL4), RANTES (CCL5), IL-8 (CXCL8), and CXCL10; and chemokine receptors (reviewed by Akdis et al. 2011; de Waal Malefyt et al. 1991a; de Waal Malefyt et al. 1991b).

IL-10 inhibits cytokine production and proliferation of $CD4^+$ T cells mainly indirectly through its effects on APCs (reviewed by Akdis et al. 2011; de Waal Malefyt et al. 1991a; de Waal Malefyt et al. 1991b). IL-10 also directly affects T cell activation by suppressing CD28, CD2 and signaling of the Inducible T-cell co-stimulator (ICOS) via the protein tyrosine phosphatase, SHP-1 (Taylor et al. 2007). In addition, IL-10 is also thought to play a role in influencing the balance between Th1 versus Th2 cytokines. As a pro inflammatory cytokine IL-10 promotes survival, proliferation, and differentiation of human B cells and increases the production of IgG (Akdis and Akdis 2009).

ADs result when there is a breakdown in multiple layers of these tolerance and regulation mechanisms. Different environmental factors and numerous genes are associated with autoimmunity.

1.4 The role played by environmental factors in development of ADs

Although a degree of predisposition to autoimmunity is present in everyone, clinically relevant AD develops only in susceptible persons. Even with a genetic predisposition most people do not develop ADs unless some environmental factor acts as a trigger (Cojocaru

2008; Racanelli et al. 2011). The evidence for this is provided by familial and twin concordance studies and also animal models of ADs such as NOD mice where genetically identical mice that develop T1D do so with different disease kinetics and some escape disease altogether (Murphy et al. 2007a). Concordance rates for ADs in monozygotic twins are 30-70% but not 100% implying that environmental factors play an important role in disease development (Simmonds and Gough 2005). The environmental triggers for ADs only act in the presence of genetic predisposition and do not affect the population at large (Cojocaru 2008). Many studies have noted that there is an association between microbial infection and the induction or exacerbation of ADs (Bachmaier et al. 1999; Miller et al. 1997). It is not known whether this is due to molecular similarity between microbial epitopes and self - antigens (molecular mimicry). Although there is evidence for molecular mimicry in some cases (Gautam et al. 1998; Panoutsakopoulou et al. 2001; Steere et al. 2001), most ADs linked to infections are related to the release of sequestered self antigens which the normal immune system ignores (Miller et al. 1997) or to the non specific inflammatory effect of infection which is thought to alter the phenotype of APCs and modify cell trafficking (Ehl et al. 1998; Horwitz et al. 1998; Keffer et al. 1991; Kivity et al. 2009). The innate immune system has also been implicated in the breakdown of self-tolerance leading to AD. An aberrant innate reaction to self-tissue might provide an activatory cytokine environment and relevant costimulation for an autoimmune response from autoreactive T cells. This is shown by the requirement of NK cell involvement in the development of experimental myasthenia gravis in mice (Shi et al. 2000).

1.5 Genetics of Autoimmunity

The genetic basis of autoimmunity is not clearly understood but genetic defects alone do not always lead to ADs. Genes that predispose to autoimmunity affect one or more of the layers of tolerance. Genetics evidently plays an important role in predisposition to ADs as is shown by the fact that some familial clustering of some ADs occurs suggesting a role for genetic susceptibility (Heward and Gough 1997). Additionally, if one of the two monozygotic twins is affected the other one is very likely to as well, whereas concordance of the disease is much less in dizygotic twin (reviewed in Heward and Gough 1997; Murphy et al. 2007a). Various animal models of autoimmunity such as the non-obese diabetic (NOD) strain of mice, which are very likely to get autoimmunity provide more evidence for the role of genetics in

predisposing to ADs. Genes that predispose to autoimmunity can be classified as follows: genes that affect auto-antigen availability and clearance, genes that affect apoptosis, genes that affect cytokine gene expression, genes that affect co stimulatory molecules and genes that affect signalling thresholds (Murphy et al. 2007a).

The genes that affect antigen availability or clearance can affect the induction of central and peripheral tolerance mechanisms leading to autoimmunity. Low or inadequate expression of self antigens in the thymus can compromise the negative selection of developing thymocytes as the T cells that are reactive to these antigens can escape deletion and will be released in the periphery where they can react to self antigens leading to AD. An example of this is mutation in the AIRE gene, which results in APECED by affecting antigen availability in the central and peripheral lymphoid organs (Anderson et al. 2000; Hanahan 1998; Heino et al. 1999; Klein et al. 2000). In addition, polymorphisms in the insulin gene affecting its regulation are associated with T1D (Barratt et al. 2004; Pugliese et al. 1997; Vafiadis et al. 1997). The disease-associated allele shows reduced expression in the thymic epithelium and increased expression in the pancreatic islet cells. This is thought to predispose to a lack of central tolerance to insulin in the developing thymocytes in the thymus (Chentoufi and Polychronakos 2002). Graves disease is also associated with polymorphisms in the thyroid stimulating hormone receptor gene the product of which is a target autoantigen in Graves disease (Dechairo et al. 2005). In terms of peripheral tolerance, hereditary deficiencies in the complement proteins such as C1q, C3 and C4, which are important in clearing apoptotic cells and complexes, have been associated with the development of SLE (Botto and Walport 2002; Pickering et al. 2000; Walport 2002).

Genes that control apoptosis such as Fas are important in limiting the duration and vigour of the immune response. Mutations in the Fas gene which is involved in induction of apoptosis predisposes to autoimmunity by compromising intrinsic peripheral tolerance mechanisms such as AICD (de Souza et al. 2010; Watanabe-Fukunaga et al. 1992 (de Souza et al. 2010; Fisher et al. 1995; Walker and Abbas 200). AICD ensures that most cells that have been activated in response to an antigen will die by apoptosis thereby limiting inflammation and excessive damage to the tissue which would lead to release of autoantigens (Zhang et al. 2004). AICD also helps maintain tolerance by keeping in check self-reactive cells in the periphery, which have escaped other tolerance mechanisms (Kabelitz and Janssen 1997; Zhang et al. 2004).

Genetic defects in costimulatory molecule expression also predispose to ADs. This is because costimulatory molecules on the T cell surface modulate activation of T cells via their TCRs. Additionally, costimulatory signals affect Treg development, homeostasis, and suppressive function independently of their effect on effector T cells (reviewed in Bour-Jordan and Bluestone 2009). Thus, costimulation plays an important role in tolerance induction at multiple levels through signals mediated by positive and negative costimulatory molecules that affect effector T cells (Bour-Jordan et al. 2011). CD28, CTLA-4, and ICOS are co-stimulatory molecules expressed on T cells which bind to homologous ligands on APCs (reviewed by Keir and Sharpe 2005). CD28 and ICOS provide positive signals whereas CTLA-4 is generally a negative regulator of T cell activation (reviewed by Chen 2004; van Berkel and Oosterwegel 2006). CTLA-4 knockout mice develop lymphoproliferative disease that results in their death by 3-4 weeks (Tivol et al. 1995). Furthermore, CTLA-4 polymorphisms have been associated with a variety of ADs including Graves disease, SLE, MS, T1D and RA (Holmberg et al. 2005; Nistico et al. 1996; Plenge et al. 2005; Ueda et al. 2003; Yanagawa et al. 1995). The odds ratio of association between the different CTLA-4 polymorphisms and ADs is between 1.1 and 1.5 (Brand et al. 2005). One of the proposed mechanisms of this predisposition is associated with the finding that the CTLA-4 risk haplotype has been linked with lower levels of a splice variant encoding a soluble form of CTLA-4 which can interact with CD80 and CD86 and block their interaction with co-stimulatory molecules such as CD28 (reviewed by Gough et al. 2005). This might cause a lack of local blocking of the effector T cells.

Similar to CTLA-4, PD-1 protein which is an important co-inhibitor of T cell activation has also been associated with ADs (reviewed in Bour-Jordan et al. 2011). The autoimmunity due to complete loss of PD1 expression in mice however is observed to be much milder than that associated with loss of CTLA-4 (Nishimura et al. 1999; Nishimura et al. 2001). Polymorphisms in the PD1 gene have also been found to be associated with RA, Graves disease, T1D, MS and SLE in humans (Kroner et al. 2005; Nielsen et al. 2003; Prokunina et al. 2002).

Defects in genes that are involved in cytokine-cytokine receptor expression or signalling can also lead to autoimmunity (Zenewicz et al. 2010). Overexpression or underexpression of the genes involved in cytokine production leading to over- or underexpression of the respective cytokines can lead to autoimmunity (O'Shea et al. 2002; Ogura et al. 2005). Cytokines are required for proper activation and recruitment of the innate and adaptive immune cells for a

regulated immunological response. Correct expression of the cytokine receptors is also required for effective signalling and polymorphisms in cytokine receptors have been associated with human ADs (reviewed in O'Sullivan et al. 2007; Zenewicz et al. 2010). For example, the IL2 receptor alpha gene has been associated with both T1D and MS and MS has also been associated with IL-7 receptor alpha.(Barrett et al. 2009; Hafler et al. 2007) Overexpression of pro-inflammatory cytokines could for example lead to improper and excessive activation of autoreactive T and B cells and lead to wide tissue damage causing release of sequestered antigens and recruitment of more autoreactive cells (Murphy et al. 2007a). On the other hand underexpression of cytokines that are involved in mediating immune suppression could lead to autoimmunity because of the defect in cytokine mediated suppression of T cells, B cells and APCs (Murphy et al. 2007a). Many cytokines have been associated with ADs. Overexpression of TNF-α IL-2, IL-7, IL-10, IL-12 has been associated with inflammatory bowel disease (IBD) (Marquez et al. 2009; van Heel et al. 2007; van Heel et al. 2002; WellcomeTrust 2007). Overexpression of INF-γ is associated with SLE (Fan and Wuthrich 1997; Harigai et al. 2008; Prud'homme et al. 1995). Additionally, underexpression of TNF-α and TGF-β have been associated with SLE (Baechler et al. 2004; Ronnblom et al. 2006).

The genes affecting signalling thresholds represent the largest category of mutations associated with predisposition to ADs (Murphy et al. 2007a). Adjusting the signalling thresholds by making the signalling more or less sensitive can equally result in autoimmunity. If thymocytes are unable to respond adequately to signals delivered via their TCR due to defects in downstream signalling molecules, this can lead to defective negative selection in the thymus and therefore ADs (Gregersen and Behrens 2006). For example, in mice it has been shown that a loss of function mutation in Zap-70 which is a molecule involved in TCR signalling results in reduced negative selection and subsequent escape of self reactive cells into the periphery leading to autoimmune arthritis in these mice (Sakaguchi et al. 2003). In contrast, increasing TCR sensitivity in the periphery can lead to greater and prolonged activation of the T cells once again resulting in autoimmunity. This can be observed in mutations in negative regulators of TCR signalling which can lead to hyperproliferation of lymphocytes and exaggerated immune responses (Bour-Jordan et al. 2011; Gregersen and Behrens 2006), for example, CTLA-4 polymorphisms, which have been linked to autoimmunity as discussed above.

In addition, genes affecting signalling thresholds can impact on individual T cell lineages, for example, the generation of T reg cells in the thymus requires TCR ligation of intermediate strength and if the TCR is hyporesponsive, the quantity and quality of T reg cells generated may be affected (Baecher-Allan et al. 2001; Davidson and Diamond 2001; Jordan et al. 2001; Suto et al. 2002). A role for genes implicated in signalling in the generation and function of particular T cell lineages is seen in involvement of Foxp3 with T reg cell generation (Rudensky 2011). Foxp3 mutations result in rare autoimmune disease, IPEX, in humans (Bennett et al. 2001). T reg cells are also critically dependent on IL-2 for their proliferation and function and a lack of IL-2 or its receptors can lead to autoimmunity (Fontenot et al. 2005a; Setoguchi et al. 2005).

1.5.1 Identification of genes involved in ADs

Human genetics has provided powerful insights into ADs. There are two approaches to identifying genes associated with ADs. First is the candidate gene approach, where a particular genetic polymorphism in a specific gene is chosen due to its known involvement in crucial immunological pathways and studies are carried out to check for any association of this gene with ADs. Either a linkage study involving a group of unrelated patients and healthy controls can be carried out to check for any association between the candidate gene and ADs or a family based study involving genetically similar individuals from different families who either have or do not have a AD can be used to identify any association between the gene and the AD (Gregersen and Behrens 2006). Alternatively, instead of picking a candidate gene, random Single Nucleotide Polymorphisms (SNPs) distributed throughout the genome can be scanned to check if a polymorphism in any part of the genome is associated with autoimmunity (Gregersen and Behrens 2006). Once again the association study can either be a linkage or a family based study.

The most common and the strongest genetic contributors to ADs are polymorphisms in the MHC alleles (Simmonds and Gough 2005). The human MHC (termed HLA) region is located at chromosome 6p21.3 and spans across a 7.2 Mb genomic segment (reviewed by Rai and Wakeland 2011; Shiina et al. 2004). The human leukocyte antigen (HLA) region contains over 400 genes many of which play an important role in antigen processing, presentation, cellular activation, inflammation and various other crucial functions of the innate and

adaptive immune systems (Simmonds and Gough 2005). The HLA region can be divided into three different parts: class I, class II and class III. The class I region encodes HLA-A, HLA-B and HLA-C molecules which are expressed on the cell surface of nucleated cells and are involved in the presentation of endogenous antigens to $CD8^+$ T cells (Simmonds and Gough 2005). The class II region encodes various membrane bound proteins expressed on the cell surfaces of B-lymphocytes, macrophages, dendritic cells and activated T lymphocytes, which are involved in the processing and presentation of exogenous antigens to $CD4^+$ T cells (Simmonds and Gough 2005). The class III region is located between class I and class II regions and encompassing genes encoding components of the complement region (C2 and C4), the heat shock protein (HSP70) and the tumour necrosis factor (TNF) (Simmonds and Gough 2005). The HLA region is very polymorphic and variations are strongly associated with almost every AD (Rai and Wakeland 2011). It is thought that variations in MHC peptide binding properties modulate T cell antigen recognition in the periphery, leading to potentially increased susceptibility to a breakdown in tolerance in the T cells (Todd. 2010). About 30% of the estimated genetic heritability for most AD is due to the polymorphisms in the HLA region and hundreds of associations between HLA alleles and ADs have been identified (Rai and Wakeland 2011; Shiina et al. 2004). For the majority of the ADs, susceptibility is linked to a MHC class II allele but in some cases particular MHC class I alleles have also been shown to have strong associations (reviewed by Elder et al. 2009 2010; Handunnetthi et al. 2010; Todd 2010. 2010). MHC class III region (complement region) has also been associated with AD (Harley et al. 2009). However, there is a strong linkage disequilibrium across the entire HLA region making interpretation of associations difficult (Rai and Wakeland 2011) .

Genes outside of the MHC regions have also been associated with ADs. For example AIRE, CTLA-4, Foxp3, Fas and PD-1 have all been associated with ADs (see above). There are many other genes which have also been found to correlate with autoimmunity, however, the most robust association with ADs outside of the MHC region has been found to be a SNP in the genes encoding for the protein tyrosine phosphatase non-receptor type 22 (PTPN22).

Several association-based genetic studies and candidate gene based studies have linked a SNP in PTPN22 to ADs such as T1D (Bottini et al 2004, Qu et al 2005), RA (Begovich et al 2004), Graves Disease (Velaga et al 2004) and SLE (Kyogoku et al 2004).

1.6 Protein Tyrosine Phosphatases

A number of molecular regulatory mechanisms exist in immune cells to maintain the fine balance between immune protection and autoimmunity. One such mechanism is protein phosphorylation, a reversible biochemical event regulating many signalling pathways. Protein phosphatases are a large class of enzymes that remove phosphate from specific residues in proteins. Dephosphorylation is an important means of resetting a phosphorylated protein back to its original state and thus potentially switching a signal off.

Initially, protein phosphatases were viewed as passive housekeeping enzymes that functioned promiscuously to reverse the action of protein kinsases. However, it is now clear that the kinases which catalyze phosphorylation and the phosphatases that promote dephosphorylation are partners, working in a coordinated fashion in the regulation of signalling responses (Alonso et al. 2004b). The kinases have been implicated in controlling the amplitude of the signalling whereas the phosphatases are thought to have an important role in controlling the rate and duration of the response (Heinrich et al. 2002; Hornberg et al. 2005).

Protein tyrosine phosphatases (PTPs) are a large family of enzymes that remove a phosphate group from phosphorylated tyrosine residues in proteins. The human genome encodes a total of 107 PTPs. Of the 107 PTPs, 11 are catalytically inactive, 81 encode active protein phosphatases, 13 dephosphorylate inositol phospholipids and 2 dephosphorylate messenger RNA (mRNA) (Alonso et al. 2004b; Mustelin et al. 2005). These enzymes are defined by the active-site signature motif (H/V)CX_5R(S/T) (reviewed by Zhang 1998).

PTPs can be divided into four major subfamilies, class I, class II, class III which use a cysteine as a nucleophile for catalysis and class IV PTPs which use an aspartic acid as a nucleophile (Figure 1.7). The class I PTPs use a cysteine-based catalytic mechanism. This is the largest sub-family and contains 38 well known "classical" PTPs and 61 "dual specific" PTPs (Andersen et al. 2004). The classical PTPs are strictly tyrosine specific and can be further divided into transmembrane receptor-like PTPs which generally have an extracellular, putative ligand binding domain, a single transmembrane region and one or two cytoplasmic PTP domains and the intracellular non-receptor PTPs which contain a single catalytic domain and various amino- or carboxy- terminal extentions. There are 21 receptor type PTPs and 17 non-receptor type PTPs in the human genome (Andersen et al. 2004). Unlike the classical

PTPs, the dual specific PTPs can utilize protein substrates that contain phospho-tyrosine as well as phospho-serine and phospho-threonine (Zhang 2001). Dual specific PTPs are much more diverse than the classical PTPs and can be divided into seven subgroups. Eleven out of the 61 dual specific PTPs are specific for the MAP kinases Erk, Jnk and p38 (Alonso et al. 2004a; Keyse 1998; Saxena and Mustelin 2000). A second subgroup referred to as "atypical dual specific phosphatase" includes 16 small enzymes (less than 250 amino acid residues) that lack MAP kinase targeting motifs. The third and fourth subgroups are the three slingshots (SSH1, SSH2 and SSH3) and the three PRLs (PRL-1, PRL-2 and PRL-3). The fifth subgroup is the CDC14 group, which contains 4 enzymes. The sixth and the seventh subgroup of the dual specific phosphatases are the PTENs (5 genes) and the myotubularins (16 genes) which specifically dephosphorylate the D3 phosphates of inositol phospholipids (Wishart and Dixon 2002).

The class II PTPs are also cysteine based and this group contains only one low molecular weight phospho tyrosine phosphatase. The class III phosphatases are also cysteine based and tyrosine/ threonine specific. This group contains three $p80^{Cdc25}$ cell cycle regulators. The class IV PTPs use an asparatate-based mechanism of catalysis and contain the four Eya proteins, which were discovered to be tyrosine specific or dual serine and tyrosine specific phosphatases (reviewed by Alonso et al. 2004b; Rayapureddi et al. 2003; Tootle et al. 2003).

The expression pattern of PTPs varies from ubiquitous to tissue specific. Most cells express 30-60% of the entire complement of PTPs. Haematopoietic and neuronal cells tend to express higher numbers of PTPs, for example T cells contain 60 to 70 different PTPs. Some PTPs are tissue specific such as PTPN22 or Lyp, which is restricted to haematopoietic cells. Other haematopoetic-restricted PTPs include PEST enriched phosphatase (PEP-mouse ortholog of Lyp), SHP-1 (Src homology 2-domain-containing-PTP1), CD45 and HePTP (Haematopoietic PTP) (Alonso et al. 2004b). Approximately 20 of the 60-70 PTPs expressed in T cells, regulate signalling events between the TCR and the transactivation of the IL-2 gene (Mustelin et al. 2005). Most of these PTPs effect TCR signalling in a negative manner but some such as CD45 and SHP-2 have positive regulatory roles (Mustelin et al. 2005).

Several human diseases have been linked to PTPs. For example, the absence of PTP1β, which is thought to act as a negative regulator for insulin signalling leads to an increased sensitivity to insulin and resistance to obesity in mice (Elchebly et al. 1999; Klaman et al. 2000). In addition, PTPα has been implicated in promoting cell transformation by activating Src family

kinases via dephosphorylation of the inhibitory carboxy-terminal phosphotyrosine on the kinase (Ponniah et al. 1999; Su et al. 1999; Zheng et al. 1992). Cdc25 phosphatases have also been implicated in cancers as they play an important role in cell cycle regulation by removing the inhibitory phosphatase from tyrosine and threonine residues of the cyclin dependent kinases. Cdc25A and Cdc25B are consequently thought to be proto-oncogenes (reviewed by Kristjansdottir and Rudolph 2004). In addition, PTPs have also been linked to several ADs. The first example of this was the discovery that the motheaten mouse had a mutation in the SHP-1 gene (Tsui et al. 1993). These mice have hyper responsive T and B cells and overactive phagocytic cells. They also develop inflammatory pathology in several tissues. In humans, a genetic polymorphism in PTPN6 (which encodes SHP-1) has not yet been linked to ADs (Mustelin et al. 2005). However, CD45, which is a positive regulator of TCR signalling, has been implicated in ADs. CD45 abnormalities were detected in T cells from patients with SLE and loss of CD45 was found in some patients with severe combined immunodeficiency (Jury et al. 2004; Tchilian et al. 2001).

Recently several studies have associated a genetic polymorphism in the gene PTPN22 to several ADs. Outside of the MHC region, this polymorphism has shown the most robust association with ADs (Burn et al. 2011).

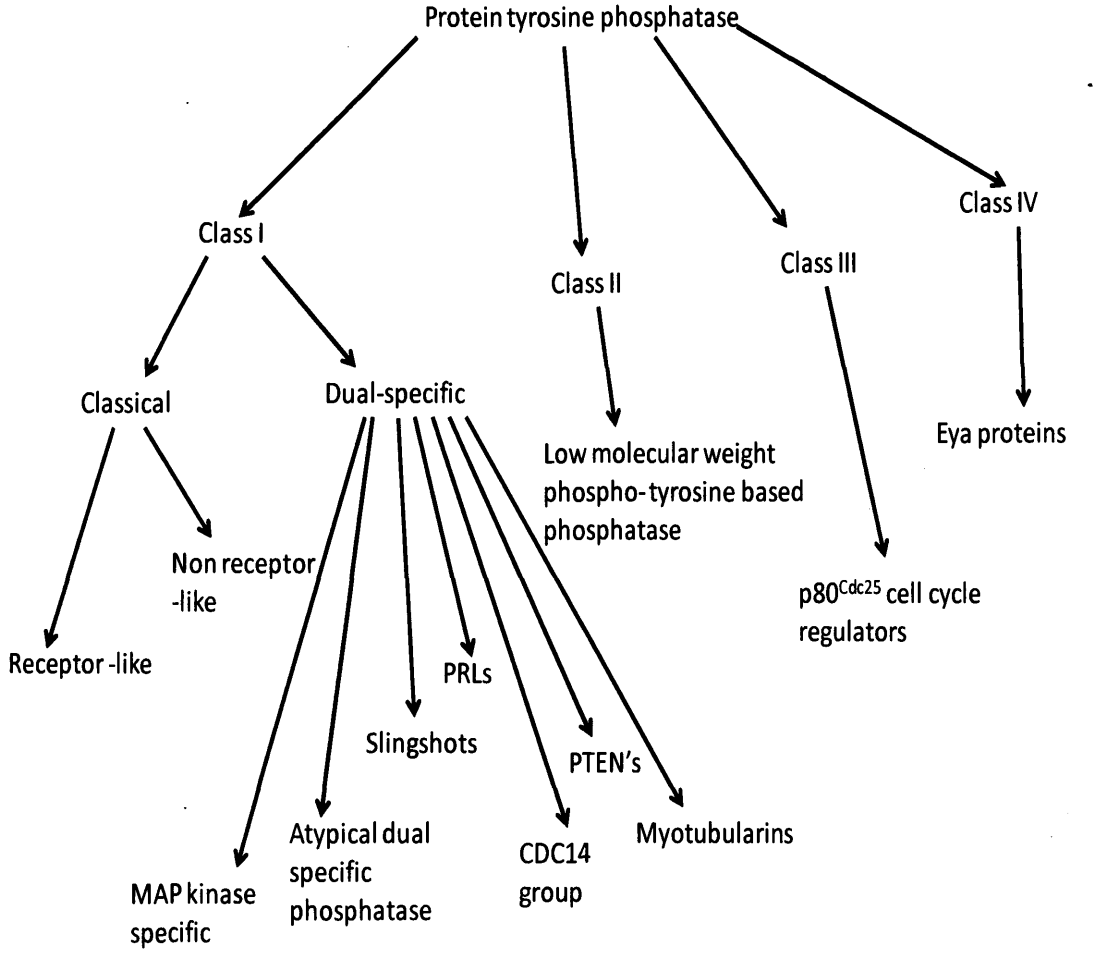


Figure 1.7 Classifications of the Protein Tyrosine Phosphatases in the human genome.

Of the 107 identified Protein Tyrosine Phosphatases (PTPs) in the human genome, 81 are active PTPs. This superfamily is divided into four major subfamilies, Class I- IV. Class I- III are cysteine based PTPs whereas class IV is an asparate based PTP. Class I PTPs are further divided into classical and dual specific phosphatases. Class II PTPs are low molecular weight phospho-tyrosine based phosphatases. Class III containes p80 Cdc 25 cell cycle regulators and Class IV contains four Eya proteins.

1.6.1 Protein Tyrosine Phosphatase Non receptor Type 22: PTPN22

PTPN22 is a member of the class I, classical PTP family and belongs to the non-receptor like subfamily. It is one of the PEST domain-containing phosphatases alongside BTP1 and PTP-PEST (Cohen et al. 1999; Matthews et al. 1992). The PTPN22 gene maps to chromosome 1p13.3- 1p13.1 and encodes an 807 amino acid residue protein referred to as lymphoid specific phosphatase (Lyp) in humans or PEST domain containing PTP (PEP) in mice (Cohen et al. 1999; Matthews et al. 1992). Lyp and PEP share 89% identity between PTP domains and 61% identity for their non-catalytic portions (Cohen et al. 1999). Lyp and PEP are both exclusively expressed in haematopoietic cells (Chow and Veillette 1995; Cloutier and Veillette 1996; Cohen et al. 1999). Lyp mainly has a cytoplasmic subcellular localization, however, some PEP has been found in the nucleus (Cohen et al. 1999; Gjorloff-Wingren et al. 2000). Lyp and Pep are approximately 105kDa and 110kDa in size respectively (Cohen et al. 1999; Gjorloff-Wingren et al. 1999). Lyp contains a 300 amino acids N-terminal PTP domain, a central region of approximately 300 amino acids of unknown function also called the interdomain, and a C-terminal portion of approximately 200 amino acids (Figure 1.8) (Cohen et al. 1999; Matthews et al. 1992). The C-terminal of Lyp contains four proline-rich-motifs termed P1-P4. P4 is part of the C-terminal homology (CTH) domain found in all members of the PEST group of PTPs (Cohen et al. 1999; Cote et al. 2002; Spencer et al. 1997).

Lyp and PEP have been found to have several binding partners. Lyp has been reported to interact with adapter molecule, Grb2, and E3 ligase, c-Cbl (Cohen et al. 1999; Hill et al. 2002). However, the best characterized binding partner of Lyp/PEP is the C-terminal Src kinase (Csk). Csk is thought to bind to P1 region of Lyp/PEP via its Src homology 3 (SH3) domain (Cloutier and Veillette 1996; Ghose et al. 2001; Gregorieff et al. 1998).

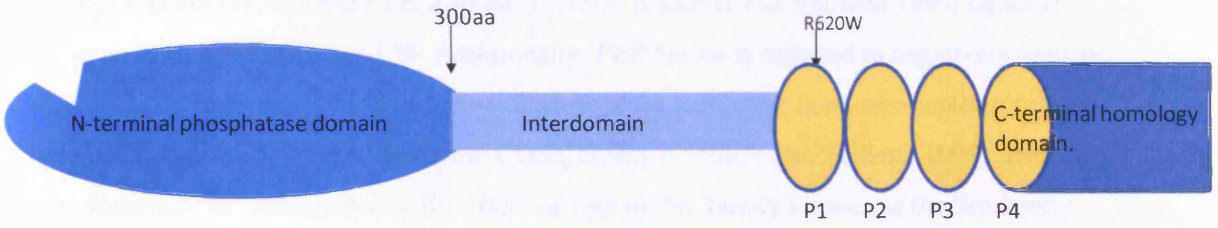


Figure 1.8 Domain structure of Lyp

Lyp has an amino terminal protein tyrosine phosphatase domain of approximately 300 amino acids, followed by approximately 200 amino acids long interdomain whose function is not yet known and approximately 300 amino acids carboxy terminal. The carboxy terminal of Lyp contains four prolin-rich-motifs termed P1- P4 (represented as orange ovals). The R620W polymorphism lies in the P1 motif of the C-terminal. P4 is part of the C- terminal homology domain.

1.6.1.1 Function of Lyp/PEP

Lyp and PEP are thought to negatively regulate TCR signalling by acting immediately downstream of the TCR. PEP has been implicated in the dephosphorylation of the positive regulatory tyrosine residue in the activation loop of the Src family kinases, Lck (Tyr 394), and FynT (Tyr417); and the PTP, Zap 70 (Tyr493) (Cloutier and Veillette 1999; Gjorloff-Wingren et al. 1999) (Figure 1.9). Additionally, PEP has been reported to negatively regulate TCR-induced phosphorylation of the tyrosine residues within the immunoreceptor tyrosine based activation motifs (ITAMs) in the CD3/ζ chains (Cloutier and Veillette 1999). However, this effect may be indirect due to the effect of Lyp on Src family kinases as the Src family kinases are responsible for the phosphorylation of the ITAMs. Lck, Zap 70, ITAMs of the CD3/ζ chains, Vav and valosin-containing protein have also been identified as potential substrates by a study using a substrate trapping mutant version of Lyp together with a mass spectrometry based peptide identification technique (Wu et al. 2006). The ability of Lyp/PEP to bind Csk may be important for Lyp/PEP function. Csk phosphorylates the C-terminal negative regulatory tyrosine in Lck and FynT, thereby negatively regulating signalling via the TCR (Cloutier and Veillette 1999; Gjorloff-Wingren et al. 1999).

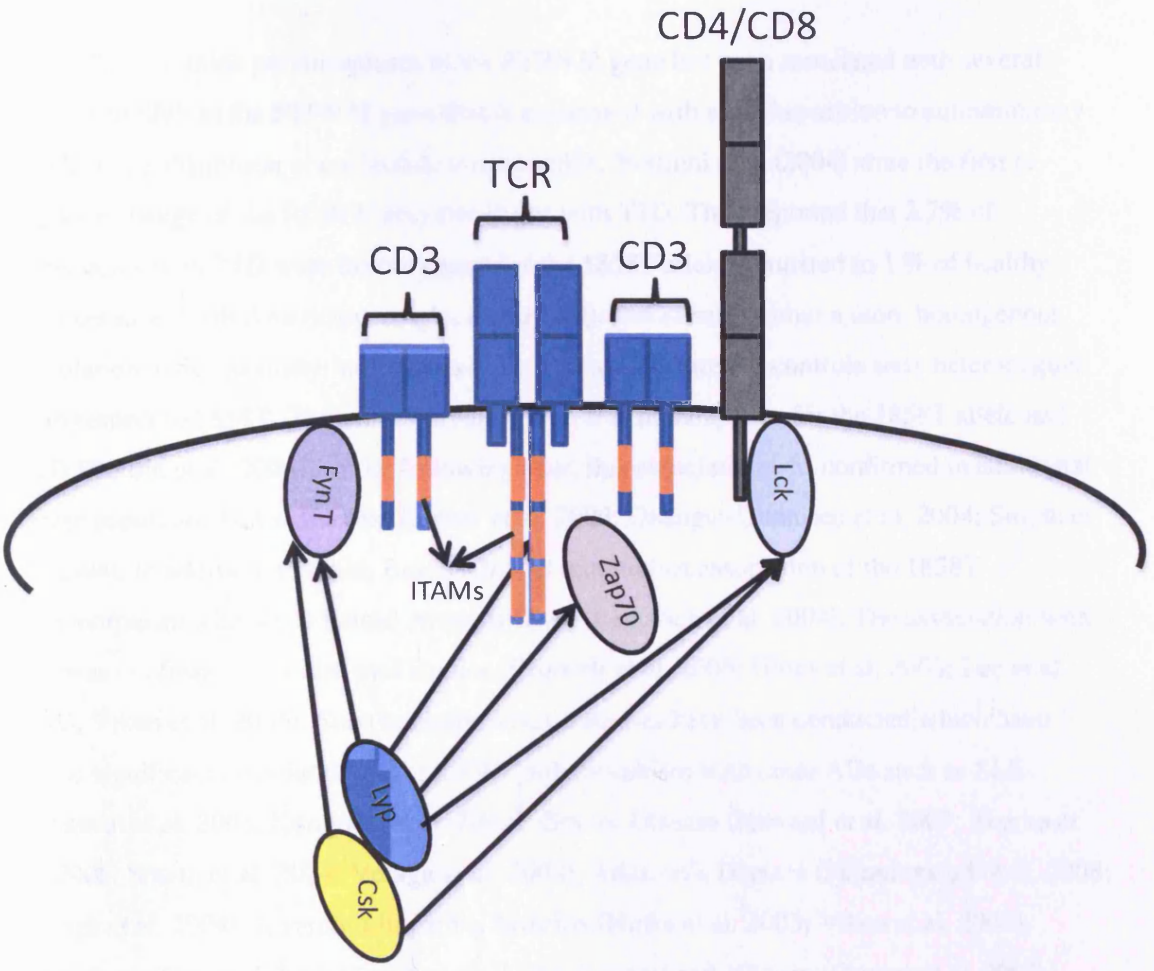


Figure 1.9 A model for the role played by Lyp/Pep in TCR signalling

Lyp acts as a negative regulator of TCR signalling by acting directly downstream of the TCR. Lyp/Pep has been shown to dephosphorylate tyrosine residues in the activatory loop of the Src family kinases, Lck, which is constitutively associated with the co-receptor and CD3 assocated protein FynT. Lyp/Pep has also been shown to dephosphorylate Zeta chain associated protin Zap-70 and ITAM motifs on the CD3 complex and the TCR Zeta chain. This Lyp mediated dephosphorylation is thought to result in the suppression of the TCR signalling. Lyp and Csk have been shown to be binding partners. Csk phosphorylates the tyrosine residues in the inhibitory loop of Src family kinases thereby inhibiting downstream TCR signalling.

1.6.1.2 Association of C1858T polymorphism in PTPN22 with ADs

A single nucleotide polymorphism in the PTPN22 gene has been associated with several ADs. The SNP in the PTPN22 gene that is associated with a predisposition to autoimmunity is a C to T substitution at nucleotide position1858. Bottinni et al (2004) were the first to report a linkage of the R620W polymorphism with T1D. They reported that 3.7% of individuals with T1D were homozygous for the 1858T allele compared to 1 % of healthy controls in a North American sample, and in an Italian sample within a more homogenous population, twice as many individuals with T1D as compared to controls were heterozygous with respect to 1858T. The only individual who was homozygous for the 1858T allele had T1D (Bottini et al. 2004). In the following year, this association was confirmed in additional larger population based studies (Ladner et al. 2005; Onengut-Gumuscu et al. 2004; Smyth et al. 2004). In addition, in 2004, Begovich et al reported an association of the 1858T polymorphism with Rheumatoid Arthritis (RA) (Begovich et al. 2004). The association with RA was confirmed by additional studies (Criswell et al. 2005; Hinks et al. 2005; Lee et al. 2005; Viken et al. 2005). Subsequently, several studies have been conducted which have found significant association of the 1858T polymorphism with other ADs such as SLE (Criswell et al. 2005; Kyogoku et al. 2004), Graves Disease (Heward et al. 2007; Skorka et al. 2005; Smyth et al. 2004; Velaga et al. 2004), Addison's Disease (Skinningsrud et al. 2008; Velaga et al. 2004), Juvenile Idiopathic Arthritis (Hinks et al. 2005; Viken et al. 2005), Myasthenia Graves (Vandiedonck et al. 2006), Generalized Vitiligo (Canton et al. 2005; LaBerge et al. 2008) and Wegener's Granulomatosis (Jagiello et al. 2005).Genome wide association study involving 14,000 British patients with common diseases confirmed the association of the C1858T SNP in PTPN22 to several ADs including T1D and Rheumatoid Arthritis (RA) (WellcomeTrust 2007). Some ADs have been found not to be associated with the C1858T polymorphism in the gene PTPN22. These include MS (Begovich et al. 2005; Criswell et al. 2005; Hinks et al. 2005), Inflammatory Bowel Diseases such as Crohn's disease (Martin et al. 2005; van Oene et al. 2005; Wagenleiter et al. 2005) and Ulcerative Colitis (Martin et al. 2005; Prescott et al. 2005), Celiac Disease (Rueda et al. 2005; Smyth et al. 2008; Viken et al. 2005), Primary Sclerosing Cholangitis (Viken et al. 2005), Primary Biliary Cirrhosis (Milkiewicz et al. 2006) and Psoriasis (Hinks et al. 2005).

The PTPN22 C1858T polymorphism has also been found to have a protective effect against certain ADs. A recent large study suggested the C1858T polymorphism in the PTPN22 gene

might have a protective role in Crohn's disease and Behcet's disease (Baranathan et al. 2007; Barrett et al. 2008). It is not clear why PTPN22 associates with certain ADs and not with others. However, it has been suggested that the PTPN22 C1858T polymorphism strongly associates with ADs with a significant autoantibody component. There is some experimental data in support of this hypothesis. For example, the PTPN22 C1858T polymorphism is associated with Graves Disease, which has a strong autoantibody component and there is the observation that association of PTPN22 C1858T polymorphism with RA is restricted to a subgroup positive for anti-cyclic citrullinated peptide antibody (Kallberg et al. 2007; McGonagle et al. 2009). However, this is not always the case as is seen in the case of Celiac Disease, which also has a strong autoantibody component but has no association with the PTPN22 C1858T polymorphism (Rueda et al. 2005; Smyth et al. 2008; Viken et al. 2005).

There is a clear geographic gradient with regard to the frequency of the disease -associated T1858 allele in Europe (Burn et al. 2011). The T1858 allele is relative rare in southern European populations with a frequency of only 2% in Italy and 6% in Spain. The frequency increases northward through Europe reaching 8% in U.K, 12% in Sweden and 15.5% in Finland. The T1858 allele is almost absent in the Asian and African American populations (Ikegami et al. 2007; Lee et al. 2009; Mori et al. 2005; Zhang et al. 2008). One explanation for such a varied geographic difference in PTPN22 T1858 allele frequency may be that the polymorphism appeared recently during evolution and/or its frequency is severely affected by selection due to its protective effect against certain infections or diseases, for example, the T1858 allele has been found to have a protective role against Tuberculosis (Gomez et al. 2005; Lamsyah et al. 2009).

PTPN22 is a general autoimmunity gene like the MHC genes and CTLA4 gene. The T1858 allele increases the risk to multiple ADs as discussed above. PTPN22 contributes approximately 2% to the familial clustering in T1D compared to an approximately 40% contribution by HLA (Concannon et al. 2005). The PTPN22 T1858 allele behaves as a dominant variant, conferring an increased risk of disease already when present in single copy (Bottini et al. 2006; Gregersen et al. 2006). The risk conferred by *PTPN22* is variable among the ADs, but it is substantial in T1D and RA, with average reported odds ratios of 1.7–2.0 per single allele copy.

1.6.1.3 How does the Lyp R620W polymorphism effect T cell activation?

The C1858T polymorphism in the PTPN22 gene that is associated with a predisposition to autoimmunity, changes amino acid residue 620 from arginine to tryptophan in the Lyp protein (Begovich et al. 2004). It was originally proposed that the R620W polymorphism results in a loss of function of the protein, Lyp, leading to a hyperactive TCR. This was because the R620W polymorphism was thought to severely disrupt the Lyp-Csk complex (Begovich et al. 2004; Bottini et al. 2004; Vang et al. 2005). The residue 620, which is located in the P1 region of Lyp is important for interaction with the SH3 domain of Csk. As PEP and Csk in complex were reported to downmodulate TCR signalling in a cooperative manner (Cloutier and Veillette 1999; Gjorloff-Wingren et al. 1999), the inability of WLyp to bind Csk was expected to impair Csk mediated negative regulation of the TCR signalling resulting in hypereactive T cells. This model was supported by the finding that although ^{R}Lyp bound strongly to the SH3 domain of Csk, ^{W}Lyp was unable to bind to Csk (Begovich et al. 2004; Bottini et al. 2004; Vang et al. 2005). The inability of Csk to bind to Lyp was thought to result in less suppression of TCR activity hence a more active TCR which could lead to autoimmunity in the periphery.

Unexpectedly however, the R620W polymorphism was found to be a gain-of function mutation by Vang et al (2005), resulting in an increase in the TCR signalling threshold rather than a decrease as was originally predicted. This study showed that when expressed in both Jurkat T cells and human primary T cells, both ^{R}Lyp and ^{W}Lyp downregulated TCR signalling in a dose dependent manner, but at equivalent expression levels ^{W}Lyp was consistently more potent at downregulating signalling via TCR. Lck mediated TCRζ chain phosphorylation, LAT phosphorylation, Lck (Tyr 394) phosphorylation and activation of the Erk2 kinase were observed to be reduced in ^{W}Lyp expressing cells compared to ^{R}Lyp. TCR mediated intracellular calcium flux was also inhibited more by ^{W}Lyp compared to ^{R}Lyp expressing cells (Vang et al. 2005). Additionally, activation of the proximal IL-2 promoter was also inhibited more by the ^{W}Lyp expressing cells in comparison to the ^{R}Lyp expressing cells. This was detected by measuring levels of luciferase activity using a system containing the luciferase reporter gene driven by the nuclear factor of activated T cells (NFAT)/ activator protein-1 (AP1) transcription factor complex. Furthermore, T cells from the peripheral blood of T1D patients heterozygous for the R620W polymorphism secreted less IL-2 in response to CD3/CD28 costimulation than T cells from patients homozygous for the

^{R}Lyp allele. Naive, memory, $CD4^{+}$, $CD8^{+}$ and $CD4^{+}CD25^{+}$ T cell subsets were all observed to be similar between the two groups excluding the possibility that the reduced IL-2 levels was due to a skewing in these subsets. Importantly, stimulation of these T cells from patients with phorbol ester and ionomycin to bypass proximal TCR signalling resulted in very similar levels of IL-2 secretion between the two groups confirming that the effect of Lyp was at the level of signalling events immediately downstream of the TCR. In this study Vang et al (2005) also measured the catalytic activites of ^{R}Lyp and ^{W}Lyp immunoprecipitated from cells and observed that ^{W}Lyp was approximately 1.5 fold more active as a phosphatase than ^{R}Lyp despite not binding Csk. In support of the Vang et al (2005) study, Aarnisalo et al (2008) also reported a reduction in IL-2 production and decreased proliferation of $CD4^{+}$ cells from T1D patients carrying the ^{W}Lyp expressing allele and a reduced intracellular calcium flux in response to TCR stimulation (Aarnisalo et al. 2008). Rieck et al (2007) in their study also observed that ^{W}Lyp was a more potent negative regulator of TCR signaling than ^{R}Lyp. They reported reduced calcium mobilization and IL-2 production by T cells from ^{W}Lyp homozygous and heterozygous subjects respectively when compared to T cells from ^{R}Lyp homozygous subjects (Rieck et al. 2007). The majority of published studies report a decreased TCR-mediated T cell activation in primary T cells from individuals homozygous or heterozygous for the ^{W}Lyp variant compared to individuals homozygous of the ^{R}Lyp variant. However, Zikherman et al (2009), by using phosphorylation of the MAPK Erk as a readout, after transfecting Jurkat cells with ^{R}Lyp or ^{W}Lyp either alone or in conjunction with Csk and then stimulating T cells through the TCR/CD3 complex, reported the R620W polymorphism as a hypomorph leading to a loss of function (Zikherman et al. 2009). Phospho-Erk was significantly increased in cells co-expressing ^{W}Lyp and Csk. In addition, it was shown that calcium mobilisation in cells co-expressing ^{W}Lyp and Csk was more efficient in comparison to ^{R}Lyp and Csk. These results imply that the ^{W}Lyp phosphatase, when co-expressed with Csk, is a loss-of-function variant leading to increased TCR signalling following stimulation (Zikherman et al. 2009). Another exception to the gain-of function hypothesis is provided by a report on Myasthenia Gravis, whereby the R620W polymorphism was associated with increased TCR-induced IL-2 production (Lefvert et al. 2008). However, this result has not been replicated by other groups also investigating the effect of R620W polymorphism in Myasthenia Gravis (Chuang et al. 2009). Further research into the effect of the R620W polymorphism on T cell activation is required to substantiate the current view of the R620W polymorphism as a "gain-of-function" polymorphism.

The molecular mechanism by which the R620W polymorphism, which is not in the catalytic region, but is found approximately 300 amino acid residues downstream from the end of the catalytic PTP domain, may result in change in the PTP activity of the protein is poorly understood. It has been speculated that the non-catalytic portion may play a regulatory role in the function of the catalytic domain. The evidence for this comes from the observation that deletion of the C-terminal half of PEP results in a five fold increase in its PTP activity (Gjorloff-Wingren et al. 1999). Furthermore, it was recently demonstrated that the activity of Lyp is modulated by an intramolecular interaction between the proximal interdomain and the catalytic domain by studying serial truncation mutants of a recombinant form of Lyp (Liu et al. 2009). This study shows that the proximal portion of the interdomain of Lyp may directly interact with the catalytic domain and reduce its activity (Liu et al. 2009). Crystallization of the catalytic domain of the phosphatase also showed that the enzyme might be autoregulated by a reversible oxidation mechanism involving a Cys residue (Tsai et al. 2009). These studies suggest an interaction between the N and C termini of the protein. Therefore, it is plausible that the R620W polymorphism disrupts the autoregulatory function of the interdomain acting on the catalytic domain of the phosphatase thereby increasing its phosphatase activity. It is also probable that binding of ligands to the C-terminal of Lyp or PEP may affect the catalytic activity of Lyp or PEP. It has been observed that although ^{R}Lyp coimmunoprecipitated with Csk, no coimmunoprecipitation was observed between ^{W}Lyp and Csk (Vang et al. 2005). In the case of ^{W}Lyp, the loss of a binding partner such as Csk might lead to an increase in PTP activity due to the possible loss of the regulatory role played by the binding of the ligand.

A second hypothesis is that the R to W polymorphism at amino acid position 620 in the protein Lyp gives the P1 region of ^{W}Lyp an increased ability to interact with other proteins and these proteins bring about a more catalytically preferential conformation of the Lyp PTP domain (Vang et al. 2008). Currently, there are no experimental data to support this hypothesis. A third hypothesis is based on the observation that Lyp is negatively regulated by Lck mediated phosphorylation of an inhibitory tyrosine residue, which might be an important regulatory mechanism influencing TCR signalling. This Lck mediated regulation of Lyp is in turn dependent on interaction between Lyp and Csk, which is thought to facilitate the interaction between Lck and Lyp. This could mean that an abnormality in the Csk mediated post-translational modification of ^{W}Lyp leads to gain of function due to the lack of Lck mediated negative regulation (Fiorillo et al. 2010). However, this observation does not explain how the R620W polymorphism leads to a more potent PTP in vitro. Another

hypothesis is that the R620W polymorphism in Lyp changes the amount of free Lyp and bound Lyp in the cells (Vang et al. 2008). Assuming that there is an equilibrium between the free Lyp and the Lyp bound to known (Csk, Grb-2, c-Cbl) and unknown proteins, any skewing of this equilibrium may have profound cellular effects. In support of this hypothesis is the fact that in mice 25-50% of PEP is found bound to Csk (Cloutier and Veillette 1996). Assuming that this might also be the case for human Lyp then it is plausible that a disruption of Lyp-Csk interaction as in the case of ^{W}Lyp results in an increase in the pool of free Lyp for binding to other proteins. The increase in free Lyp coupled with the altered ligand specificity of the ^{W}Lyp may contribute to making ^{W}Lyp a more active PTP leading to a stronger inhibition of TCR signalling (Vang et al. 2008).

An altered subcellular localization of the protein Lyp due to new set of interacting partners of Lyp because of the R620W polymorphism may be another explanation for the observed increased inhibitory effect of ^{W}Lyp on TCR signalling (Vang et al. 2008). Lastly, alteration in the substrate specificities and/or substrate affinities of the ^{W}Lyp compared to ^{R}Lyp may be responsible for the augmented TCR signalling observed with the ^{W}Lyp expressing cells (Vang et al. 2008). All or some of these explanations or something other may lead to the augmented PTP activity observed in ^{W}Lyp. A thorough investigation is required to clarify how the polymorphisms lead to a more active PTP.

1.6.1.4 How does an apparent reduction in TCR stimulation lead to autoimmunity?

Based on the notion that Lyp W620 is a "gain-of function" polymorphism, it is hypothesised that the ^{W}Lyp isoform suppresses TCR signalling more potently than the ^{R}Lyp isoform. Although a decrease in TCR signalling resulting in autoimmunity is counterintuitive there is some evidence for a decrease in TCR signalling leading to autoimmunity in some ADs (Stanford et al. 2010). Reduced TCR signaling has been reported in T cells from NOD mice and in peripheral T cells from T1D patients (Buchs and Rapoport 2000; Zhang et al. 1998). Studies of SKG mice have provided additional evidence for an association between decreased TCR signalling and autoimmunity (Sakaguchi et al. 2003). It has been demonstrated in this and other mouse models that attenuation of TCR signalling due to a "loss of function" mutation of Zap70, one of the substrates of Lyp, results in dysregulated thymic selection and a spontaneous AD similar to RA (Hsu et al. 2009; Sakaguchi et al. 2003; Siggs et al. 2007).

There are several theories as to how a decrease in TCR signalling due to the R620W polymorphism could result in autoimmunity (Figure 1.10). One of the favoured hypotheses is that it may result in the survival of auto-reactive cells that would normally be deleted by negative selection in the thymus. Alternatively, the impact of ^{W}Lyp on TCR signalling may have an effect on the development and functioning of T regulatory cells (Figure 1.10). It may be the case that fewer T reg cells are generated in ^{W}Lyp expressing individuals compared to individuals who are homozygous for the ^{R}Lyp isoform. Alternatively, there may be no difference in the number of T reg cells generated but their TCR signalling may be suppressed and therefore they may not be as effective in suppressing effector T cells as T reg cells expressing exclusively the ^{R}Lyp isoform. This hypothesis is supported by the finding that PEP is constitutively expressed in the thymus and PTPN22 knockout mice show anomalies in thymic selection like Zap-70 mutant mice. Normalisation of signalling in the thymus of the Zap-70 mutant SKG mice is able to rescue the phenotype of the SKG mice (Sakaguchi et al. 2003) and therefore it is reasonable to assume that normalisation of the signalling in the PTPN22 knockout mice would also rescue the reported anomalies of thymic selection.

Other mechanisms not involving a thymic selection defect by which the R620W polymorphism may result in autoimmunity have also been proposed. For example, an increase in activity of the Lyp in effector T cells might negatively impact the activity or expansion of the peripheral T reg cells in carriers of WLyp (Marson et al. 2007). In addition, decreased TCR signalling leading to a decreased IL-2 production by effector T cells may impair expansion of T reg cells. This is supported by the observation that T reg cells are highly dependent on IL-2, and decreased IL-2 levels has been shown to impair expansion of T reg cells in mouse T1D, and neutralisation of IL-2 in vivo induces autoimmunity in mice through a T reg mediated mechanism (Setoguchi et al. 2005; Tang et al. 2008; Yamanouchi et al. 2007). The PTPN22 gene has also been recently identified as being a target gene of Foxp3. (Marson et al. 2007). This study also showed that T reg cells express a lower amount of the phosphatase compared to effector T cells suggesting the existence of a direct mechanism by which the gain of function of Lyp can affect T reg function.

TCR signalling strength is also an important regulator of naive T cell differentiation and it is possible that a decrease in TCR signalling may affect the polarisation of naive T cells into specialised T helper (Th) subsets, for example by favouring the polarisation into Th1 subset in ^{W}Lyp carriers (Stanford et al. 2010). In T1D Th1 cells have been associated with the disease pathology. This hypothesis is supported by the observation that peripheral T cells

from ^{W}Lyp expressing individuals have a different cytokine secretion pattern with reduced production of IL-10 (Rieck et al. 2007).

Alternatively, other mechanisms are also worth considering as the R620W polymorphism is only associated with ADs that have an autoantibody component. T follicular helper (Tfh) cells characterized by stable expression of CXCR5 receptor, play an important role in regulating humoral immunity by providing "help" to B cells and regulating their proliferation, immunoglobulin class switching and B cell homing into germinal centres (GC). Alternatively it could be the case that the R620W polymorphism affects B cell differentiation or tolerisation directly (Figure 1.10).

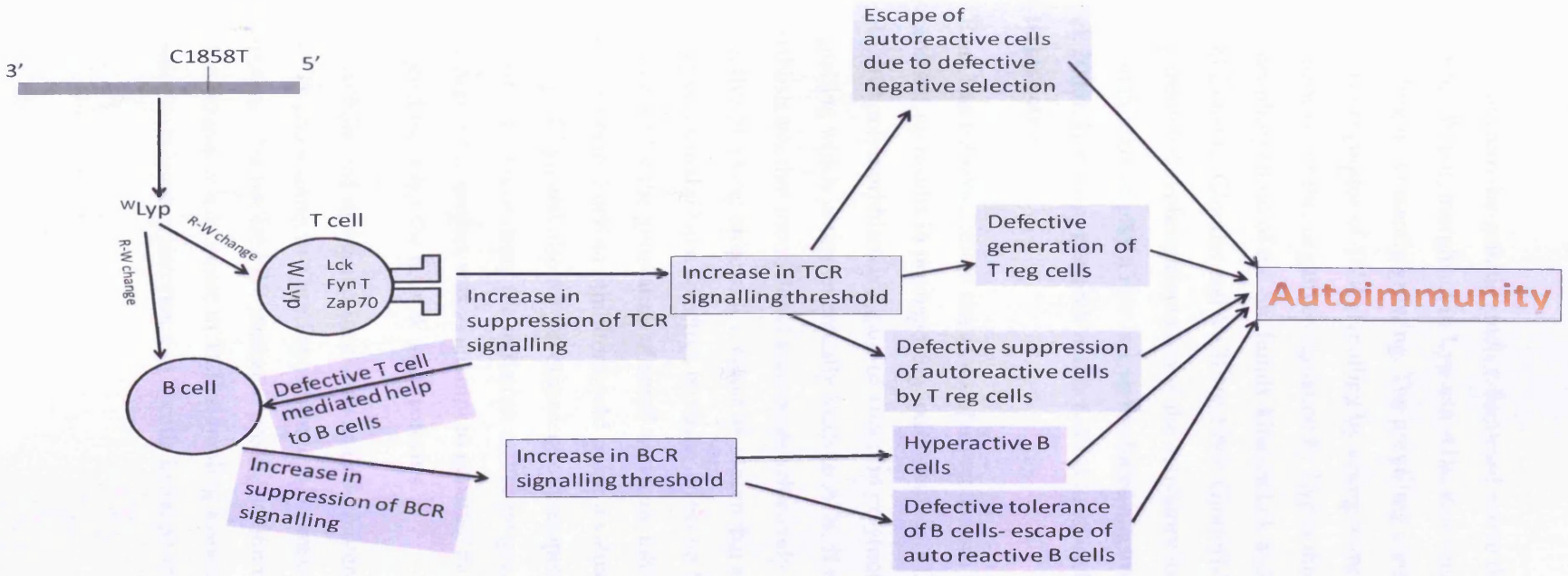


Figure 1.10 Schematic diagrams showing how R620W Lyp polymorphism might predispose to autoimmunity

The R620W polymorphism associated with several ADs has been found to be a "gain of function" resulting in an increase in phosphatase activity of Lyp. This results in an increase in Lyp mediated suppression of TCR signalling increasing the TCR signalling thresholds. It has been hypothesied that the increase in TCR signalling thresholds may result in impaired negative selection in the thymus resulting in escape of autoreactive T cells and/or defective generation of T reg cells in the thymus and/or impaired suppression of autoreactive T cells by the T reg cells in the periphery resulting in autoimmunity. Increase in suppression of TCR signalling might also affect the function or development of T follicular helper (Tfh) cells which in turn may impact B cell development and maturation. The lack or reduced help provided by Tfh cells to B cells might lead to production of hyperactive B cells or may impair B cell negative selection leading to autoimmunity. ^{W}Lyp may also directly suppress BCR signalling impairing B cell negative selection and tolerisation resulting in predisposition to autoimmunity.

1.7 Project aims

It is clear from the genetic studies discussed above that there is a robust association between the R620W polymorphism in Lyp and ADs. Knowledge about the function of Lyp in T cell signalling is constantly growing. The prevailing consensus to date is that Lyp acts as a negative regulator of TCR signalling by acting immediately downstream of the TCR. The mechanism for this negative regulation by Lyp is thought to be due to Lyp mediated dephosphorylation of the Src family kinases Lck and FynT, Zap70, and the ITAMs in the CD3/ζ chains (Cloutier and Veillette 1999; Gjorloff-Wingren et al. 1999; Wu et al. 2006). Lyp mediated dephosphorylation of the activatory loop of the negative regulatory kinase Csk is also thought to play a role in Lyp mediated negative regulation of TCR signalling (Vang et al. 2005). However, research in this field is at an early stage and several questions remain unanswered.

Even less is known about the molecular mechanism of how the R620W polymorphism in the protein Lyp results in predisposing individuals to ADs. The current consensus is that the R620W polymorphism is a gain-of-function polymorphism resulting in a decrease in TCR signalling which in turn eventually leads to ADs. If this is true, it would be of great interest to establish whether monoclonal antibodies selectively targeting the ^{W}Lyp isoform could have selective blocking effects. If it could be shown that such an antibody exclusively targeting the ^{W}Lyp isoform did have selective blocking effect on PTP *in vitro*, this would provide confidence for the generation of small molecule inhibitors designed to selectively block the ^{W}Lyp isoform. Such an inhibitor could prove a valuable reagent for further investigating the biology of Lyp and also for development of therapeutic strategies aimed at reducing the severity of ADs or delay the initiation and/or progress of ADs. To explore this hypothesis the first aim of the project was to attempt to generate monoclonal antibodies specifically recognising either the R or W Lyp isoforms.

The cellular and molecular mechanisms underpinning the increase in Lyp phosphatase activity and leading to a predisposition to ADs are currently unknown. The most popular hypothesis for the R620W mediated predisposition to ADs is an impairment of thymic selection due to a decrease in TCR signalling associated with the ^{W}Lyp isoform eventually leading to release of autoreactive T cells in the periphery which go on to result in

autoimmunity. Impairment of TCR signalling in the thymus may also affect the generation of natural T reg cells, which play a major role in mediating peripheral tolerance.

There are also several other hypotheses proposed to explain the molecular and cellular mechanisms by which the R620W polymorphism in Lyp leads to AD. However, there is currently no direct evidence to support any of these. Nevertheless, some studies have investigated the functional effect of the R620W polymorphism on T cells. These studies have explored the potential effect of the R620W polymorphism in TCR mediated cytokine production and calcium mobilization when comparing T cells expressing each of the two polymorphic forms of Lyp. One of these described studies looked at the effect of overexpression of Lyp isoforms by nucleofection of primary human T cells (Vang et al. 2005). Nucleofection is a non-viral electroporation based transfection method which utilises a device that delivers unique electrical parameters in order to introduce genes directly into the nucleus of the cells being transfected. This method can be highly toxic to cells, especially T cells. Hence, a better more stable method of transfecting T cells is required which can potentially be used to transduce primary T cells. Therefore, the second aim of this project was to generate recombinant Lyp expressing lentivirus plasmids that could be used to generate recombinant lentivirus particles expressing the ^{R}Lyp or the ^{W}Lyp isoforms. The recombinant Lyp expressing lentiviruses were subsequently used to transduce T cells to generate stable permanently transduced T cell lines. Three previous studies have concentrated on the differences in cytokine production by T cells from patients who are either heterozygous or homozygous for the ^{R}Lyp isoform (Aarnisalo et al. 2008; Rieck et al. 2007; Vang et al. 2005). In the above study no purification of different T cell subsets was carried out. As a consequence, the previous studies did not take into account possible skewing of T cell subsets and therefore it cannot be unambiguously stated that the differences observed in cytokine expression are due to the intrinsic effects of different Lyp isoforms on T cell function. Therefore the third aim of this project was to generate $^{R/W}$Lyp transduced homogenous populations of cells by transducing leukaemic T cell lines with the recombinant $^{R/W}$Lyp lentiviruses and the fourth aim of this project was to study the functional effects of over-expression of the $^{R/W}$Lyp isoforms in leukaemic T cells. Knowledge of how the overexpression of the ^{R}Lyp and ^{W}Lyp isoform affects the function of a monoclonal population of T cells would allow further investigation and dissection of pathways that might be affected by Lyp.

The proposed hypothesis was that overexpressing R and W Lyp isoforms in T cells would lead to a decrease in activation status and cytokine production by the T cells expressing exogenous Lyp isoforms compared to controls not expressing exogenous Lyp and this reduction will be more profound in ^{W}Lyp expressing T cells compared to ^{R}Lyp expressing T cells.

The specific aims of the project were:

- To generate monoclonal antibodies specific against the R and the W isoforms of Lyp.
- To generate recombinant lentivirus plasmids encoding the two isoforms of Lyp.
- To generate $^{R/W}$Lyp expressing recombinant lentiviruses.
- To use the $^{R/W}$Lyp expressing recombinant lentiviruses to transduce leukaemic T cell lines to generate T cell lines permanently expressing either the R or the W isoforms of Lyp.
- To assess the TCR activation status of the leukaemic T cells expressing either the R or the W isoforms of Lyp.
- To assess cytokine expression by the leukaemic T cells expressing either the R or the W isoforms of Lyp.

Chapter 2

Materials and Methods

2.1 Reagents and consumables:

2.1.1 Antibiotics

Antibiotic solutions were sterile filtered through a 40µl syringe filter and stored in stocks at either -20°C or at 4°C for continuous use.

Ampicillin

Ampicillin was purchased from Sigma Aldrich [Gillingham, Dorset, U.K.] and reconstituted in dH_2O to give a 100mg/ml stock.

2.1.2 Antibodies

The primary antibody solutions, anti-Lyp, anti-GFP and anti-actin were aliquoted and stored at -20°C. The fluorescent and the HRP conjugated secondary antibodies were stored at 4°C for continuous use.

Immunoblotting antibodies

The goat anti-Lyp polyclonal antibody was purchased from R&D systems [Abingdon, U.K.]. The rabbit anti-actin polyclonal antibody was purchased from Sigma Aldrich [Gillingham, Dorset, U.K.]. The horseradish peroxidase (HRP) conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Bio-Rad [Bio-Rad, Hercules, CA]. The HRP conjugated rabbit anti-goat antibody was purchased from Sigma Aldrich [Gillingham, Dorset, U.K.]

Fluorescent conjugated anti-rat and anti-human antibodies for detecting protein expression

Anti-rat CD2 ^{PE} antibody was purchased from Serotec [Oxford, U.K.]. Aqua live/dead^{AmCyan} stain was purchased from Invitrogen [Paisley, U.K.]. Anti human CD69 ^{PE} antibody was purchased from eBioscience [Hatfield, U.K.].

2.1.3 Cell media and associated reagents

Roswell Park Memorial Institute medium (RPMI), Dulbecco modified Eagle's minimal essential media (DMEM), L-glutamine, penicillin, streptomycin, sodium pyruvate and 0.5% trypsin in HBSS were purchased from Gibco, Invitrogen [Paisley, U.K].

2.1.4 Chemical reagents

Tris was obtained from Sigma and Tween 20 and Nonidet P-40 (NP-40) were supplied by BDH [Poole, Dorset, U.K]. All other reagents were purchased from Fisher Scientific [Leicester, U.K] unless stated otherwise.

2.1.5 Distilled water (dH_2O)

Distilled water (dH_2O) was obtained from a Millipore reverse osmosis system followed by filtration through two ion exchange resin columns using a Millipore Milli-Q system.

2.1.6 Foetal calf serum (FCS)

Foetal calf serum (FCS) was purchased from Gibco, Invitrogen [Paisley, U.K] and was heat inactivated at 56°C for an hour.

2.1.7 MACs Buffer

MACs Buffer (0.5% BSA (Sigma), 2mM EDTA (Sigma), GIBCO PBS (Invitrogen)) was sterile filtered using a 0.2μm bottle top filter and store at 4°C.

2.1.8 Plasmid DNA

Plasmids for DNA sequencing and screening by restriction digestion were purified using an Invitrogen plasmid Mini-prep kit. Plasmid DNA for DNA sub-cloning was purified using a Qiagen Gel Extraction kit. Plasmids for transfection were prepared using an Invitrogen PureLink HiPure Plasmid filter Maxiprep kit and then stored at -20°C in TAE buffer.

2.1.9 Tissue culture plastic

All tissue culture flasks (T25, T75 and T180), tissue culture plates (6, 24 and 96 wells) and petri- dishes were supplied by Nunc [Thermo Fisher Scientific].

2.2 Cell based techniques

2.2.1 Cell counting by trypan blue exclusion

10µl of cell suspension was mixed with 10µl of 0.1% trypan blue in PBS, loaded on to an improved Neubauer haemocytometer [Weber Scientific International Limited, Lancing, U.K.] and viable cells counted at 100 times magnification under white light.

2.2.2 Cryopreservation of cell lines

Cells were removed from tissue culture and washed with PBS by centrifugation at 1800rpm for 3 minutes at 4°C. Cells were re-suspended in freezing buffer (90% FCS and 10% DMSO [Sigma]) to give 0.5 – 2 x 10^6 cells per ml, and 1ml aliquots dispensed in to cryo-vials. The cryo-vials were placed in a Nalgene 5100 cryo-freezing container [Merc Laboratory Supplies, Dorset, U.K.] for 24 hours at -80°C before being transferred to liquid nitrogen. Cells were removed from cryopreservation by thawing rapidly at 37°C (water-bath) and then washing in PBS by centrifugation at 1500rpm for 5 minutes at 4°C, before being placed in culture.

2.2.3 Flow cytometer

A FACS Calibur flow cytometer [BD Biosciences, San Jose, CA] fitted with an argon-ion (488nm) and red diode laser (635nm) and capable of four channels of fluorescent detection was used throughout this study. Acquired data was analysed using Summit [Dako Colorado, Inc] or FlowJo software (Tree Star Inc, Ashland, OR].

Single colour immunoflourescent staining

1 x 10^6 cells were pelleted in 5ml FACS tubes by centrifugation at 1600 rpm for 5 minutes. The pellets were washed once with 300µl cold PBS and centrifuged at 1600 rpm for 5 minutes. For anti Rat CD2 and anti human CD69 staining, cells were resuspended in 30µl of

1/10 diluted anti Rat $CD2^{PE}$ or anti Rat $CD69^{PE}$ antibody. For SLY tetramer (HLA-A*0201 biotinylated monomer was refolded with SLYNTVATL peptide and tetramerised using Streptavidin-PE [Invitrogen]) staining. 2.5µg SLY tetramer was used per stain. Cells were incubated for 15 minutes in the dark at room temperature. Cells were washed twice by adding 200µl of PBS and centrifuged at 1600rpm for 5 minutes. Supernatant was removed and the cells were resuspended in 300µl of PBS for immediate analysis or fixed with paraffin formaldehyde (PF). For fixation, cells were resuspended in 300µl of 1% PF and incubated at 4°C in the dark until analysis could be performed. Negative control wells (consisting of cells not expressing rat CD2 for anti Rat CD2 staining and unstimulated cells for anti CD69 staining) were included in all experiments. For anti CD69 and anti tetramer staining, unstained cells were also used as an additional control. In addition, for tetramer staining, a diabetes peptide tetramer (ALWGPDPAAA peptide, tetramerised using Streptavidin-PE [Invitrogen]) was also used as a negative control. Where GFP was the fluorochrome to be detected, cells were washed twice with PBS and resuspended in either 300µl of PBS for immediate analysis or 300µl of 1% PF and stored at 4°C in the dark until acquisition and analysis be performed.

2.2.4 Protein analysis

Immunoblotting techniques were used to detect the levels of Lyp expression in 293T cells that had been transfected with Lyp Lentivirus and Lyp pGEM plasmids, and also to screen hybridoma clones for antibody production against denatured full length Lyp. Actin was used as an internal control.

Detergent lysis of cells

Cells were removed from culture, washed and counted. The cells were then pelleted and the supernatant was carefully removed. The pelleted cells were re-suspended in an appropriate volume of ice cold detergent lysis buffer (1ml lysis buffer per 10^7 cells). Either NP-40 (150mM NaCl, 50mM Tris-HCL pH 8.0, 25mM NaF, 1mM sodium orthovanadate 0.5% Nonidet P40) or RIPA (150mM NaCl, 50mM Tris pH 8.0, 1% NP-40, 0.5% Deoxycholic acid, 0.1%SDS) detergent lysis buffer containing protease inhibitors (10µg/ml Aprotinin, 10µg/ml Leupeptin, 10µg/ml Pepstatin A, 1mM EDTA, 1mM Phenylmethylsulfonylfluoride), which were added immediately prior to lysis, was used. Cells were allowed to lyse for 30

minutes on ice and unlysed cells/nuclear material removed by centrifugation (Beckham GS15R) at 1300rpm for 10 minutes at 4°C. The supernatant was collected and mixed with 6X sample buffer (0.35M Tris pH 6.8, 1.65M Dithiothreitol, 33% glycerol, 10% SDS, 0.12 mg/ml bromophenol blue [Sigma Aldrich]. The cell lysate was then heated for 10 minutes at 70°C and if not immediately required for analysis stored at -20°C.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were resolved using NuPAGE Novex 4-12% Bis-Tris pre-cast gels [Invitrogen] mounted in a XCell SureLock Mini-Cell [Invitrogen] which was filled with NuPAGE 1x MOPS SDS running buffer [Invitrogen], according to the manufacturer's instructions. SDS-PAGE samples were boiled for 10 minutes at 70°C and microfuged at 13000rpm for 30 seconds. Samples were loaded into the wells of the gel by careful pipetting using gel tips. For size discrimination of the resolved proteins, 10µl of SeeBlue Plus 2 prestained standard protein ladder [Invitrogen] was loaded into one well. The gels were resolved at 200 Volts for 1 hour.

Immunoblotting

Polyvinylidenedifluoride (PVDF) membranes [Immobilon-P, Millipore, Bedford, MA] were cut to 7.5cm x 8cm size, wet with methanol for 10 seconds, rinsed in dH_2O and equilibrated in 1x NuPAGE transfer buffer (5% of 20x NuPAGE transfer buffer and 10% Methanol in dH_2O) until required. Two pieces of filter paper were also cut to 7.5cm x 8cm in size and placed in the 1x NuPAGE transfer buffer until required. Five blotting pads were soaked in 400mls of 1x NuPAGE transfer buffer until required. Following electrophoresis for one hour at 200 Volts, the gel was removed from the gel cassette trimmed and the gel, PVDF membrane, filter paper and blotting pads assembled according to manufactures instructions [Invitrogen XCell II Blot Module]. The gel/membrane/blotting pads assembly was carefully placed into the Invitrogen XCell II blot module and the Gel Tension Wedge inserted and locked into position. The lower buffer chamber was filled with 1x NuPAGE transfer buffer until the gel/membrane assembly was covered and the outer buffer chamber was filled with 650 ml dH_2O. The lid was placed into position and the gel blotted at 30 Volts for 1 hour 30 minutes.

Immuno-detection by Enhanced Chemiluminesence (ECL)

The non-specific binding sites of the PVDF membrane were blocked by placing it in blocking buffer (PBS containing 3% non-fat milk) overnight at 4°C. The membranes were then incubated for two hours in primary antibody at a dilution of 1 in 1600 for Lyp and 1 in 1000 for Actin in blocking buffer, by constant rocking at 4°C. The PVDF membrane was then rinsed briefly in PBS solution followed by a 5minute wash in wash buffer (0.05%Tween 20 in PBS) and two 5 minute washes in PBS, at room temperature by constant rocking. Secondary antibodies, anti-goat (for Lyp) and anti-rabbit (for Actin) were added at a dilution of 1 in 3000 in blocking solution and incubated for 1 hour by gentle rocking at room temperature. The membranes were rinsed then washed once in washing buffer for 5 minutes, followed by one wash in PBS for 5 minutes at room temperature by constant rocking. The membranes were then covered with a 1:1 mixture of ECL reagents 1 and 2 [Amersham] and allowed to react for 1 minute before being sealed in a polyethene sleeve. The membranes sealed in the polyethene sleeve were subsequently exposed to photographic film for 10 seconds to 20 minutes and the photographic film developed automatically using a Compact X2 Processor [X-Ograph Ltd., Wiltshire, U.K.]

2.3 Molecular Biology

In order to over-express Lyp, a lentiviral system known to be effective for transducing T cells was used. To achieve over-expression of R620 and W620 Lyp using lentiviruses, two parallel lentivirus plasmids expressing the R620 and W620 isoforms of Lyp were generated, and to allow potential identification of transduced cells, truncated rat CD2 and GFP were selected as reporter genes. The reporter gene was required to be expressed from the same mRNA as the gene of interest, Lyp, therefore a self-cleaving peptide, 2A, was introduced for the purpose. The generation of $^{R/W}$Lyp-2A-CD2 or $^{R/W}$Lyp-2A-GFP lentivirus plasmids first required the assembly of $^{R/W}$ Lyp, self-cleaving 2A peptide and CD2/GFP cassettes. A pUC x-2A-y vector was used for this purpose.

All DNA manipulations were carried out using autoclaved tips, tubes, vials and solutions. All polymerase chain reaction (PCRs) were performed on a Biometra Tpersonal PCR machine [Thistle Scientific].

Buffer	Contents
1 x TBE (1in 5 dilution of 5x stock).	5x TBE: 108g Tris, 55g Boric acid [Sigma Aldrich], and 40mls of 0.5M EDTA pH 8.0
Agarose gel loading dye	3.6mM Bromophenol Blue and 1.2M Sucrose
Lauria-Bertani (LB)	1% Tryptone Soya Broth [Oxoid, Basingstoke, U.K], 0.5% Yeast extracts [Oxoid] and 1% NaCl [Sigma Aldrich], buffered to pH 7.0 then autoclaved and allowed to cool at room temperature.
LB Ampicillin broth	100μg/ml final ampicillin was added to cooled (below 50°C) LB broth, immediately prior to use.
LB Ampicillin plates	1.5% Agar granules added to LB broth, autoclaved and cooled to 50°C before the addition of the ampicillin (100μg/ml final concentration)

Table 2.1 Buffers and Broths

2.3.1 PCR

A number of different PCRs were carried out to molecularly clone the different cDNA sequences for the generation of the recombinant Lentivirus and other plasmids for this project. PCRs were primarily conducted out using Phusion polymerase [Finnzymes, Finland]. The only exceptions to this were the mutagenesis PCRs, which were carried out using Pfu Turbo [Stratagene]. All the PCR primers used for amplification, mutagenesis and sequencing of the DNA were obtained from MWG Biotech. The primers were reconstituted according to manufacturer's instructions using dH_2O to achieve a concentration of 100pmol/μl and then diluted to 25pmol/μl.

Primer name	Primer Sequence	Description
Lyp 5' *XbaI* mutagenesis primer	$^{5'}$CCAGAAAGAACTCTGGAGTCCTTCTTTC$^{3'}$	G→C mutation to remove the internal *XbaI* site in Lyp cDNA.
Lyp 3' *XbaI* mutagenesis primer	$^{5'}$GGTCTTTCTTGAGACCTCAGGAAGAAAG$^{3'}$	G→C mutation to remove the internal *XbaI* site in Lyp cDNA.
CD2 5'*XbaI* mutagenesis primer	$^{5'}$CTTGAGGATTCTGGAGATGGTCTC$^{3'}$	G→C mutation to remove the internal *XbaI* site in the truncated rat CD2 cDNA.
3'CD2-*XbaI* mutagenesis primer	$^{5'}$ GAACTCCTAAGACCTCTACCAGAG $^{3'}$	G→C mutation to remove the internal *XbaI* site in the truncated rat CD2 cDNA.
5'Lyp-*KpnI* mutagenesis primer	$^{5'}$CTCCAAGTGGTACAAGTTCTAAGATGTC$^{3'}$	A→T mutation to remove the internal *KpnI* site in Lyp cDNA.
3'Lyp-*KpnI* mutagenesis primer	$^{5'}$GAGGTTCACCATGTTCAAGATTCTACAG$^{3'}$	A→T mutation to remove the internal *KpnI* site in Lyp cDNA.

5'Lyp-tryp mutagenesis	$^{5'}$CTT CCT GTA TGG ACA CCT G$^{3'}$	C⟶T mutation to convert the Arginine form of Lyp into a Tryptophan form
3'Lyp-tryp mutagenesis	$^{5'}$G AAG GAC ATA CCT GTG GAC$^{3'}$	C⟶T mutation to convert the Arginine form of Lyp into a Tryptophan form
Lyp 5'primer with Kozak sequence.	$^{5'}$AAA *TCT AGA CCA GCC ATG* GAC CAA AGA GAA ATT CT$^{3'}$	Lyp amplification primer incorporating *XbaI* site and Kozak consensus sequence
Lyp 3'primer with Myc tag	$^{5'}$TGA ACC TTA *TAA CTT GTT TTT GAA TAA AGA CTT CTT CTA* GAC *GAG CTC* AAA$^{3'}$	Lyp amplification primer incorporating c-myc tag and *XhoI* site.
Lyp 5'primer with new Kozak sequence.	$^{5'}$AAA *TCT AGA GCC GCC ACC* ATG GAC CAA AGA GAA ATT CT$^{3'}$	Lyp amplification primer incorporating *XbaI* site and most used Kozak consensus sequence
Lyp 3'primer without myc tag	$^{5'}$GGTGGTTGA ACC TTA TAA *GAGCTC*AAA$^{3'}$	Lyp amplification primer incorporating *XhoI* site.

3' CD2 sequencing primer for *XbaI* site mutagenesis	$^{5'}$ CA GAA CCT TCC TTG TCT ACA $^{3'}$	CD2 sequencing primer to sequence *XbaI* site directed mutagenesis
3' Lyp *XbaI* mutagenesis sequencing primer	$^{5'}$ GA TAG GAC TGT GGT ACC TTT $^{3'}$	Lyp sequencing primer to sequence *XbaI* site directed mutagenesis
5' primer for CD2 amplification	$^{5'}$AAA *GGT ACC* ATG AGA TGT AAA TTC$^{3'}$	5' primer incorporating *KpnI* site for amplification of truncated rat CD2 sequence
3' primer for CD2 amplification	$^{5'}$CTTCTCCTTTTTTGCC**ATT***CGCCGGCG*AA^{3}	3' primer incorporating *NotI* site for amplification of truncated rat CD2 sequence
5'Lyp primer for amplification of the missing Lyp DNA sequence	$^{5'}$AAA*GAGCTC*ATCTGGGATGTA$^{3'}$	5'Lyp primer incorporating a *SacI* site for amplification of the missing DNA

3'Lyp primer for amplification of the missing Lyp DNA sequence	5' GATTTGTGGTTT*ATGCAT*AAA 3'	3'Lyp primer incorporating a *BsaAI* site for amplification of the missing DNA sequence
5'primer for Lyp amplification	5'AAA*GGTACC* ATGGACC AAAGAGA AATT CTG3'	5'Lyp amplification primer incorporating a *KpnI* site for cloning Lyp into "y" site of pUC GFP-2A-y plasmid
3' primer for Lyp amplification	5'GTGGTGGTTGAACCTTATAA**ATT***CGCCGG CG*AAA 3'	3'Lyp amplification primer incorporating a *NotI* site for cloning Lyp into "y" site of pUC GFP-2A-y plasmid
5' primer for GFP amplification	5'AAA*TCTAGAGCCGCCACC*ATGGTGAGCAA GGGC3'	5'GFP amplification primer incorporating a *XbaI* site for cloning GFP into "x" site of pUC x-2A-y plasmid

3' primer for GFP amplification	$^{5'}$ CTGCTCGACATGTTC*GAGCTC*AAA $^{3'}$	3'GFP amplification primer incorporating a *XhoI* site for cloning GFP into "x" site of pUC x-2A-y plasmid
5'RFP amplification primer	$^{5'}$AAA *TCT AGA GCC GCC ACC* ATG GCC TCC TCC GAG AAC$^{3'}$	5' RFP amplification primer incorporating the Kozak sequence and *XbaI* site.
3'RFP amplification primer	$^{5'}$ GCGGTGGTGGACAAGGAC*GAGCTC*AAA $^{3'}$	3'RFP amplification primer incorporating the *XhoI* site.
5' Kozak GFP primer	$^{5'}$AAA*TCTAGAGCCGCCACC*ATGGTG AGCAAGGGC$^{3'}$	5' primer for GFP amplification incorporating *XbaI*, site to generate **GFP**-2A-**Lyp** plasmid.

3' Kozak GFP primer	5'AAA*CTCGAG*CTTGTACAGCTCGTC3'	3' primer for GFP amplification incorporating *XhoI* site to generate **GFP**-2A-**Lyp** plasmid.
5'Lyp *KpnI* amplification primer	5'AAA*GGTACC*ATGGACCAAAGAGAAATTC TG3'	5' primer for Lyp amplification incorporating *KpnI* site to generate **GFP**-2A-**Lyp** plasmid.
3'Lyp *NotI* amplification primer	5'AAA*GCGGCCGC***TTA**AATATTCCAAGTTG GTGGTG3'	3' primer for Lyp amplification incorporating *NotI* site to generate **GFP**-2A-**Lyp** plasmid.
5' myc tag-2A sequencing primer	5'CAG GCC CAA TCT ATA GAA3'	5' sequencing primer to sequence the 2A-myc tag junction
5'pUC Lyp-2A-GFP/CD2 sequencing primer 1	5'CAAGGCGATTAAGTTGGGTA3'	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids

5'pUC Lyp-2A-GFP/CD2 sequencing primer 2	$^{5'}$GGTAGAACTATCCCTGATAA$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 3	$^{5'}$GCTGAAAAAAGGAAATCTGA$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 4	$^{5'}$CATTAGTTCAAACGCAGGAA$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 5	$^{5'}$TGCACCCTGCTAAATCAAGC$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 6	$^{5'}$TTGGAATCTCAACCACATGA$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 7	$^{5'}$CTACATCCCTCTTCTCTTATTA$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids

5'pUC Lyp-2A-GFP/CD2 sequencing primer 8	$^{5'}$ACCAAGCAAGAGTGTAAAAC$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 9	$^{5'}$CAGAATCTGTTCAGTCAAAT$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 10	$^{5'}$ACGGCCACAAGTTCAGCGTG$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 11	$^{5'}$CGAGCTGAAGGGCATCGACT$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids
5'pUC Lyp-2A-GFP/CD2 sequencing primer 12	$^{5'}$TCCTGCTGGAGTTCGTGACC$^{3'}$	Sequencing primer to sequence entire puC Lyp-2A-GFP/CD2 plasmids

5' Lyp genotyping primer	$^{5'}$TCACCAGCTTCCTCAACCACA$^{3'}$	5' primer for genotyping R620W polymorphism in Lyp
3' Lyp genotyping primer	$^{5'}$ TTTAAGGCAACTTCGTTGTTATAG $^{3'}$	3' primer for genotyping R620W polymorphism in Lyp

Table 2.2 PCR primer sequences used in the generation of recombinant plasmids.

The primer name is shown alongside the sequence with any modifications to the cDNA that were introduced or the introduced of restriction sites and Kozak consensus sequence in the right hand column. Restriction endonuclease sites, Kozak consensus sequences and c-myc tag sequences are shown in the primer sequence in italics, any introduced stop codons are shown in bold and single base pair mutations in the cDNA sequence are underlined.

Amplification PCR using Phusion

A typical 50µl PCR reaction using Phusion polymerase [Finnzymes, Finland] contained the following reagents:

50ng of DNA template,

2.5mM of each dNTP [Invitrogen],

5X HF Phusion buffer [Finnzymes, Finland],

25pmoles of forward primer [MWG Biotech],

25pmoles of reverse primer [MWG Biotech],

dH_2O to take the reaction volume to 50µl ,

and 0.5µl of Phusion polymerase [U/µl Finnzymes, Finland].

The PCR was performed on a Biometra Tpersonal PCR machine [Thistle Scientific]. Each PCR consisted of the following cycles:

Initial denaturing at 98°C for 3 minutes.

30 cycles of:

Denaturing : 98°C for 15 seconds.

Annealing: 55°C for 15 seconds (annealing temperature is dependent upon the specific primer used).

Extension: 72°C for 1 minute.

Followed by a final extension at 60°C for 4 minutes.

Pfu Turbo PCR

Pfu Turbo polymerase [Stratagene] was used for "Quick change" *in vitro* mutagenesis PCR. This reaction consisted of:

50ng of DNA template,

2.5mM of each dNTP [Invitrogen],

10X *Pfu turbo* buffer[Stratagene],

x pM of forward primer [MWG Biotech], (primer concentration dependent on the primer sequence and worked out using the following formula

y pM of reverse primer [MWG Biotech],

dH_2O to take the reaction volume to 50µl,

and 0.5µl of *Pfu Turbo* [U/µl Stratagene].

The PCR was performed on a Biometra Tpersonal PCR machine [Thistle Scientific]. Each PCR consisted of the following cycles:

Initial denaturing at 95°C for 30 seconds.

12 cycles of:

Denaturing : 95°C for 30 seconds.

Annealing: 55°C for 60 seconds (annealing temperature is dependent upon the specific primer used).

Extension: 68°C for 12 minute (extension time is dependent on the size of the plasmid containing the template DNA)

2.3.2 Genotyping

Genotypping for the C1858T SNP was conducted using PCR restriction fragment length polymorphism. The genomic DNA was extracted using a Relia Prep™ gDNA tissue Miniprep system [Promega, U.K.]. A set of forward and reverse oligos shown in Table 2.2 previously used by (Bottini et al. 2004) were used to amplify a 215bp sequence of the genomic DNA by PCR. The PCR reaction was carried in a 50µl reaction using Phusion polymerase [Finnzymes, Finland] containing the following reagents:

50 ng of genomic DNA,

2.5 mM of each dNTP [Invitrogen],

5X HF Phusion buffer [Finnzymes, Finland],

25 pM of forward primer [MWG Biotech],

25 pM of reverse primer [MWG Biotech],

dH_2O to take the reaction volume to 50 µl ,

and 0.5 µl of Phusion polymerase [U/µl Finnzymes, Finland].

The PCR was performed on a Biometra Tpersonal PCR machine [Thistle Scientific]. Each PCR consisted of the following cycles as described by (Bottini et al. 2004):

30 cycles of:

Denaturing : 95°C for 30 seconds.

Annealing: 60°C for 30 seconds

Extension: 72°C for 30 seconds.

Followed by a final extension at 60°C for 4 minutes.

The amplified 215bp fragment of the PTPN22 gene sequence encompassing the C1858T polymorphism was genotyped by *XcmI* restriction endonuclease digestion as described in section 2.3.5.

2.3.3 Agarose gel electrophoresis

PCR products and DNA fragments were separated and visualised by agarose gel electrophoresis. 1% (w/v) agarose [Invitrogen] in 1x TBE buffer was melted in a microwave oven and allowed to cool before the addition of 0.4µg/ml of ethidium bromide. The agarose was poured into a gel casting tray, an appropriate size comb inserted and allowed to set. After PCR amplification using Phusion polymerase, 5µl of the sample was loaded onto a 1% (w/v) agarose gel containing ethidium bromide (0.4µg/ml) with 5µl of loading buffer. In addition, 3µl of 1Kb Plus DNA ladder [Invitrogen] was also loaded onto the gel. The gels were run at 120 Volts for 60 minutes. The DNA within the gel was visualised using a UV transilluminator and the images captured on a digital camera using a Bio Rad gel documentation system.

2.3.4 Purification of PCR products

PCR products were purified using an Invitrogen PCR purification kit prior to restriction endonuclease digestion. The manufacturer's protocol was used for the PCR purifications. In brief, the DNA was bound to a silica membrane, washed and then eluted using 50µl of elution buffer or TAE. The concentration was determined by absorbance at 260nm.

2.3.5 Restriction endonuclease digestion

The PCR products and plasmids were digested using the relevant restriction endonucleases. All the restriction endonucleases were purchased from New England Biolabs [Hitchin, Herts, U.K]. PCR products and plasmid vectors were restricted using restriction endonucleases to produce complimentary DNA ends before ligation reactions were performed. A typical 50µl restriction digest reaction contained 5µl of 10 x NEB Buffer, 20-30µl of cDNA and 1µl of

NEB restriction endonuclease (if a double digest was required, 1µl each of the two restriction endonucleases). The reactants were then briefly mixed and incubated for 2 hours at 37°C.

2.3.6 Purification of restricted PCR products and vectors

The restricted PCR products and plasmid vectors were purified from agarose gels using a Qiagen QIAquick gel purification kit [Qiagen Crawley, West Sussex, U.K]. To purify the restricted PCR products and restricted plasmid vectors from an agarose gel, the product was loaded into a 1% agarose gel with ethidium bromide, the gel slice containing the DNA band was excised with a clean sharp scalpel, the size of the gel slice minimized by removing excess agarose and the DNA extracted according to the manufacturer's instructions. Briefly, the agarose was dissolved in sodium iodide, the DNA bound to a silica matrix, washed and eluted into 50µl of elution buffer. The concentration was determined by absorbance at 260 nm.

2.3.7 Ligation

Purified restricted inserts and plasmid vectors were ligated using T4 DNA Ligase [New England Biolabs]. Per recombinant plasmid, four to five ligation reactions with a varying vector to insert ratio (usually 1:0, 1:3, 1:3.5, 1:4) were set up. Typically, a 10µl ligation reaction containing 1µl of 10 x Ligase reaction buffer, 0.5µl T4 DNA ligase, 1µl vector, Xµl insert (a 3 fold molar excess of insert calculated by (3x ((mass (ng) of vector x size (kb) of insert)/size (kb) of vector) was used) was prepared, mixed briefly and incubated for 15 to 20 minutes at room temperature.

The ligated DNA was transformed into bacteria directly from the ligation mix.

2.3.8 Transformation

The ligated plasmid DNAs were transformed into 50µl aliquots of competent *Escherchia Coli* strain, DH5α, using heat shock for 20 seconds at 42°C. Transformed bacteria were then grown without selection in 950 µl of LB medium for 1 hour at 37°C to allow synthesis of plasmid-encoded antibiotic resistant proteins. The bacteria were then centrifuged at 13000 rpm for 2 minutes, 950µl of the supernatant removed, the pellet re-suspended in remaining 50µl of LB and streaked on to LB agar plates containing 100 µg/ml of ampicillin and allowed to grow for 16 hours at 37°C.

2.3.9 Plasmid selection

Following transfection by heat shock, 1ml cultures were centrifuged to pellet the bacteria which was then re-suspended in 50µl of LB broth and streaked onto LB-ampicillin plates and allowed to grow for 16 hours in an incubator at 37°C. Bacterial cultures from frozen stocks were directly streaked onto LB-Ampicillin plates and allowed to grow for 16 hours in an incubator at 37°C.

2.3.10 Bacterial colony screening

Bacterial colonies were screened by restriction digestion analysis of DNA isolated from cloning and identity confirmed by DNA sequencing. Ampicillin resistant bacterial colonies were picked from the ampicillin agar plate and grown for 16-18 hours in 5mls of LB broth containing 100 µg/ml of ampicillin. The plasmid DNA was extracted for each clone and the DNA quantified using a spectrophotometer. Subsequently, the DNA was screened by restriction endonuclease digestion. Typically, 5 µl of purified DNA was digested as described above but in a 15 µl total volume using specific restriction endonuclease(s), which were dependent on the recombinant being screened.

2.3.11 Plasmid DNA isolation

Following a restriction endonuclease digestion screen, the positive LB broth cultures of single colonies picked from agar plates and inoculated in 5 ml LB-Ampicillin (100 µg/ml) broth were subsequently, used as starter cultures to inoculate a much larger LB broth culture. Typically, 1ml of starter culture was used to inoculate 500 mls of LB-Ampicillin broth and incubated overnight at 37°C on an orbital shaker. Bacteria were collected by centrifugation at 6000 rpm for 10 minutes at 4°C on a Sorvall Evolution centrifuge. Plasmids were isolated from 500 ml LB-culture using an Invitrogen PureLink HiPure Plasmid filter Maxiprep kit according to manufactures protocol. In brief, the bacteria were lysed and the DNA bound to a silica matrix and following a wash, the DNA was eluted in elution buffer then precipitated using isopropanol and 70% ethanol and re-constituted in 500 µl of TBE buffer. The DNA was stored at -20°C unless immediately required.

2.3.12 Storage of bacterial clones

500 µl of a 5ml starter culture was mixed in a 1:1 ratio with glycerol freezing buffer (65% glycerol, 0.1M $MgSO_4$ and 0.23M Tris pH 8.0) and stored at -80°C.

2.3.13 DNA sequencing

The recombinant plasmids generated were DNA sequenced using the Big Dye TM Terminator Cycle Sequencing Kit [Perkin Elmer]. ClustalW2 software available online at [http://www.ebi.ac.uk/Tools/msa/clustalw2/] was used to align the sequences. The sequencing reaction contained:

200 to 300ng of plasmid DNA,

2.5pmol of either forward or reverse sequencing primer,

1µl of Big Dye 3.1 [Applied Biosystems] containing fluorescent tagged dideoxynucleotides (ddNTPs).

3µl of Big Dye 3.1 buffer and

dH_2O to make the reaction volume up to 15µl

The sequencing reaction was performed on a Biometra Tpersonal PCR machine [Thistle Scientific] and consisted of 25 cycles of the following steps: 96°C for 15 seconds, 50°C for 30 seconds and 60°C for 4 minutes.

Following the reaction, the DNA was precipitated by adding 35 µl of 100% ethanol, incubating on ice for 5 minutes and subsequently collected by centrifuging for 10 minutes at 13000 rpm. The DNA was then washed once again with 50 µl of 70% ethanol. The DNA pellet was left to air dry for 5 minutes before sequencing by the DNA sequencing facility of Cardiff University [Central Biotechnology Services, Henry Wellcome Research Institute, Cardiff University] using a ABI prism 3130X1 Genetic Analyzer.

2.3.14 Plasmid vectors

The plasmid vectors used within this project, pUC x-2A-y, pUC x-2A-GFP, Lenti-SxW, pGEM-4Z, pCS2 and Lenti SeW were kindly provided by Mr Matthew Ketterling [Department of Infection, Immunity and Biochemistry]. The Lenti-CD2 plasmid which was used as a control was generated in the department by Dr Laurence Pearce [Department of

Infection, Immunity and Biochemistry]. All the recombinant pUC and lentivirus plasmids generated in this project were designed using Vector NTI® Software, Invitrogen.

2.3.15 "Quick Step" *in vitro* mutagenesis

"Quick step" *in vitro* mutagenesis was used to remove internal *XbaI* and *KpnI* sites from the Lyp coding sequence, an *XbaI* site from the rat CD2 coding sequence and to execute the Arginine to Tryptophan change at position 620 of Lyp. For each modification, sense and anti-sense primers (Table 2.2) incorporating a change in a single nucleotide were designed and ordered from MWG biotech. Each mutagenesis PCR reaction was carried out using high fidelity Pfu turbo polymerase [Stratagene] and specific cycling parameters (initial denaturing at 95°C for 30seconds, 12 cycles of 95°C for 30seconds, 55°C for 60seconds, 68°C for 12 minutes). DpnI digestion was performed by adding 1µl of DpnI to 25µl of PCR product and incubating at 37°C for two hours to cleave the methylated parental DNA. DpnI digested DNA was then transformed into DH5α competent cells. Colonies were picked and grown in 5ml cultures followed by extraction of the plasmid DNA using an Invitrogen mini prep kit. The mutant DNA was screened by restriction endonuclease digest and confirmed by sequencing.

2.4 Tissue culture and lentivirus production

2.4.1 Culture conditions

Cells were cultured in either RPMI or DMEM (4.5g/L glucose and L-Glutamine), with 10% heat-inactivated FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate unless mentioned otherwise. Cells were maintained at 37°C in a Heraeus incubator with a humidified atmosphere of 5% CO_2. Adherent and non-adherent cells were grown to 50-90% confluence before being split. When splitting non-adherent cells, the tissue culture flask was tapped twice on the side to release the slightly sticky cells. The cells were then removed from the flask, filtered and returned to the tissue culture plastic. Adherent cells were split by incubating the cells with 0.5% trypsin in HBSS. The media was removed from the cells, and the cells were rinsed once with PBS and incubated with warm trypsin for 5-7 minutes. Cells were then removed from the plastic, filtered, washed with PBS by centrifugation and finally passed through a 29G needle to create a single cell suspension

before being returned to the tissue culture plastic. Cells grown in tissue culture plates or petri-dishes were placed in a ventilated plastic container with a small reservoir filled with PBS

Cell Line	Description	Maintenance
293 T (HEK)	Human Embroynic Kidney cell line derived from adenoviral transformation of kidney cells from a healthy aborted fetus. 293T cells transfect very easily and have been used for lentivirus production.	Cultured in 10% FCS-DMEM (D10) media and split 1:10, 2 times a week.
E6.1 (Jurkat)	Human leukaemic T cell lymphoblast cell line, clonal derivative of Jurkat T cell line. The Jurkat cell line was established from the peripheral blood of a 14 year old boy. Clone E6.1 cells produce large amounts of IL-2 after stimulation with phorbol esters and either lectins or monoclonal antibodies against the T3 antigen (both types of stimulants are needed to induce IL-2 production).	Cultured in 10% FCS-RPMI (R10) media and split 1:3, 2-3 times a week.
E6.1 gag^{+}	E6.1 cells infected with lentivirus particles generated from recombinant plasmid containing pSLY -TCR Alpha.T2A.beta (kindly provided by Dr John Bridgeman, Department of Infection, Immunity and Biochemistry, Cardiff University)	Cultured in 10% FCS-RPMI (R10) media and split 1:3, 2-3 times a week.

H9 (HuT78)	H9 cell line is a clonal derivative of HuT 78 cell line which was derived from peripheral blood of a 50 year old male patient with Sezary syndrome.	Cultured in 10% FCS-RPMI (R10) media and split 1:3, 2-3 times a week.
H9 gag^+	H9 cells infected with lentivirus particles generated from recombinant plasmid containing pSLY – TCR Alpha.T2A.beta. (H9 gag^+ cell line was kindly provided by Dr John Bridgeman, Department of Infection, Immunity and Biochemistry, Cardiff University)	Cultured in 10% FCS-RPMI (R10) media and split 1:3, 2-3 times a week.

Table 2.3 Description of established cell lines used.

2.4.2 Lentivirus production

Lentiviruses are diploid single stranded RNA viruses belonging to the retrovirus family. Lentiviruses are composed of two copies of RNA, a nuclear capsid, a membrane associated matrix, a capsid, envelope proteins, enzymes such as Integrase, Reverse Transcriptase, Protease and accessory proteins, Vip, Vpr, Vpu, Nef, Tat, and Rev (Buchschacher and Wong-Staal 2000). Lentiviruses are capable of infecting both dividing and non-dividing cells because their pre-integration complex consisting of matrix, vpr and integrase contains a localisation sequence that allows them to manipulate nuclear import machinery of a cell, allowing them to enter an intact nuclear membrane (Buchschacher and Wong-Staal 2000). Replication deficient HIV-1 based lentiviruses were produced using a three-plasmid transfection system (Buchschacher and Wong-Staal 2000). A packaging cell line was trainsiently transduced with three plasmids. First plasmid encodes the gene of interest flanked by a self inactivationg viral LTR and psi sequence (packaging signal) and driven by spleen focus forming virus (Sffv) promoter known to be proficient at driving gene expression in T cells. A second and third plasmids, pCMVdelta8.91 (coding the *gag/pol and rev* proteins) and pMD2G (coding the vesicular stomatitis virus G protein), respectively. pCMV delta8.91 and

pMD2G lack the psi sequence therefore cannot be packaged into a virions. Only the first plasmid encoding the gene of interest contains the psi sequence and is packaged into virions.

A total of 6 x10^6 293T cells were cultured in a T75 flask with complete DMEM medium 24 hrs prior to transfection with plasmid DNA. A few minutes prior to transfection, the media was replaced with 8mls of fresh medium. A lipid based transfection kit, Effectine [Qiagen], was used according to manufacturer's instructions to produce lentivirus particles. Briefly, to transfect cells in a T75 flask, 1µg of Lenti Lyp-2A-GFP/CD2 plasmid was mixed with 0.5µg of pMD2G plasmid and 0.75µg of pCMVdelta8.91 plasmid and 300µl of EC buffer was added to the mixture followed by 16µl of the Enhancer reagent, which condenses the plasmid DNAs. The plasmid DNAs, EC buffer and Enhancer mixture was then vortexed for 1 second and incubated for 2-5 minutes at room temperature. Subsequently, 60µl of Effectine reagent was added to the DNA, EC buffer, Enhancer mixture, vortexed for 5-10 seconds and incubated for 5 - 10 minutes at room temperature. The Effectine reagent coats the condensed DNA in lipid, which allows it to enter the cells. After 5 - 10 minutes of incubation, 3 ml of fresh complete DMEM medium was then added to the DNA transfection complexes before being added to the T75 flask. Cells were maintained for 72 hours at 37°C in a Heraeus incubator with a humidified atmosphere of 5% CO_2.

Viral harvest was carried out at 48hrs and 72hrs post transfection. The media supernatant was harvested at 48 hrs and replaced with 8mls of fresh DMEM medium, followed by another harvest at 72 hrs. Cell debris was removed from the supernatant by centrifugation at 3000 rpm for 5 minutes. The harvested virus was subsequently concentrated by centrifugation at 600 000 rpm for 2hrs at 4ºC in a Beckman Coulter Optima™ L-100 XP ultracentrifuge. The virus pellet was then resuspended in 300 - 500µl of DMEM media with no additives and stored at -80°C in 70µl aliquots.

2.4.3 Viral transduction of T cell lines

The $H9^{gag+}$ and $E6.1^{gag+}$ cell lines were each transduced with either ^{R}Lyp-2A-CD2 or ^{W}Lyp-2A-CD2 or CD2 lentivirus particles to generate the $H9^{gag+\ R}$Lyp-2A-CD2, $H9^{gag+\ W}$Lyp-2A-CD2, $H9^{gag+}$CD2, $E6.1^{gag+\ R}$Lyp-2A-CD2, $E6.1^{gag+\ W}$Lyp-2A-CD2 and $E6.1^{gag+}$CD2 cell lines. Viral transduction was carried out by adding 64µl of concentrated lentivirus particles drop-wise to each well of a 24 well tissue culture plate containing 2 x 10^5 cells per well in

500µl of complete RPMI media. The cells were then incubated for 48-72 hours at 37ºC before the transduction efficiency was checked by flow cytometry.

2.5 T cell based functional assays

2.5.1 Enrichment of rat CD2 expressing cells using anti PE magnetic beads

Lentiviral transduced cells were harvested into a 15ml Falcon tube and centrifuged. The supernatant was removed and the cells were washed once with 1ml of PBS. Cells were then incubated for 15-10 minutes in the dark with 15µl of anti-rat CD2 PE antibody per 10^7 cells. Cells were then washed once and rat CD2 expressing cells enriched according to manufacturer's instructions. Briefly, cells were resuspended in MACs buffer and stained with anti-PE biotin conjugate beads. A MS column was prepared and cells were washed once and passed through the column. Rat CD2 positive cells, which were bound to the column, were harvested into a sterile 15 ml falcon tube. This rat CD2 enriched cell fraction was then expanded in culture.

2.5.2 T cell activation

SLY (SL9) gag peptide

The SLY peptide sequence encoding SLYNTVATL was purchased from Severn Biotech Ltd [Kidderminister, Worcs, U.K.]. The SLY peptide is HLA-A2 specific. The peptide was reconstituted in DMSO to give a stock concentration of 5mg/ml and stored in 30µl aliquots at -20ºC.

The HLA-A2 restricted B cell line, C1R, was used as a source of antigen presenting cells (APCs) for the activation of T cells using the SLY peptide. A day prior to the activation, the T cell lines (E6.1 cells were HLA-A3 and H9 cells were HLA-A1 restricted) were rested overnight at a density of 1 x 10^6 cells per ml in complete RPMI media supplemented with 2% FCS (R2). The following day, 45 000 APCs per well, were pulsed with varying concentration of peptide (from 10^{-3}M to 10^{-9}M) or left unpulsed (control) for one hour in R2 media at 37ºC. Following an hour incubation, the 96 well plate was washed once with complete R2 media

and 150,000 T cells in 100µl were added to each of the well at a 1:3 APCs: T cells ratio. The cells were then incubated for 24 hours at 37 ºC. After 24 hours, the supernatants were harvested and stored at -80 ºC for cytokine analysis. The cells were then harvested, washed and incubated with anti-human $CD69^{PE}$ antibody and analysed by flow cytometry for CD69 expression.

2.5.3 IL-2, IL-10 and MIP-1β ELISAs

IL-2 ELISAs

The levels of IL-2 in culture supernatents was analysed using an IL-2 ELISA kit [R&D systems, Abingdon, U.K]. The IL-2 ELISA was carried out on supernatants followings peptide stimulation of all eight cell lines, $H9gag^{+}$, $H9^{gag+\,R}$Lyp-2A-CD2, $H9^{gag+\,W}$Lyp-2A-CD2, $H9^{gag+}$rCD2 cell lines and $E6.1gag^{+}$, $E6.1^{gag+\,R}$Lyp-2A-CD2, $E6.1^{gag+\,W}$Lyp-2A-CD2 and $E6.1^{gag+}$rCD2 cell lines. ELISA plates were coated with 100µl of 1 µg/ml anti-human IL-2 capture antibody diluted in 1X reagent diluent [R&D systems, Abingdon, U.K] and incubated overnight at room temperature. Next day, the wells were aspirated and washed three times with PBS + 0.05% Tween-20 (Sigma). To each well was then added 300µl of 1x Reagent dilutent and the plates incubated at room temperature for 2 hours. After incubation, the wells were aspirated and washed three times as previously. 100µl of diluted samples were added in duplicates. $E6.1^{gag+}$ (Jurkat) cell supernatants were diluted 1in 2.5 in reagent dilutent for the IL-2 assay whereas $H9^{gag+}$ (Hut78) cell supernatants were diluted 1 in 4 with reagent dilutent. Control standards were also added starting with 1000 pg/ml with doubling dilutions down to 15.6 pg/ml diluted in the 1x reagent dilutent and samples incubated for 2 hours at room temperature. After incubation the wells were aspirated and washed three times as previously described. To each well was added 100µl of a 1 in 180 diluted IL-2 detection antibody. The plates were once again incubated at room temperature for 1.5 hours after which the plates were aspirated and the wells washed three times as previously described. To each well was then added 100 µl of a 1:10,000 diluted strepavidin HRP after which plates were incubated at room temperature for 1 hour. The wells were then aspirated and washed three times as previously described. 100 µl of colour substrate [R&D systems, Abingdon, U.K] was added to each well and the colour allowed to develop for approximately 2-5 minutes in

the dark. The reaction was stopped by the addition of 50 µl of stop solution [R&D systems, Abingdon, U.K] to each well before reading the plate at 450-650nm

IL-10 ELISAs

The levels of IL-10 in culture supernatents was analysed using an IL-10 ELISA kit [BioLegend, Cambridge, U.K]. The IL-10 ELISA was carried out on supernatants following peptide stimulation of H9gag^{+}, H9$^{gag+\ R}$Lyp-2A-CD2, H9$^{gag+\ W}$Lyp-2A-CD2 and H9^{gag+}rCD2 cell lines. The IL-10 ELISA was carried out according to manufacturer's instructions. Briefly ELISA plates were coated with 100µl of IL-10 capture antibody diluted in PBS and incubated overnight at room temperature. Next day, the wells were aspirated and washed three times with PBS + 0.05% Tween-20. To each well was then added 300µl of 1x Reagent dilutent [R&D systems, Abingdon, U.K] and the plates incubated at room temperature for 2 hours. After incubation the wells were aspirated and washed three times as previously described and 100µl of H9^{gag+} (Hut78) cell supernatants diluted 1 in 10 with reagent dilutent was added in duplicates. Control standards were also added starting with 500pg/ml of IL-10 with doubling dilutions down to 15.6 pg/ml diluted in the 1x reagent dilutent. Samples were then incubated for 2 hours at room temperature. After incubation, the wells were aspirated and washed three times as previously described. To each well, was added 100µl of 1 in 200 diluted IL-10 detection antibody. The plates were once again incubated at room temperature for 1 hour after which the plates were aspirated and the wells washed three times as previous. To each well was added 100 µl of diluted HRP conjugated antibodies, for IL-10 this was avidin HRP conjugate diluted 1:10,000 in reagent dilutent and the plates were incubated at room temperature for 30 minutes. The wells were then aspirated and washed three times as previously described. 100 µl of colour substrate [R&D systems, Abingdon, U.K] was then added to each well and the colour allowed to develop for approximately 2-5 minutes in the dark. The reaction was stopped by the addition of 50 µl of stop solution [R&D systems, Abingdon, U.K] to each well before reading the plate at 450-650nm

MIP-1β ELISAs

The levels of MIP-1β in culture supernatents were analysed using a MIP-1β ELISA kit [R&D systems, Abingdon, U.K]. The MIP-1β ELISA was carried out on supernatants following peptide stimulation of E6.1gag^{+}, E6.1$^{gag+\ R}$Lyp-2A-CD2, E6.1$^{gag+\ W}$Lyp-2A-CD2 and E6.1^{gag+} rCD2 cell lines. The MIP-1β ELISA was carried out according to manufacturer's instructions. Briefly, ELISA plates were coated with 100µl MIP-1β capture antibody diluted in 1x reagent dilutent [R&D systems, Abingdon, U.K] and incubated overnight at room temperature. Next day, the wells were aspirated and washed three times with PBS + 0.05% Tween-20. To each well was then added 300µl of 1x Reagent dilutent and the plates incubated at room temperature for 2 hours. After incubation the wells were aspirated and washed three times as previously described and 100µl of 1in 2.5 diluted samples in reagent dilutent added in duplicates. Control standards were also added starting with 1000 pg/ml of MIP-1β with doubling dilutions down to 15.6 pg/ml diluted in the 1x reagent dilutent. Samples were incubated for 2 hours at room temperature. After incubation, the wells were aspirated and washed three times as previously described. To each well was added 100µl of 1in 200 diluted MIP-1β detection antibody. The plates were once again incubated at room temperature for 1.5 hours after which the plates were aspirated and the wells washed three times as previously described. To each well was then added 100 µl of 1 in 200 diluted strepavidin HRP after which plates were incubated at room temperature for 1 hour. The wells were aspirated and washed three times as previously described. 100 µl of colour substrate [R&D systems, Abingdon, U.K] was added to each well and the colour allowed to develop for approximately 2-5 minutes in the dark. The reaction was stopped by the addition of 50 µl of stop solution [R&D systems, Abingdon, U.K] to each well before reading the plate at 450-650nm.

2.6 Monoclonal Antibody Production

2.6.1 Animals

All mice used for hybridoma generation (both sources of antibody producing B cells and feeder cells) were of the Balb/c strain and were bred in house at the Biomedical Services Unit (BSU) located at the School of Medicine, Heath Park, Cardiff University.

2.6.2 Preparation of antigens

11 amino acid long peptides encompassing the R620 (peptide B-PLPV**R**TPESFC) or the W620 (peptide A-PLPV**W**TPESFC) isoforms of PTPN22 were synthesised (in house by Ms Miriam Vigar) and checked by mass spectroscopy[Central Biotechnology Services, Henry Wellcome Research Institute, Cardiff University]. The peptides were coupled to bovine serum albumin (BSA) using an m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS) heterobifunctional cross-linker and the conjugation checked by mass spectroscopy and Coomassie blue stained protein gel.

2.6.3 Coupling of Lyp peptides to bovine serum albumin (BSA)

1g of bovine serum albumin (BSA) was dissolved in 100mls of 0.01M phosphate buffer pH7 (1 in 10 dilution of 0.1M stock solution consisting of 57.7mls of 1M Na_2HPO_4 42.3mls of 1M NaH_2PO_4 and 900mls of H_2O). 5mg of heterobifunctional cross-linker m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS) was dissolved in 333.3µl of Dimethylformamide (DMF). The MBS and BSA solutions were mixed by adding 140µl of MBS solution in 1ml of BSA solution and rotated for 30 minutes at room temperature. Subsequently, 0.05M Phosphate buffer pH6 (1 in 2 dilution of 0.1M stock solution consisting of 12.0mls of 1M Na_2HPO_4 88.0mls of 1M NaH_2PO_4 and 900mls of H_2O) was used to purify the BSA/MBS solution using low pressure liquid chromatography. Finally, 500µl of H_2O was added to the purified BSA/MSA solution.

2.8mg of each peptide (2.8mg of peptide A and 2.8mg of peptide B) were dissolved in 76µl of DMF each. 1ml of purified BSA/MBS solutions was rapidly added to each of the dissolved peptide solutions and mixed. 8.25µl of 2M NaOH was added immediately to both the peptide A/BSA/MBS and peptide B/BSA/MBS solutions. Next, the peptide/BSA/MBS solutions were rotated for 3 hours at room temperature before adding 2.25mls of 0.1M ammonium bicarbonate to each of the two peptide/BSA/MBS solutions. Subsequently, the peptide/BSA/MBS solutions were snap frozen by rotating the falcon tubes containing the solutions in a dry ice/ethanol bath to form a thin frozen layer sheet around the circumference of the falcon tubes. The samples were then lyophilised overnight and stored at -20°C until

required. The coupled proteins were checked by mass spectroscopy [Central Biotechnology Services, Henry Wellcome Research Institute, Cardiff University] as well as protein gels with coomassie blue staining.

2.6.4 Immunisation schedule

BSA conjugated ^{R}Lyp and ^{W}Lyp peptides were used to immunise mice. Three mice per peptide were initially immunised subcutaneously with 100μg of peptide/carrier conjugate mixed 1:1 with complete Freund's adjuvant. The mice were subsequently boosted with 100μg of peptide/carrier conjugate mixed 1:1 with incomplete Freund's adjuvant at 2 weeks, 4 weeks and again at 6 weeks. The antibody titre in the sera directed against ^{R}Lyp and ^{W}Lyp peptides was measured 12 days after the last immunisation using an Enzyme Linked Immunosorbent Assay (ELISA). Four days prior to fusion and harvest of splenocytes, the mouse selected for this purpose was boosted intra-peritoneally for a final time with 50μg of conjugated peptide in PBS.

2.6.5 Measuring the polyclonal antibody titre in sera of immunised mice

Blood was used to measure the antibody titre in the serum of the six immunised mice. Firstly, the mouse was restrained and the tip of the tail sprayed with ethyl chloride BP [Acorus Therapeutics Limited, Durham, U.K.] and allowed to numb. A small section of the tail was removed at its tip and 30-50μl of blood taken by aspirating with a Gilson P200 pipette fitted with a small pipette tip. If blood was required at the end of an experiment, it was taken via a cardiac puncture. The mouse was placed in a chamber, which was subsequently filled with anaesthetic gas (isofluorane) using oxygen as a carrier gas. Once the mouse showed signs of anaesthesia it was transferred to a face mask with a lower concentration of anaesthetic gas. A 28G needle was inserted below the sternum and a cardiac withdrawal of blood performed with a syringe. The blood was then left to clot for 10 minutes on ice, centrifuged at 1300rpm for 10 minutes at 4°C and the serum collected.

2.6.6 Preparation of feeder cells for hybridomas

Peritoneal macrophages from Balb/c mice were used as feeder cells for hybridomas. Peritoneal macrophages from one Balb/c mouse produced enough feeder cells for ten 96 well plates of hybridomas.

Typically, mice were killed by asphyxiation in a rising concentration of CO_2, followed by cervical dislocation. Peritoneal macrophages were harvested by flushing the peritoneal cavity with 10mls of sterile cold RPMI using a syringe and a 21 gauge needle, gently massaging and withdrawing as much RPMI as possible into the syringe. The content of the syringe was then transferred into a sterile falcon tube and washed twice with cold RPMI by centrifuging at 1600 rpm for 4 minutes at 4°C.

For growing primary hybridoma clones directly after fusion and during the first round of cloning, the peritoneal cell pellet was re-suspended in the required volume (100mls per ten 96 well plates) of warm Hypoxanthine Aminopterin Thymidine (HAT) supplemented with complete RPMI 1640 media and plated at 100µl per well into the required quantity of 96 well plates (ten directly after fusion and 30 during first round of cloning).

The peritoneal macrophage feeder cells used for the second round of limiting dilution cloning were re-suspended in warm HT supplemented complete RPMI 1640 medium and in warm 20% FCS RPMI 1640 medium during the third round of limiting dilution cloning and expansion.

2.7 Generation of hybridomas.

2.7.1 Harvesting the splenocytes.

Four days after the final i.p boost the immunised mouse was killed by asphyxiation in a rising concentration of CO_2. The spleen was excised using a sterile dissection kit and the fat was removed. Splenocytes were harvested by gently pressing the spleen in 5 ml of sterile cold RPMI with the back of a sterile syringe until only the intact fibrous capsule remained. The splenocytes were filtered into a sterile Falcon tube and washed thrice in 20mls of cold RPMI by centrifugation at 1600rpm for 4 minutes at 4°C. The splenic cell pellet was then finally re-suspended in 20mls of warm RPMI 1640 media (no additives).

2.7.2 Preparation of the myeloma cells

The human myeloma cell line SP2/0 used for hybridoma production was a kind gift from Dr Claire Harris [Department of Medical Biochemistry and Immunology, Henry Wellcome Research Institute, Cardiff University]. The SP2/0 cells were maintained in complete RPMI 1640 medium. Human myeloma cells SP2/0 were passaged one day prior to fusion and 1x 10^7 cells harvested the next day whilst in log phase of growth. Subsequently, the SP2/0 cells were washed thrice in 30mls of sterile cold RPMI by centrifugation at 1600rpm for 4minutes at 4°C. The SP2/0 myeloma cell pellet was then re-suspended in 20mls of warm RPMI 1640 media (no additives).

2.7.3 Cell fusion

The re-suspended splenic cells and SP2/0 cells were mixed by adding the 20mls of re-suspended splenic cells to the Falcon tube containing the 20mls of re-suspended SP2/0 myeloma cells. The mix was then washed once by centrifugation at 1600rpm for 4 minutes at room temperature. The supernatant was carefully removed leaving the cell pellet as dry as possible. The pellet was gently flicked to re-suspend the splenic, SP2/0 cells and 1ml of pre-warmed polyethylene glycol 1500 (PEG) [Roche Diagnostic, Indianapolis, USA] was added drop-wise from a syringe with a 19 gauge needle over a 1-2 minute period with constant agitation of the cells by flicking and rotating the Falcon tube. The cells were then allowed to stand for 30 seconds after which 20mls of warm RPMI media (no additives) was added drop-wise over 1-2 minute period whilst gently agitating the cells by flicking and rotating the falcon tube. A further 10mls of warm RPMI media was added and the cells were centrifuged at 1200rpm for five minutes at room temperature. The cell pellet was then re-suspended in 98mls of warm HAT RPMI 1640 media and plated (100µl per well) into the prepared 10 x 96 well plates with feeder cells. The 96 well plates containing the primary hybridoma clones were incubated in a humidified atmosphere of 5% CO_2 at 37°C and left to grow for seven days. The hybridoma clones were fed 75µl per well of warm HAT RPMI 1640 media on day seven and left to grow until day ten when they were screened by a peptide based ELISA and 15 of the most positive clones cloned by limiting dilution cloning.

2.7.4 Cell culture

Primary hybridoma clones generated immediately following fusion (described below) and hybridomas during the first round of limited dilution cloning, were cultured in complete RPMI 1640 medium supplemented with 1% Hypoxanthine Aminopterin Thymidine (HAT) [Gibco]. Hybridomas during the second round of cloning were weaned off HAT supplement and cultured in RPMI 1640 medium supplemented with 1% Hypoxanthine Thymidine (HT) [Gibco]. Hybridomas during the third round of cloning were cultured in complete RPMI 1640 medium with 20% FCS and established monoclonal hybridomas whilst and after expansion were cultured in complete RPMI 1640 with 15% FCS.

2.7.5 Producing monoclonality

Peptide based ELISA screen

The hybridomas were screened for antibody production against ^{R}Lyp or ^{w}Lyp peptide using a peptide based ELISA. 96-well ELISA plates [Greiner bio-one] were coated overnight at 4°C with 50µg/ml of ^{R}Lyp or ^{w}Lyp peptide, washed once in wash buffer (0.05% Tween 20 in PBS) and blocked for an hour at 37°C with 50µl of blocking solution (5% Milk in PBS). The 96-well ELISA plates were then washed three times with wash buffer and incubated for 2 hours at room temperature with 75µl of hybridoma supernatant followed by three more washes with the wash buffer. Subsequently, 50µl of a secondary goat anti-mouse IgG (H+L) antibody conjugated with HRP [Bio-Rad] was added and the ELISA plates were incubated for an hour at room temperature. Finally the ELISA plates were washed three times with wash buffer and developed using TMB substrate reagent [BD] and measured at 450nm using a spectrophotometer.

Limiting Dilution Cloning

One day prior to cloning, mouse feeder cells were isolated and the required number of 96 well plates (this number depends on the number of wells to be cloned with two 96 well plates required per well to be cloned) prepared with 100µl per well of feeder cells in appropriate medium (the medium used depends on the stage of cloning, first stage cloning uses HAT RPMI1640, second stage cloning uses HT RPMI 1640 and any other stage cloning uses 20%

FCS RPMI1640). Hybridoma clones in the 10 to 15 most promising wells (based on the strength of the positive signal on the ELISA screen) were picked for further cloning to produce monoclonality. The cells in these chosen wells were re-suspended and viable cells counted using the trypan blue exclusion method. 3000 cells were counted and transferred into 6mls of appropriate medium, mixed gently and 200µl of this solution transferred to each well across the first three columns (the limited dilution cloning was done in triplicates) using a multichannel pipette so that each well in the first three column should now contain ≈ 100 cells each in 300µl. Subsequently, the contents of each of these wells were mixed by pipetting up and down using a multichannel and 100µl from each of these wells were transferred to each of the wells in the next three columns (100µl from wells in column 1, 2, 3 is transferred to column 4, 5, 6 respectively). This process was repeated across the two 96 well plates (100µl from column 4, 5, 6 was mixed and transferred to 7, 8, 9 respectively and so on until the end of the second 96 well cloning plate). 100µl of appropriate media was then added to wells 4 to 12 in the first 96 well cloning plate and wells 1-9 in the second 96 well cloning plate were (wells in the first three and the last three columns contain 200µl per well). The clones were then left to grow for 4 days at 37°C at 5% CO_2. On day four, the clones were fed 75µl per well of the appropriate medium and left to grow for further 6 days when they were screened and cloned again until they were monoclonal. After the third round of limiting dilution cloning they are generally expected to be monoclonal.

Expanding the established monoclonal hybridomas

After the third round of cloning, the hybridoma clones were screened by peptide based ELISAand18 of the most positive clones (2 clones from each set of cloning plates) were re-suspended by gentle pipetting and expanded onto a 24 well plate. Subsequently, when the hybridoma cells in the 24 well plate reached 70-80% confluence, they were once again expanded onto 6 well plates, then onto T15 flasks, followed by T25 flasks, then finally onto T75 flasks.

2.7.6 Characterization of the monoclonal hybridomas

ELISA screen

The individual monoclonal hybridoma supernatants were tested using peptide based ELISAs for cross-reactivity between the two Lyp isoforms, $^{R}620$ and $^{W}620$. Two rows of the 96 well ELISA plate were coated with the $^{w}620$ (peptide A) or $^{R}620$ (peptide B) and the ELISA screen performed according to the above protocol.

Immunoblotting screen

Established monoclonal hybridoma supernatants were screened by immunoblotting to check if any of the monoclonal antibodies produced by the different monoclonal hybridoma clones recognised the denatured Lyp protein. Typically, a SDS-Polyacrylamide Gel Electrophoresis was performed with RAMOS or DAUDI (B cell lines that are known to produce Lyp) or Lenti-GFP-2A-Lyp transfected 293 T cells lysates, untransfected 293 T cell lysates were used as a negative control for Lyp detection. Subsequently, the proteins were transferred onto PVDF membrane. The PVDF membrane was then cut into half and the bottom half (below75kDa) was immunoblotted for Actin. The top half of the membrane was cut into strips (one well = one strip) and incubated in individual monoclonal hybridoma supernatants to test if any of the supernatant could recognise the denatured full length Lyp protein. The remaining steps of the immunoblotting were performed as described above.

2.8 Statistical analysis

The level of differences in cytokine production between the panel of transduced E6.1^{gag+} (Jurkat) T cells and between the panel of transduced H9 $^{gag+}$ (Hut78) T cells was compared by Two-way ANOVA using the PRISM software and are shown to the 95% ($P < 0.05$) , 99% ($P < 0.01$) or 99.9% ($p < 0.001$) confidence level.

Chapter 3

The generation of polymorphism specific anti-Lyp monoclonal antibodies

3.1 Introduction

There are currently no reported antibodies specific for the R620 or W620 isoforms of PTPN22. However, there are potential benefits from raising antibodies against both the isoforms of PTPN22. For example, if the W620 polymorphism leads to an increase in PTP activity resulting in predisposition to ADs, then it is plausible that an antibody selectively directed against the W620 isoform may have a blocking activity. If it could be shown using *in vitro* assays that a monoclonal antibody generated against the ^{W}Lyp isoform had a blocking effect exclusively against the ^{W}Lyp isoform, this would indicate that there might be some structural differences between the R and the W isoform of Lyp that could be targeted. This would provide proof of principle for generation of a reagent such as a small molecule inhibitor that may be able to selectively target the ^{W}Lyp isoform. In principle, such a small molecule inhibitor may potentially decrease the severity of AD or even defer the initiation process. Therefore, in this chapter the expertise in monoclonal antibody production by the research group led by Dr Claire Harris was utilised in an attempt to generate specific monoclonal antibodies against the ^{R}Lyp and ^{W}Lyp isoforms.

B cells are an important part of the adaptive immune system. The secretion of antibodies, which bind pathogens or their toxic products in the extracellular space of the body, is the main effector function of B cells in adaptive immunity (Murphy et al. 2007a). The technique of monoclonal antibody production exploits this specific antibody-producing feature of the B cells. However, because B cells have a limited life span monoclonal antibody production requires a method of immortalising the antibody producing B cells. The advent of hybridoma technology by Kohler and Milstein in (1975) allowed the establishment of hybrid cell lines that could be grown indefinitely to produce large amounts of antibody of a desired specificity (Kohler and Milstein 1975). In this technique a myeloma cell is physically united with a B cell whose antigen specificity is known, creating a hybrid cell (hybridoma). The generation of a hybridoma requires cell fusion in which the plasma membranes of a B cell and a myeloma cell are joined such that the cytoplasm is combined. Therefore, monoclonal antibody production involves fusion of a myeloma with antibody producing B cells from the spleen

(splenocytes) of an animal immunised with the antigen of interest. Following fusion, limiting dilution cloning of the hybridoma cells ensures the monoclonality of the antibody producing hybridoma. Consequently, the generation of monoclonal antibody requires both *in vivo* (for generation of specific antibody secreting B cells) and *in vitro* (for fusion and limiting dilution cloning) procedures.

3.2 Mouse anti human $^{R/W}$Lyp monoclonal antibody

Eleven amino acid peptide sequences encompassing either the R 620 isoform (PLPV**R**TPESFC) or the W620 isoform of Lyp (PLPV**W**TPESFC) were kindly synthesized by Ms Miriam Vigar. Subsequently, each of the peptides was coupled to BSA using MBS hetero-bifunctional cross-linker as described in the Materials and Methods. The conjugation was checked by mass spectroscopy (data not shown) and Coomassie blue stained protein gel (Figure 3.1).

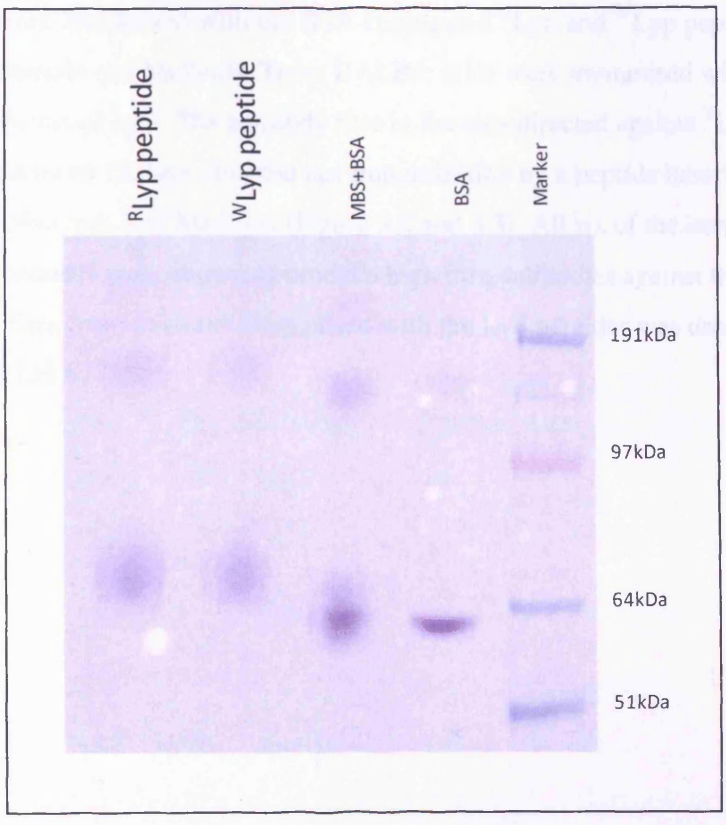


Figure 3.1 Coomassie blue protein staining of BSA-Lyp peptide conjugates

Each of the peptide i.e the R or the W isoform of Lyp was coupled to BSA using an MBS hetero-bifunctional cross-linker. Coomassie blue staining of the BSA alone, MBS and BSA, ^{W}Lyp Peptide, BSA and MBS conjugate and ^{R}Lyp peptide, BSA and MBS conjugate was carried out to check that the conjugation was successful.

3.3 Generation of anti-peptide antibody secreting B cells

BALB/c mice were immunised with the BSA conjugated ^{R}Lyp and ^{W}Lyp peptides as described in Materials and Methods. Three BALB/c mice were immunised with each of the two peptide isoforms of Lyp. The antibody titre in the sera directed against ^{R}Lyp and ^{W}Lyp peptides was measured 12 days after the last immunisation by a peptide based ELISA screen as described in Materials and Methods (Figure 3.2 and 3.3). All six of the immunised mice (three for each peptide) were shown to produce high titre antibodies against the ^{R}Lyp and the ^{W}Lyp peptides. Sera from mice not immunised with the Lyp peptides was used as a negative control for the ELISA.

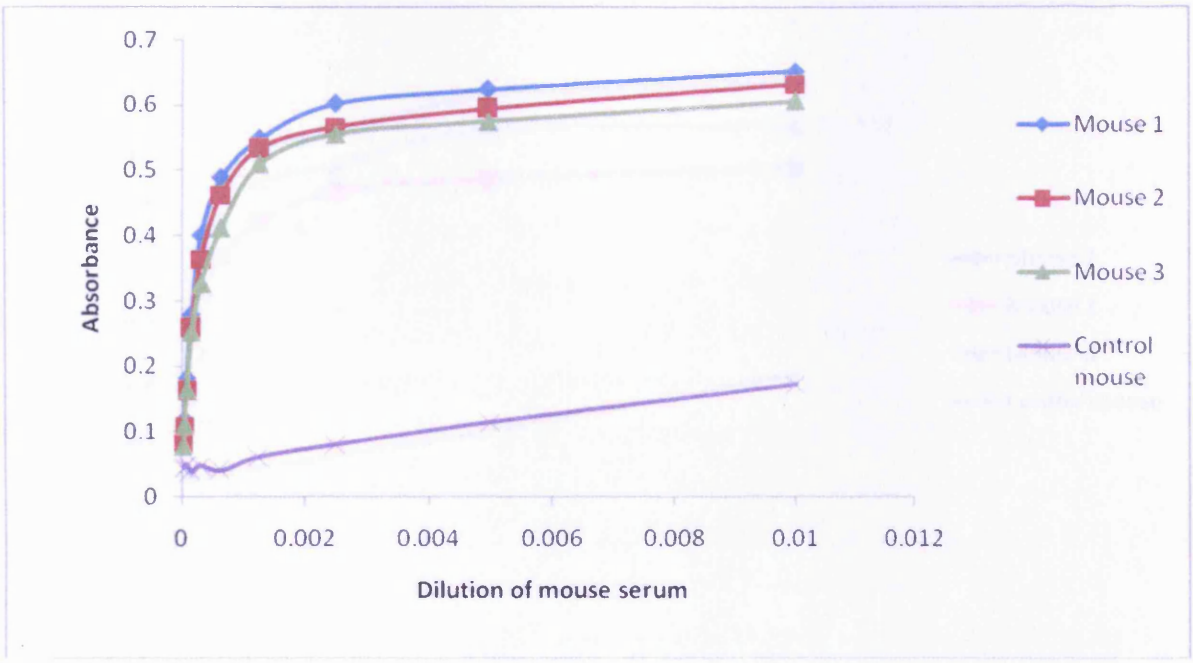


Figure 3.2 Pre-fusion ELISA screen of sera from mice immunised with ^{R}Lyp peptide

The three ^{R}Lyp peptide immunised mice and a control unimmunised mouse were tail bled, the collected blood allowed to clot and serum prepared. A peptide based ELISA screen was used to determine the antibody titre of the anti-^{R}Lyp peptide antibodies in the sera of the immunised and the control mice. Above, the volume/volume dilution of the sera was plotted against absorbance at 450 nm. All three of the ^{R}Lyp peptide immunised mice were seen to produce high titre (six fold increase compared to the control mouse) antibody against the ^{R}Lyp peptide.

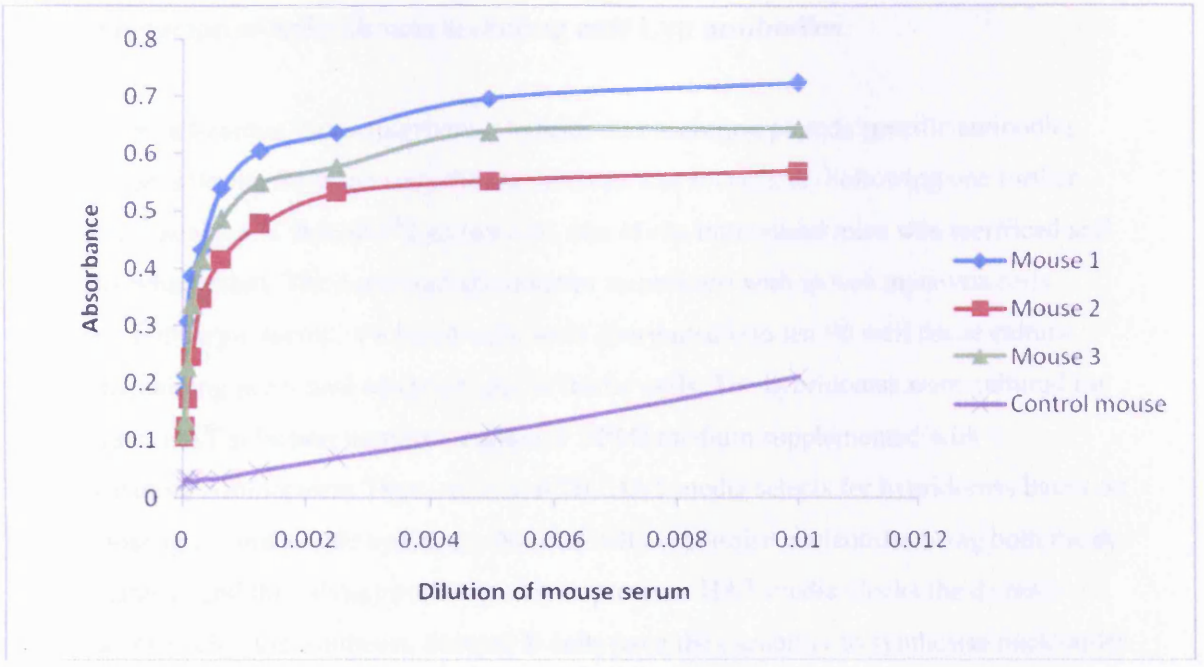


Figure 3.3 Pre-fusion ELISA screen of sera from mice immunised with ^{W}Lyp peptide

The three ^{W}Lyp peptide immunised mice and a control unimmunised mouse were tail bled, the collected blood allowed to clot and serum prepared. A peptide based ELISA screen was used to determine the antibody titre of the anti-^{W}Lyp peptide antibodies in the sera of the immunised and the control mice. Above, the volume/volume dilution of the sera was plotted against absorbance at 450 nm. All three of the ^{W}Lyp peptide immunised mice were seen to produce high titre (six fold increase compared to the control mouse) antibody against the ^{W}Lyp peptide.

3.4 Generation of hybridomas secreting anti Lyp antibodies

Fusion was attempted thrice to generate hybridomas secreting peptide specific antibodies using three different mice but only the last attempt was successful. Following one further intra-peritoneal boost with the ^{R}Lyp isoform, one of the immunised mice was sacrificed and the spleen harvested. The harvested splenocytes were fused with mouse myeloma cells (SP2/0). Following fusion, the fused cells were distributed into ten 96 well tissue culture plates containing peritoneal macrophages as feeder cells. The hybridomas were cultured for ten days in HAT selection medium (complete RPMI medium supplemented with Hypoxanthine Aminopterin Thymidine (HAT)). HAT media selects for hybridomas based on their capacity for nucleotide synthesis. Normal cells synthesise nucleotides using both the de novo pathway and the salvage pathway. Aminopterin in HAT media blocks the de novo pathway of nucleotide synthesis. Normal B cells have the capability to synthesise nucleotides using the salvage pathway but cannot survive long in an in vitro culture unless immortalised. The myeloma cell lacks the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) essential for functioning of the salvage pathway. Therefore, when grown in HAT media, myeloma cells die due to lack of nucleotide synthesis. Hybridomas which have the capability to use the salvage pathway and are immortalised, are the only cells that are able to grow in HAT selection media. Subsequently after ten days of culture in HAT medium, the culture supernatant was tested for anti ^{R}Lyp antibody production by ELISA.

3.5 Screening of the antibody producing hybridoma cells by ELISA

Supernatants from the hybridoma clones were screened for anti ^{R}Lyp peptide antibody production by direct ELISA. Hybridoma supernatant was incubated on an ELISA plate coated with ^{R}Lyp peptide (the same peptide as was used to immunise the mouse). Bound antibody was detected with an HRP conjugated anti mouse antibody. Hybridoma clones giving a positive signal by ELISA were further cloned three times by limiting dilution cloning on peritoneal macrophages as feeder cells. After three rounds of cloning the antibody producing hybridoma cells were accepted to be monoclonal. Three hybridomas producing specific monoclonal antibodies were identified using this technique.

3.6 Characterization of the monoclonal antibody generated against ^{R}Lyp peptide

Supernatants collected from the three monoclonal cell lines were used to characterize the properties of each of the monoclonal antibodies. The antibody was checked for cross reactivity with the ^{W}Lyp peptide by peptide based ELISA whereby the plate was coated with ^{W}Lyp peptide instead of the ^{R}Lyp peptide. None of the monoclonal antibodies were found to be polymorphism specific. When tested by ELISA screening against the ^{W}Lyp peptide, all the antibodies gave a positive signal (Figure 3.4). Immunoblotting was carried out to determine whether any of the monoclonal antibodies produced recognised the denatured intact Lyp protein. From Figure 3.5 it can be seen that antibody from hybridoma clones 12 and 18 detects proteins in the 293 T cell lysates, one of which seems to be of similar size to Lyp. Supernatant from hybridoma clone 12 which was used to probe for Lyp in untransduced 293 T cells (therefore not expressing the protein Lyp) also detected proteins in the cell lysates making it difficult to interpret the result. However, upon further examination it is clear that the proteins detected by the different monoclonal antibodies are almost identical in the negative control and the samples (Figure 3.5). As the supernatant from hybridoma 12 detects the same pattern of protein in transduced and non transduced (not expressing Lyp) 293T cells it is unlikely that the proteins detected correspond to Lyp. Therefore, in summary none of the monoclonal antibodies appear to recognise denatured human Lyp by immunoblot (Figure 3.5).

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.213	0.17	0.028	0.213	0.251	0.205	0.102	0.095	0.1	0.101	0.313	**0.017**
B	0.159	0.117	0.313	0.246	0.114	0.086	0.11					0.219
C	0.228	0.124	0.314	0.246	0.227	0.195	0.189	0.157	0.179	0.209	0.245	**0.017**
D	0.265	0.169	0.314	0.23	0.261	0.089	0.184					0.147
E												
F												
G												
H												
I												

^{R}Lyp Peptide

^{W}Lyp Peptide

Figure 3.4 ELISA based screening of the monoclonal antibodies produced for peptide cross-reactivity

All of the monoclonal antibody producing clones generated by immunisation of the mice with ^{R}Lyp peptide were screened for cross-reactivity with the ^{W}Lyp peptide. Each well represents an antibody producing clone. Rows A and B (top 2 rows) were coated with unconjugated ^{R}Lyp peptide whereas rows C and D (bottom two) were coated with unconjugated ^{W}Lyp peptide. Negative controls were incubated with serum from a mouse not immunised with the Lyp peptides and are shown in bold. Positive controls wells were incubated with serum from an ^{R}Lyp immunised mouse and are highlighted by underlining. Since the mice were immunized with ^{R}Lyp peptide, all the established clones will be positive when screened against ^{R}Lyp peptide (rows A and B) however, clones specific for the ^{R}Lyp peptide should not produce a positive signal when screened against ^{W}Lyp peptide (rows C and D).

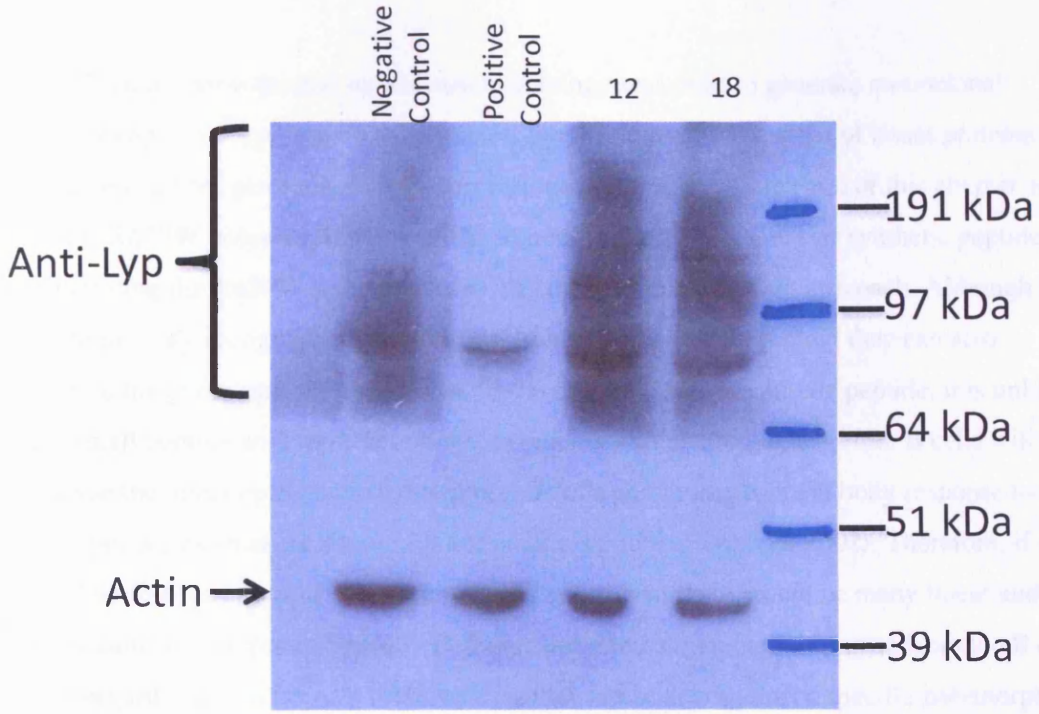


Figure 3.5 Immunoblot analysis of the monoclonal antibody generated against anti-Lyp peptide for recognition of denatured Lyp protein.

Untransfected 293T cells (negative control) and 293T cells transfected with Lyp were lysed in NP-40 lysis buffer, reduced and equivalent numbers of cells loaded into each lane of a 10% SDS-PAGE gel. The SDS-PAGE gel was transferred to a PVDF membrane and the top half of the membrane was probed for Lyp protein with supernatant from each of the seven monoclonal anti-Lyp hybridoma clones or with goat anti-human Lyp polyclonal antibody (positive control). Negative control was probed with supernatant from clone 12 for primary antibody and a anti-mouse HRP secondary antibody. The bottom PVDF membrane was probed for housekeeping protein Actin as a loading control.

3.7 Discussion

In this Chapter conventional hybridoma technology was used to generate monoclonal antibodies against ^{R}Lyp and ^{W}Lyp peptides. Synthetic peptides instead of intact proteins were used as antigen for generation of the monoclonal antibodies. As the aim of this chapter was to generate R620W polymorphism specific monoclonal antibodies, use of synthetic peptides incorporating the R620W polymorphism was thought to be the best approach. Although B cells dominantly recognise conformational epitopes in an intact protein they can also recognise linear epitopes in the protein. In the case of a small synthetic peptide, it is unlikely that a small peptide will form or contain a conformational epitope, therefore B cells will only recognise the linear epitopes in the peptide. B cells can produce an antibody response to linear epitopes as small as about five amino acid residues (Tribbick 2002). Therefore, if using intact Lyp (807 amino acid in length) as an immunogen, there would be many linear and conformational epitopes in the protein that could elicit an antibody response from the B cells. In that regard it would be very difficult to isolate antibodies against a specific polymorphism, corresponding to a single amino acid change in the Lyp protein, by immunisation with the intact Lyp. The use of synthetic peptide as an immunogen allowed the potential targeting of a particular region of Lyp for monoclonal antibody production. As the aim of this chapter was to generate monoclonal antibodies against a specific single amino acid change as a result of the R620W polymorphism in Lyp, synthetic peptides were chosen as immunogens instead of the intact Lyp protein. The use of a small synthetic peptide instead of an intact protein would also drastically limit the epitopes recognised by the B cells thereby increasing the chance of generating ^{R}Lyp or ^{W}Lyp specific antibodies. Therefore a stretch of 11 amino acids incorporating the R620W polymorphism was selected for the generation of the synthetic peptide to be used as an immunogen for the production of polymorphism specific monoclonal antibodies.

The next step was to prepare the antigen for immunisation of the mice. A small synthetic peptide is not usually sufficiently immunogenic on its own. Therefore the synthetic peptides were coupled to carrier proteins to boost the immunogenic response. The synthetic ^{R}Lyp and ^{W}Lyp peptides were conjugated to Bovine serum albumin (BSA) by using MBS as a bi-functional linker. BSA is a 59kDa carrier protein routinely used for coupling to synthetic peptides to generate anti-peptide antibodies (Lateef et al. 2007). A carrier protein is a relatively large molecule capable of stimulating an immune response on its own. The

synthetic peptide coupled to the carrier protein acts as a hapten and antibodies specific for the hapten can be produced (antibodies against the carrier protein are also produced). The conjugation was successfully carried out and the BSA-MBS-peptide conjugates were used to immunise six mice, three for each Lyp peptide. After three rounds of immunisation, blood samples were obtained from the mice by tail tipping. The obtained sera were screened for anti-Lyp peptide antibody production by peptide based ELISA and all of the six mice were found to contain the sufficient antibody titre (usually 5-6 fold increase compared to control is considered sufficient). Three mice were sacrificed at different times, their spleens harvested and the B cells fused with myeloma cells in an attempt to generate monoclonal antibody secreting hybridomas. However, only the final attempt was successful in generating hybridomas secreting monoclonal antibody. Of the remaining three mice, two succumbed to death and one was sacrificed. Subsequently, one of the mouse immunised with ^{R}Lyp peptide was boosted with soluble antigen intra-peritoneally, sacrificed three days later, spleen removed and splenocytes harvested. The splenocytes were fused with myeloma cells to generate hybridomas. The hybridoma clones were screened for anti ^{R}Lyp peptide antibody production by ELISA and the positive clones selected and further cloned three times by limiting dilution to generate monoclonal hybridoma clones.

Hybridoma clones producing mouse anti-Lyp peptide monoclonal antibodies were selected and checked for cross reactivity against ^{W}Lyp peptide. All the monoclonal hybridoma clones generated by immunisation with the ^{R}Lyp peptide were found to be reactive against the ^{W}Lyp peptide as well as ^{R}Lyp peptide. A mouse immunised with an 11 amino acid residue synthetic peptide can generate an antibody response to the peptide when its B cells recognise a linear epitope as small as only five amino acid residues. If the particular immunogenic linear epitope in the ^{R}Lyp peptide that was recognised by an individual antibody producing B cell did not contain the region with the R to W single amino acid change then the antibody generated would not be specific against the region incorporating the polymorphism. As all other ten amino acids are exactly the same for both the peptides excepting the single amino acid change due to the SNP the majority of the anti-peptide antibodies produced will recognise both the peptides. The fact that none of the hybridoma clones generated secreted peptide specific antibody could be due to a random chance that the epitope recognised by the B cell used for fusion did not include the single amino acid change or it may be the case that the amino acid residue, the R to W change in the peptide sequence did not form an epitope for B cell recognition.

Subsequently, anti-Lyp peptide antibodies produced by the hybridoma clones were tested for reactivity against denatured Lyp protein by immunoblotting. It was observed that none of the antibodies from the three hybridoma clones recognised the denatured Lyp protein. Some of the hybridoma clones were found to secrete antibody that showed a non-specific binding pattern to other proteins in the 293T cell lysates. One of the proteins detected approximated to the size of Lyp, however the same protein was also detected in the negative control lysates which did not express Lyp and was therefore attributed to non specific binding of the antibody to other proteins present in the cell lysates. The main disadvantage of generating a monoclonal antibody by immunisation with a synthetic peptide is that the antibody may not recognise the full length protein. This is because an antibody produced in response to a simple linear peptide will most likely recognise a linear epitope. However, proteins form secondary and tertiary structures in their native conformation and therefore, the linear epitope recognised by the antibody produced to the peptide may not be accessible or recognisable to the monoclonal antibody when the protein is in its native conformation. However, when the protein is denatured it should revert to a linear conformation the linear sequence of the peptide used to generate the monoclonal antibody may be accessible to the monoclonal antibody and therefore recognised by the antibody. The 11 amino acid peptide sequence chosen as an immunogen to produce the polymorphism specific monoclonal antibody was predicted to be highly hydrophilic and to have a high turn tendency in the protein. Peptide sequences that have an increased likelihood of forming turns and loop structures are generally found on protein surfaces connecting other areas of secondary structure and are therefore likely to be accessible to the antibody. In addition, high predicted hydrophilicity means that the region of Lyp encompassing the R/W620 peptides is likely to be solvent exposed and thus accessible to antibody. These properties increase the chances of the producing a monoclonal anti- peptide antibody recognising a native or denatured protein. However, even after SDS-PAGE the epitopes may not have been in a linear conformation, therefore may not be recognised by the monoclonal anti-peptide antibody.

In summary, while it is relatively simple to produce monoclonal antibodies directed against synthetic peptide, it is difficult to generate monoclonal antibodies against peptides which recognise the native protein and antibodies which can distinguish two peptides differing by only one amino acid. The generation of a polymorphism specific monoclonal antibody would require the amino acid at position 620 of Lyp to be part of an epitope recognised by the

antibody producing B cell. Unfortunately, the successful generation of anti-peptide antibodies does not necessarily mean that the antibodies will recognise a protein containing the same stretch of amino acid sequence as found in the peptide. In order for this to occur the amino acid in the peptide sequence of the intact protein must be orientated to the antibody in a similar way to how the amino acid is orientated in the synthetic peptide. This orientation depends on the conformation of the peptide in solution, the conformation of the peptide sequence in the protein and the accessibility of the stretch of the peptide sequence in the protein. All of this is not impossible to achieve, however in this instance it was unsuccessful.

Chapter 4

Generation of the recombinant lentivirus cDNAs encoding isoforms of Lyp

4.1 Introduction

In vitro functional assays have suggested that the R620W polymorphism in Lyp is a gain-of function mutation, resulting in an increase in TCR signalling threshold rather than the decrease that was originally predicted (Aarnisalo et al. 2008; Rieck et al. 2007; Vang et al. 2005). Based on the notion that ^{W}Lyp may be a gain-of function polymorphism, it has been hypothesized that the ^{W}Lyp isoform suppresses TCR signalling more potently than the ^{R}Lyp form, resulting in the survival of auto-reactive cells that would normally be deleted by negative selection in the thymus. Alternatively, the impact of ^{W}Lyp on TCR signalling may be on the development and functioning of T reg cells. It may be that fewer T reg cells are generated in ^{W}Lyp expressing individuals compared to individuals who are homozygous for the ^{R}Lyp isoform. Additionally, there may be no difference in the number of T reg cells generated but their TCR signalling may be suppressed and therefore they may not be as effective in suppressing effector T cells as T reg cells expressing exclusively the ^{R}Lyp isoform. It is possible that the effect of this polymorphism may be different in distinct T cell subsets.

The ^{W}Lyp isoform behaves in a dominant manner as individuals heterozygous for this form of Lyp have an increased predisposition to autoimmune disease. Therefore, it is plausible that introducing exogenous ^{W}Lyp into homozygote wild type T-cell populations will partially mimic the situation in heterozygote individuals and allow examination of the effect of the R620W polymorphism. Over-expression studies conducted previously have used the technique of nucleofection to introduce exogenous ^{R}Lyp or ^{W}Lyp into T cells (Vang et al. 2005). Nucleofection is a non-viral electroporation based transfection method which utilises a nucleofector device that delivers unique electrical parameters in order to introduce genes directly into the nucleus of the cells being transfected. This method can be highly toxic to cells, especially T cells. The aim of this project was to study the effect of over-expression of Lyp isoforms in T cell subsets. As this would potentially involve the study of relatively small populations of cells it was considered necessary to use a non-toxic gene delivery system that

would not result in cell death. Thus, it was proposed that lentiviral based vectors be used to deliver Lyp to different primary T cell populations. Lentiviruses, a family of retroviruses, are one of the most efficient gene delivery vectors that infect dividing and non-dividing cells with high efficiency, resulting in long term stable expression of the transgene. Furthermore and of relevance to this study, lentiviruses are known to be capable of infecting primary T cells.

Before the impact of over-expression of R620 and W620 Lyp using lentiviruses could be assessed it was first necessary to generate cDNAs expressing the two isoforms of Lyp. To allow potential identification of transduced cells, truncated rat CD2 and GFP were selected as reporter genes. The rationale behind designing plasmids expressing GFP was to allow easy visual detection and estimation of transduction efficiency whilst rat CD2 would allow rapid and economical isolation of transduced cells. The reporter gene was required to be expressed from the same mRNA as the gene of interest, Lyp, therefore a self-cleaving peptide derived from a *Thosea asigna* virus (TaV) known as 2A was used for the purpose (Donnelly et al. 2001; Radcliffe and Mitrophanous 2004). The 2A technology is the most efficient multiple transgene co-expression strategy currently available. Internal ribosomal entry sites (IRESs) sequences can also be used to co-express multiple transgenes, however the large sizes of the IRESs (~ 0.5kb) and the difficulties of ensuring a well-balanced co-expresssion makes them inferior to 2A technology (de Felipe 2004; Radcliffe and Mitrophanous 2004). 2A peptides are ~19 amino acid sequences that direct its own separation from the growing polypeptide chain during translation. 2A peptides occur in many viral genomes and are critical elements in the control of their protein biogenesis (Doronina et al. 2008). The small 2A peptide interacts with the exit tunnel of the ribosome to induce the "skipping" of the last peptide bond at the C-terminal of 2A, however the ribosome is able to continue translating the second protein after releasing the first protein fused in its C-terminal to 2A (de Felipe 2004). TaV drived 2A has been found to have over 99% self processing ability (Donnelly et al. 2001).

Figure 4.1 The Thosea asigna virus 2A peptide sequence

Shown above is the 2A sequence derived from the Thosea asigna virus (TaV). The black filled arrow indicates the site of the self-processing. The 2A arrow indicates the sequence retained in the C- terminal of the upstream protein (Lyp) and the 2B arrow indicates the amino acid retained at the N-terminal of the downstream protein (GFP or truncated rat CD2).

4.2 Overview of the cloning strategy

The generation of $^{R/W}$Lyp-2A-CD2 or $^{R/W}$Lyp-2A-GFP lentivirus cDNA plasmids first required the assembly of contiguous $^{R/W}$Lyp, self-processing 2A peptide and rat CD2/GFP cDNA cassettes. The pUC19 x-2A-y and pUC19 x-2A-GFP vectors (Figures 4.2 and 4.3), previously designed and generated in the Department, were used for this purpose. It was preferable to clone the reporter gene downstream of the gene of interest, therefore the cassette was assembled so that Lyp was cloned into the "x" or *XbaI/XhoI* sites and a truncated rat CD2 or GFP reporter gene was introduced into the "y"or *KpnI/NotI* sites of the pUC19 x-2A-y plasmid. The assembled Lyp-2A-GFP/CD2 cDNA cassettes were then extracted by *BamHI* restriction digestion and cloned into the *BamHI* site of the lentivirus vector (Lenti SxW) (Figure 4.4) to generate the lentivirus plasmids encoding the two isoforms of Lyp.

4.3 Assembly of $^{R/W}$Lyp-2A-GFP/CD2 cDNA cassettes

4.3.1 Cloning of truncated rat CD2 into the "y" site of the pUC19 x-2A-y vector

The pUC19 x-2A-y plasmid (Figure 4.2) was used as a vector for the generation of the pUC19 $^{R/W}$Lyp-2A-CD2 plasmids. A truncated rat CD2 cDNA sequence was cloned into the "y" or *KpnI, NotI* site of the pUC19 x-2A-y vector to generate a pUC19 x-2A-CD2 plasmid (Figure 4.4A).To do this a rat CD2 sequence was first PCR amplified from a rat CD2 cDNA template using specific sense and anti-sense primers (Materials and Methods). The vector and the PCR amplified insert were double restriction digested using *KpnI* and *NotI* restriction enzymes and gel purified. The vector DNA was also Shrimp Alkaline Phosphatase, (SAP) treated and purified before a ligation was performed using T4 DNA ligase. The DNA was transformed into DH5α competent cells and individual ampicillin resistant colonies screened using *KpnI, NotI* restriction digestion to identify the colonies containing the recombinant plasmids. The sizes of the truncated rat CD2 sequence and the pUC19 x-2A-y vector are 612bp and 2763bp, respectively. Of the four colonies screened all four were found to be recombinant plasmids as shown by the presence of DNA fragments at 612bp representing the truncated rat CD2 cDNA and at approximately 2.8kb encoding for the pUC19 vector (Figure 4.5B).

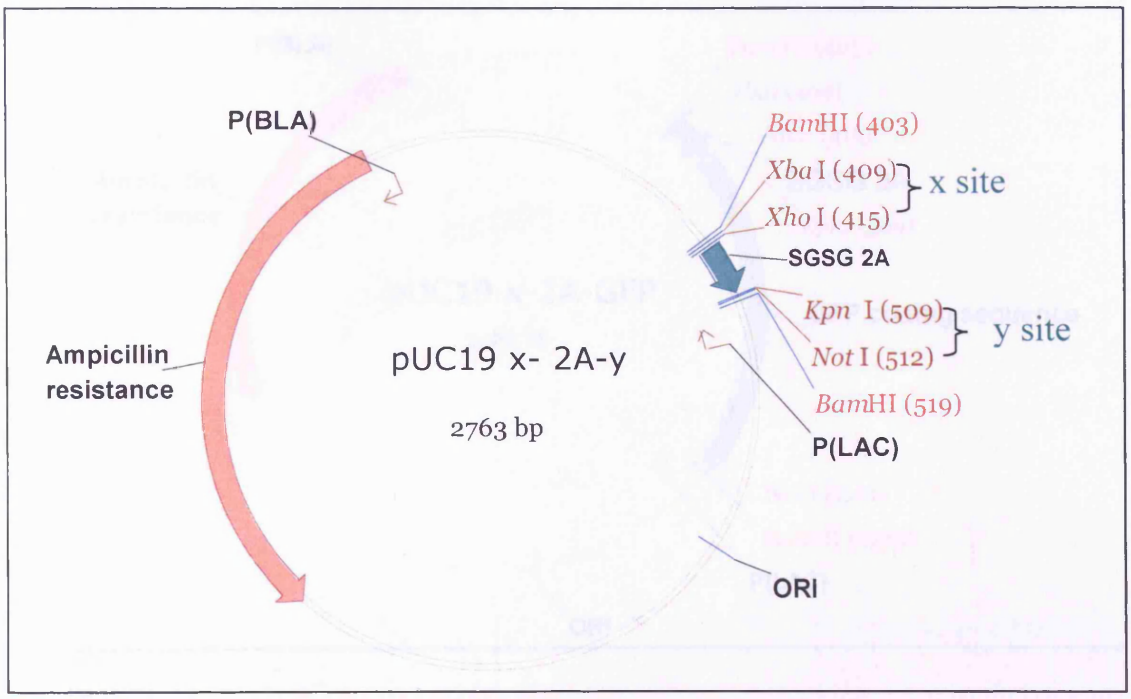


Figure 4.2 Schematic of the plasmid vector used to assemble "Lyp-2A-CD2" cassettes
The restriction sites used for cloning are shown in maroon (subcloning sites for cDNA cassette assembly) and red (flanking *BamHI* site for subsequent restriction of assembled cDNA cassettes and cloning into lentivirus plasmid vector (Lenti SxW)).

The pUC19 x-2A-y plasmid vector, containing a self processing 2A peptide sequence in between the two sets of cloning sites, was used to assemble $^{R/W}$Lyp-2A-CD2 cDNA cassettes in the pUC19 plasmid for subsequent cloning into the lentivirus plasmid. To generate the pUC19 $^{R/W}$Lyp -2A-CD2 plasmid , the Lyp cDNA was cloned into the *XbaI/XhoI* or "x" site and the truncated rat CD2 cDNA was cloned into the *KpnI/NotI* or "y" site.

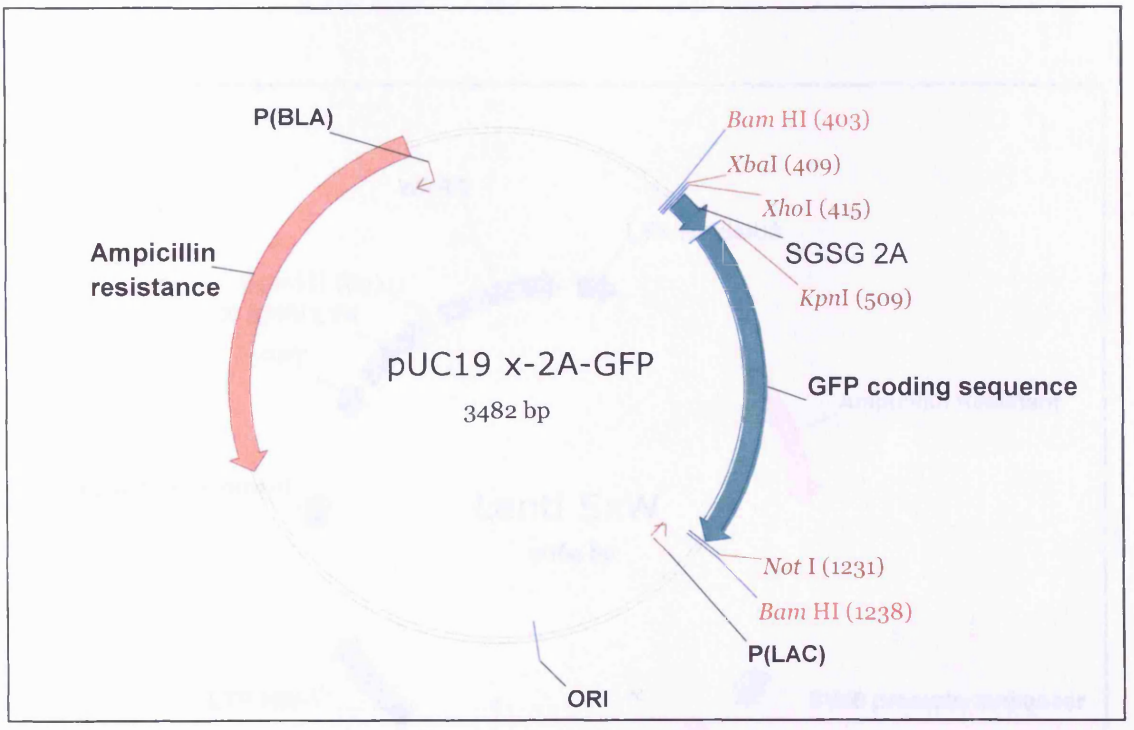


Figure 4.3 Schematic of the plasmid vector used to assemble "Lyp-2A-GFP" plasmids

A pUC19 x-2A-GFP plasmid, already containing the self processing 2A peptide and the green fluorescent protein (GFP) downstream of the 2A sequence was used for the assembly of $^{R/W}$Lyp-2A-GFP cDNA cassettes before cloning into the lentivirus plasmid.

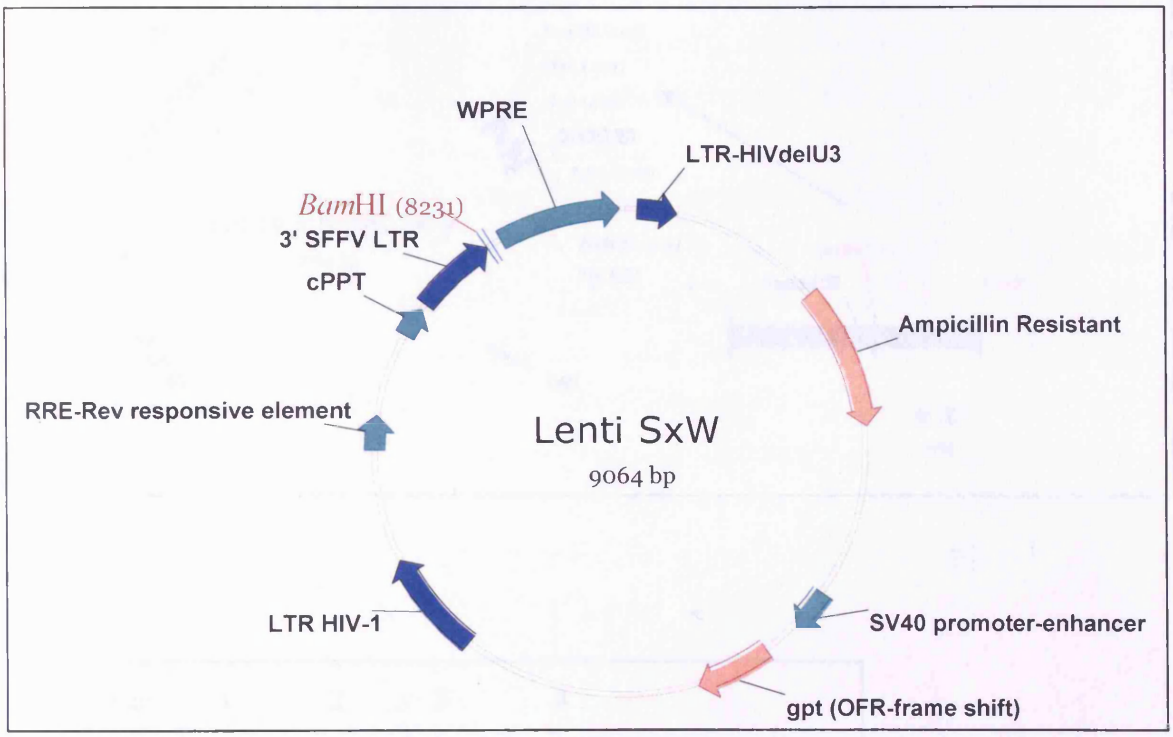
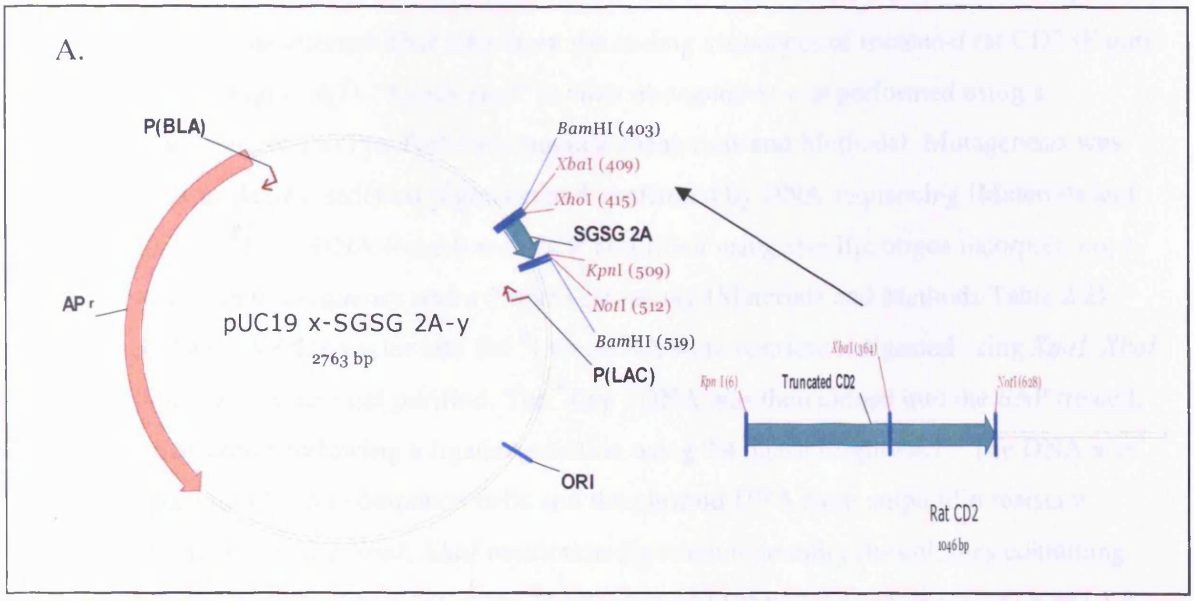


Figure 4.4 Schematic of the plasmid vector used to generate Lenti-Lyp plasmids

The $^{R/W}$Lyp-2A-CD2, $^{R/W}$Lyp-2A-GFP and GFP-2A-$^{R/W}$Lyp cDNA cassettes were each cloned into the *BamHI* site of the lentivirus plasmid vector (Lenti SxW) to generate Lenti-$^{R/W}$Lyp-2A-CD2, Lenti-$^{R/W}$Lyp-2A-GFP and Lenti- GFP-2A-$^{R/W}$Lyp cDNA plasmids respectively. In the Lenti SxW plasmid vector, Spleen focus forming virus promoter (Sffv) facilitates the transgene ($^{R/W}$Lyp-2A-CD2 or $^{R/W}$Lyp-2A-GFP or GFP-2A-$^{R/W}$Lyp) transcription and Woodchuck hepatitis regulatory element (WPRE) enhances the transcription levels of the transgene.



B.

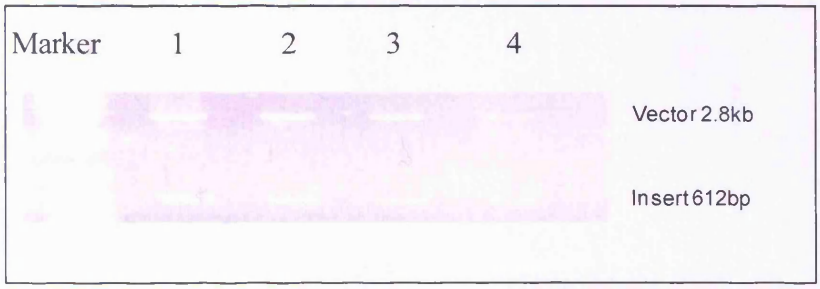


Figure 4.5 Generation of the pUC x-2A-CD2 plasmids

A. Schematic diagram showing the strategy of cloning the truncated rat CD2 cDNA into the pUC x-2A-y plasmid vector to generate a recombinant pUC x-2A-CD2 plasmid.

B. Colony screening by *NotI*, *KpnI* restriction digestion of the plasmid DNA from ampicillin resistant colonies to identify colonies containing recombinant pUC19 x-2A-CD2 plasmids. This Figure shows that all four colonies screened contain recombinant pUC x-2A-CD2 plasmids as they all contain the 612 bp rat CD2 insert.

4.3.2 Cloning of ^{R}Lyp into the "x" site of the pUC19 x-2A-CD2 vector

The cloning of a ^{R}Lyp cDNA into the "x" site of the pUC19 x-2A-CD2 vector first required the removal of the internal *XbaI* sites from the coding sequences of truncated rat CD2 (Figure 4.6) and Lyp (Figure 4.7). "Quick step" *in vitro* mutagenesis was performed using a Stratagene protocol and Pfu Turbo polymerase (Materials and Methods). Mutagenesis was checked by an *XbaI* restriction digestion and confirmed by DNA sequencing (Materials and Method). The ^{R}Lyp cDNA (insert) was PCR amplified using specific oligos incorporating a 5'Kozak consensus sequence and a 3'myc epitope tag (Materials and Methods Table 2.2). The pUC19 x-2A-CD2 vector and the ^{R}Lyp cDNA were restriction digested using *XbaI*, *XhoI* restriction enzymes and gel purified. The ^{R}Lyp cDNA was then cloned into the SAP treated, gel purified vector following a ligation reaction using T4 ligase (Figure 4.7). The DNA was transformed into DH5α competent cells and the plasmid DNA from ampicillin resistant colonies screened using *XbaI*, *XhoI* restriction digestion to identify the colonies containing recombinant plasmids. The sizes of the ^{R}Lyp insert and the pUC19 vector are 2.6kb and 3.4 kb, respectively. Of the eight colonies screened only colony number four contains recombinant pUC19 ^{R}Lyp-2A-CD2 plasmid (Figure 4.8).

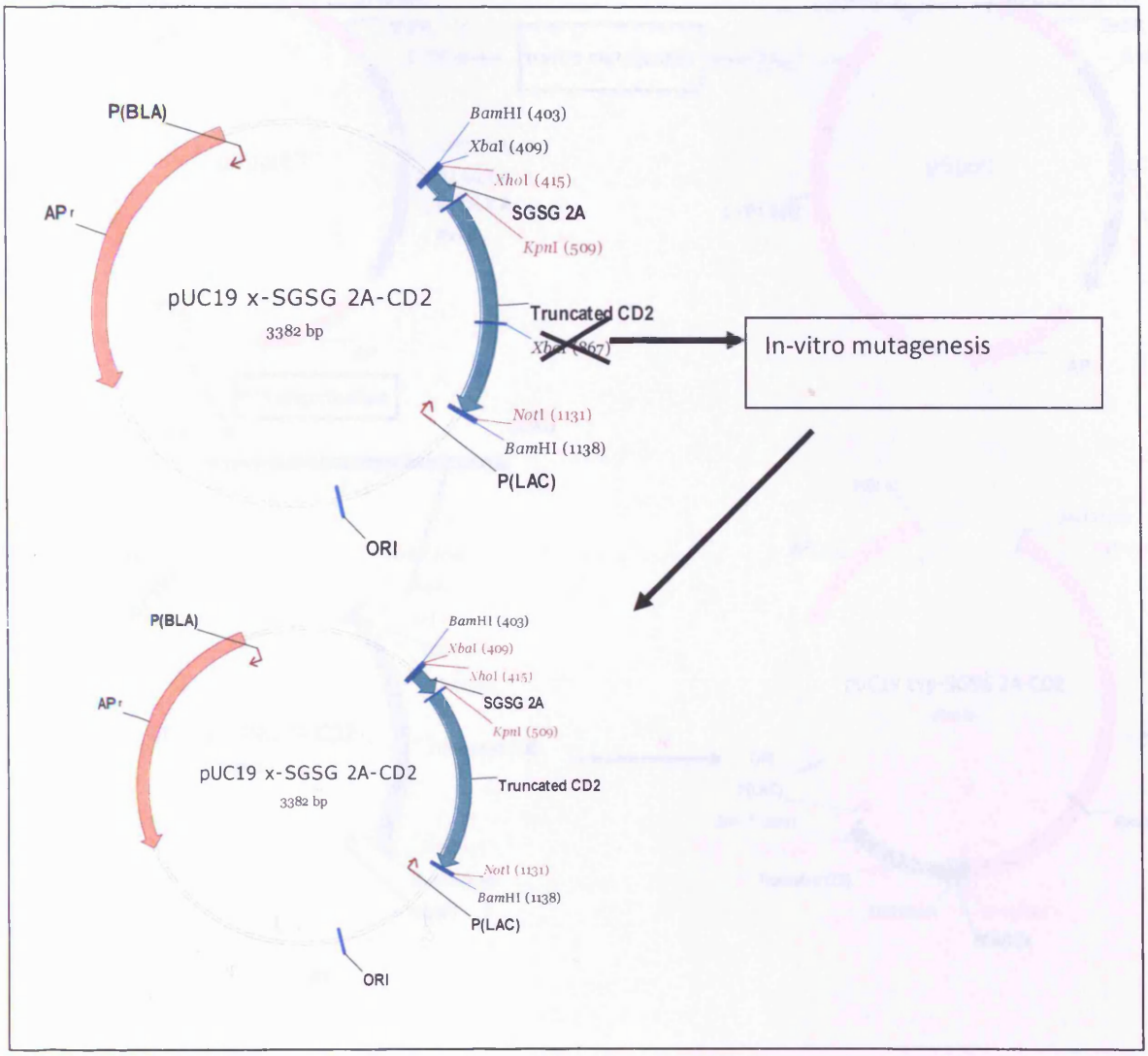


Figure 4.6 Strategy for deletion of the internal *XbaI* site

Schematic diagram showing the removal of the internal *XbaI* site from the rat CD2 cDNA sequence. The internal *XbaI* site from the rat CD2 cDNA needed to be removed to facilitate the cloning of the upstream gene, Lyp, into the *XbaI, XhoI* site of the pUC x-2A-CD2 plasmid. The internal *XbaI* site from the rat CD2 cDNA was removed by quick step *in-vitro* mutagenesis.

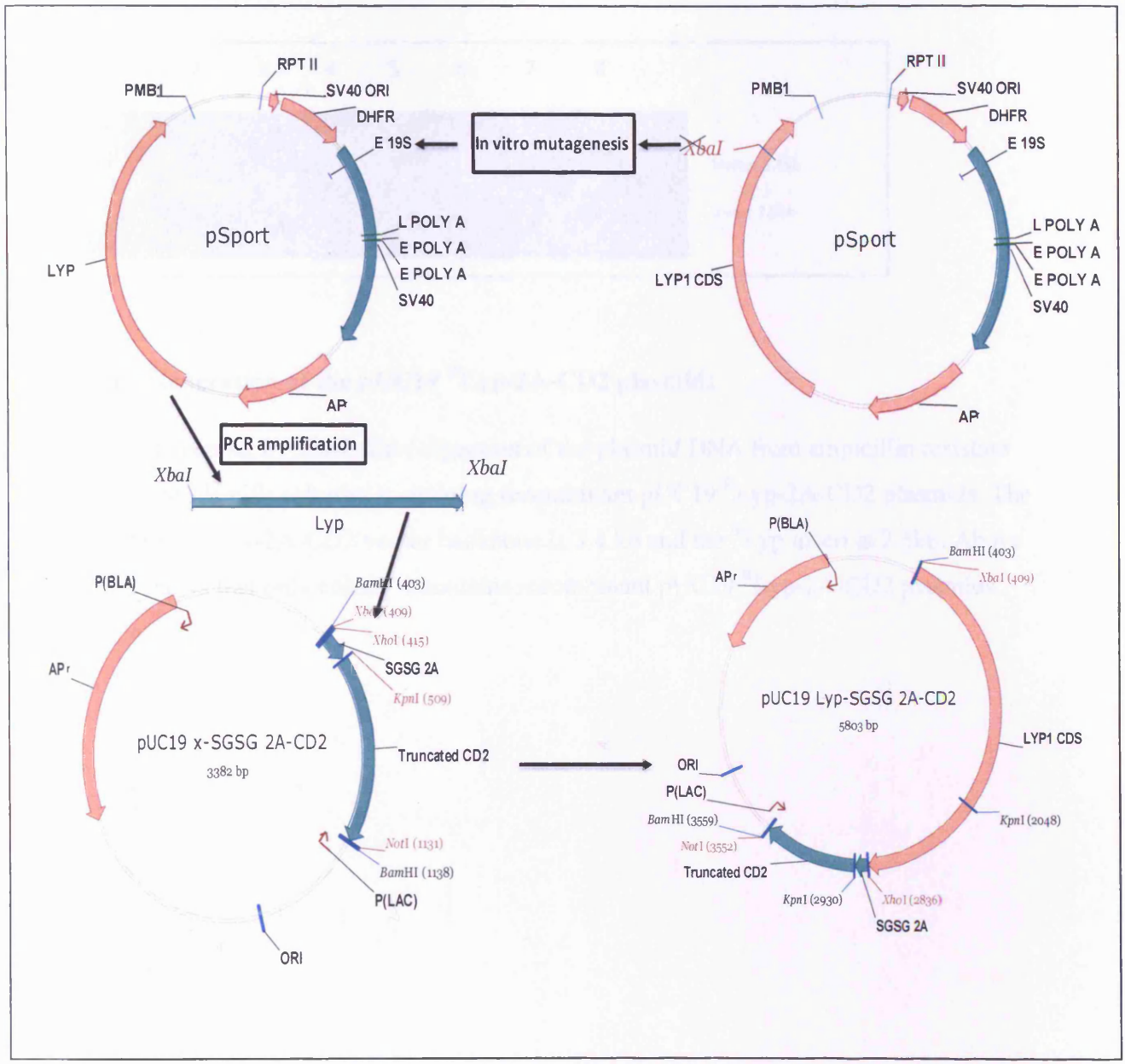


Figure 4.7 Cloning strategy for the generation of the pUC19 ^{R}Lyp-2A-CD2 plasmid

Schematic diagram showing the strategic removal of internal *XbaI* site from the ^{R}Lyp cDNA, followed by PCR amplification of the ^{R}Lyp cDNA and subsequently cloning of the mutated ^{R}Lyp into the *XbaI*, *XhoI* site of the pUC19 x-2A-CD2 vector (top right to bottom right) to generate recombinant pUC19 ^{R}Lyp-2A-CD2 plasmid.

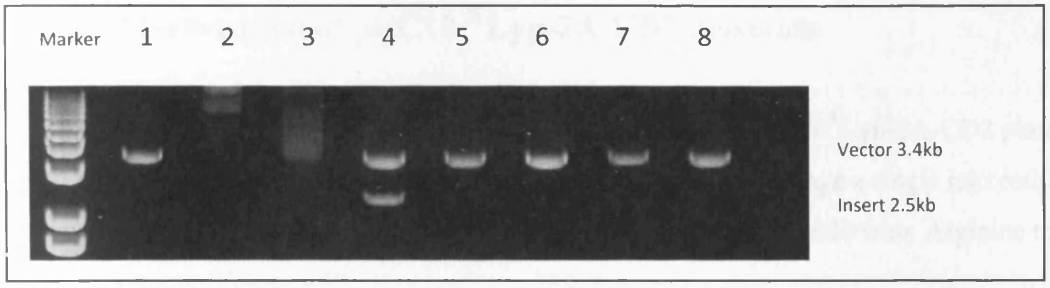


Figure 4.8 Generation of the pUC19 ^{R}Lyp-2A-CD2 plasmids

Colony screening by *XbaI*, *XhoI* digestion of the plasmid DNA from ampicillin resistant colonies to identify colonies containing recombinant pUC19 ^{R}Lyp-2A-CD2 plasmids. The size of the pUC x-2A-CD2 vector backbone is 3.4 kb and the ^{R}Lyp insert is 2.5kb. Above Figure shows that only colony 4 contains recombinant pUC19 ^{R}Lyp-2A-CD2 plasmids.

4.3.3 Generation of pUC19 ^{W}Lyp-2A-CD2 plasmids

The pUC19 ^{W}Lyp-2A-CD2 plasmid was generated from pUC19 ^{R}Lyp-2A-CD2 plasmid using Quick step *in vitro* mutagenesis (Materials and Method) to change a single nucleotide at position 1858 which in turn changes the amino acid at position 620 from Arginine to Tryptophan (Figure 4.9).

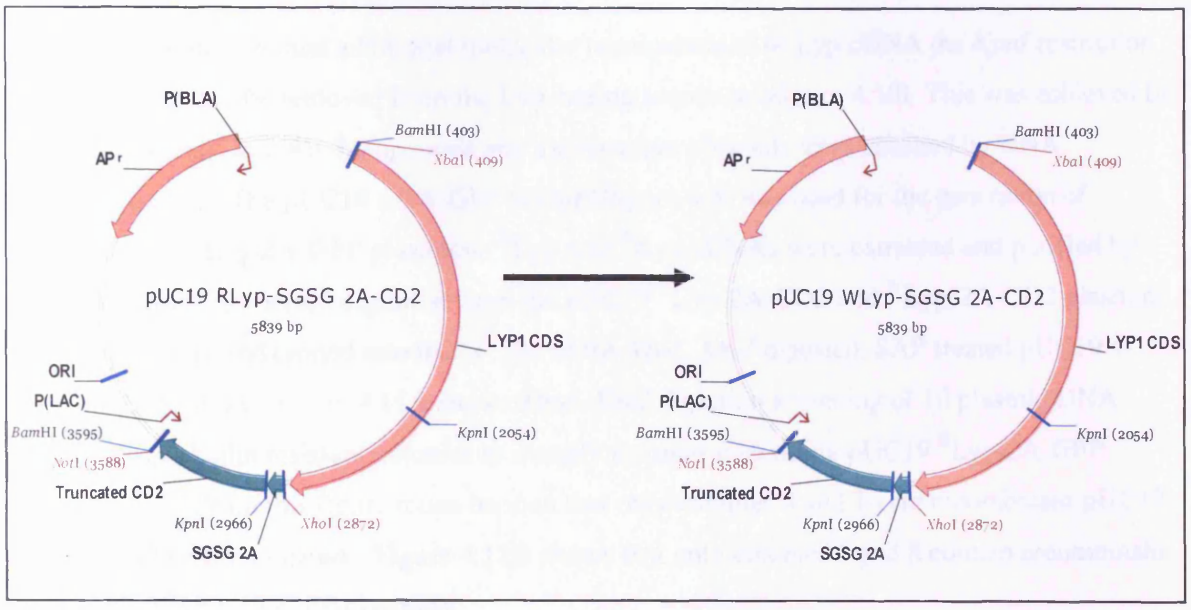


Figure 4.9 Schematic diagrams showing the generation of pUC19 ^{W}Lyp-2A-CD2 plasmids

Schematic diagram showing the generation of pUC19 ^{W}Lyp-2A-CD2 plasmid from the pUC19 ^{R}Lyp-2A-CD2 plasmid by using site directed in vitro mutagenesis to change the Arginine at amino acid position 620 of Lyp to Tryptophan.

4.3.4 Generation of the pUC19 $^{R/W}$Lyp -2A-GFP plasmids

To facilitate potential additional molecular manipulations of Lyp cDNA the *KpnI* restriction site needed to be removed from the Lyp coding sequence (Figure 4.10). This was achieved by "Quick step" *in vitro* mutagenesis and the resultant plasmids were checked by DNA sequencing. The pUC19 x-2A-GFP vector (Figure 4.3) was used for the generation of pUC19 $^{R/W}$Lyp-2A-GFP plasmids. ^{R}Lyp and ^{W}Lyp cDNAs were extracted and purified by *XbaI*, *XhoI* restriction digestion from the pUC19 ^{R}Lyp-2A-CD2 and ^{W}Lyp-2A-CD2 plasmids, respectively and cloned into the 'x' site of the *XbaI*, *XhoI* digested, SAP treated pUC19 x-2A-GFP vector. Figure 4.11A shows *XbaI*, *XhoI* digestion screening of 10 plasmid DNA from ampicillin resistant colonies to identify colonies containing pUC19 ^{R}Lyp-2A-GFP plasmids. From this figure it can be seen that only colonies 4 and 10 are recombinant pUC19 ^{R}Lyp-2A-GFP plasmids. Figure 4.11B shows that only colonies 1 and 8 contain recombinant pUC19^{W}Lyp-2A-GFP plasmids.

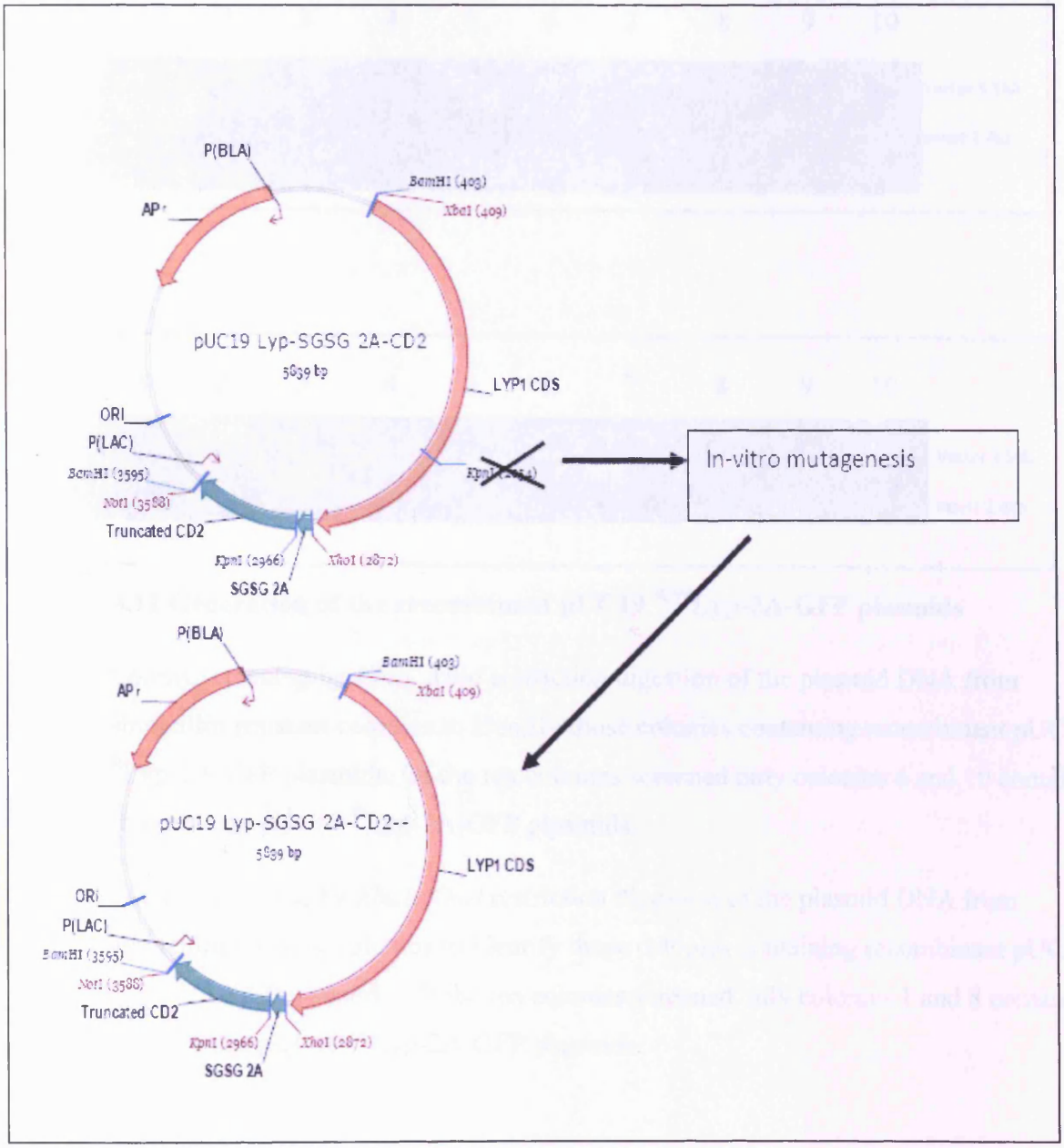


Figure 4.10 Strategy for *KpnI* site removal from Lyp sequence

Schematic diagram showing the strategic removal of internal *KpnI* site from the Lyp cDNA by site directed *in vitro* mutagenesis to generate a *KpnI* mutant Lyp for easier future manipulations.

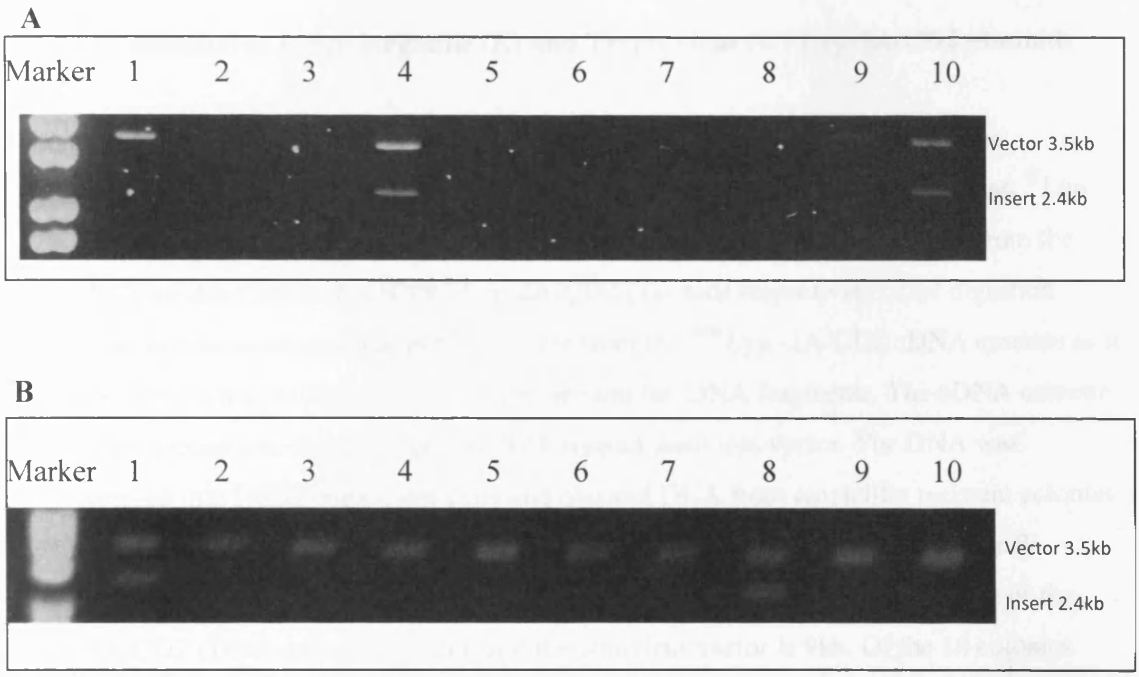


Figure 4.11 Generation of the recombinant pUC19 $^{R/W}$Lyp-2A-GFP plasmids

A. Colony screening by *XbaI*, *XhoI* restriction digestion of the plasmid DNA from ampicillin resistant colonies to identify those colonies containing recombinant pUC19 ^{R}Lyp-2A-GFP plasmids. Of the ten colonies screened only colonies 4 and 10 contains recombinant pUC19 ^{R}Lyp-2A-GFP plasmids.

B. Colony screening by *XbaI*, *XhoI* restriction digestion of the plasmid DNA from ampicillin resistant colonies to identify those colonies containing recombinant pUC19 ^{W}Lyp-2A-GFP plasmids. Of the ten colonies screened only colonies 1 and 8 contains recombinant pUC19 ^{W}Lyp-2A-GFP plasmids.

4.4 Generation of the lentivirus plasmids

4.4.1 Generation of Lenti- Arginine (R) and Tryptophan (W) Lyp-2A-CD2 plasmids

Lentivirus vector (Lenti-Sffv-x-WPRε or Lenti SxW) (Figure 4.4) was used for the generation of the Lenti-$^{R/W}$Lyp-2A-CD2 plasmids (Figure 4.12). ^{R}Lyp-2A-CD2 and ^{W}Lyp-2A-CD2 cassettes were extracted and purified by *BamHI, AseI* double digestion from the pUC19 ^{R}Lyp-2A-CD2 and pUC19 ^{W}Lyp-2A-CD2 plasmids respectively. *AseI* digestion allows a clear separation of the pUC19 vector from the $^{R/W}$Lyp -2A-CD2 cDNA cassette as it further restricts the pUC19 plasmid vector into smaller DNA fragments. The cDNA cassettes were then cloned into *BamHI* digested, SAP treated lentivirus vector. The DNA was transformed into DH5α competent cells and plasmid DNA from ampicillin resistant colonies was screened using a *BamHI* digestion to identify recombinant colonies and an *EcoRI* digestion was performed to determine the orientation of the cloned insert. The size of the Lyp-2A-CD2 cDNA cassette is 3.2kb and the lentivirus vector is 9kb. Of the 10 colonies screened using *BamHI* digestion, 1-9 were recombinant Lenti-^{R}lyp-2A-CD2 colonies, whereas colony no 10 contained a self-ligated vector only (Figure 4.13A). *EcoRI* digestion was performed to check the orientation of the insert cloned in the vector. If the insert had been cloned in the correct orientation, DNA fragments of sizes 139bp, 2283bp and 9326bp would have been expected whereas cloning in the incorrect orientation would have given DNA fragments of 139bp, 1786bp and 9823bp. From Figure 4.13B it can be seen that only colony 4 contained plasmid DNA whereby the cDNA cassette has been inserted in the correct orientation as it is the only clone that has a plasmid that releases a DNA fragment at 2283bp upon *EcoRI* digestion. Figure 4.13A shows that all 6 colonies screened contained recombinant Lenti ^{W}Lyp-2A-CD2 cDNAs and from Figure 4.13B it can be deduced that colonies 1, 4 and 6 contain plasmid DNA whereby the cDNA cassette has inserted in the correct orientation.

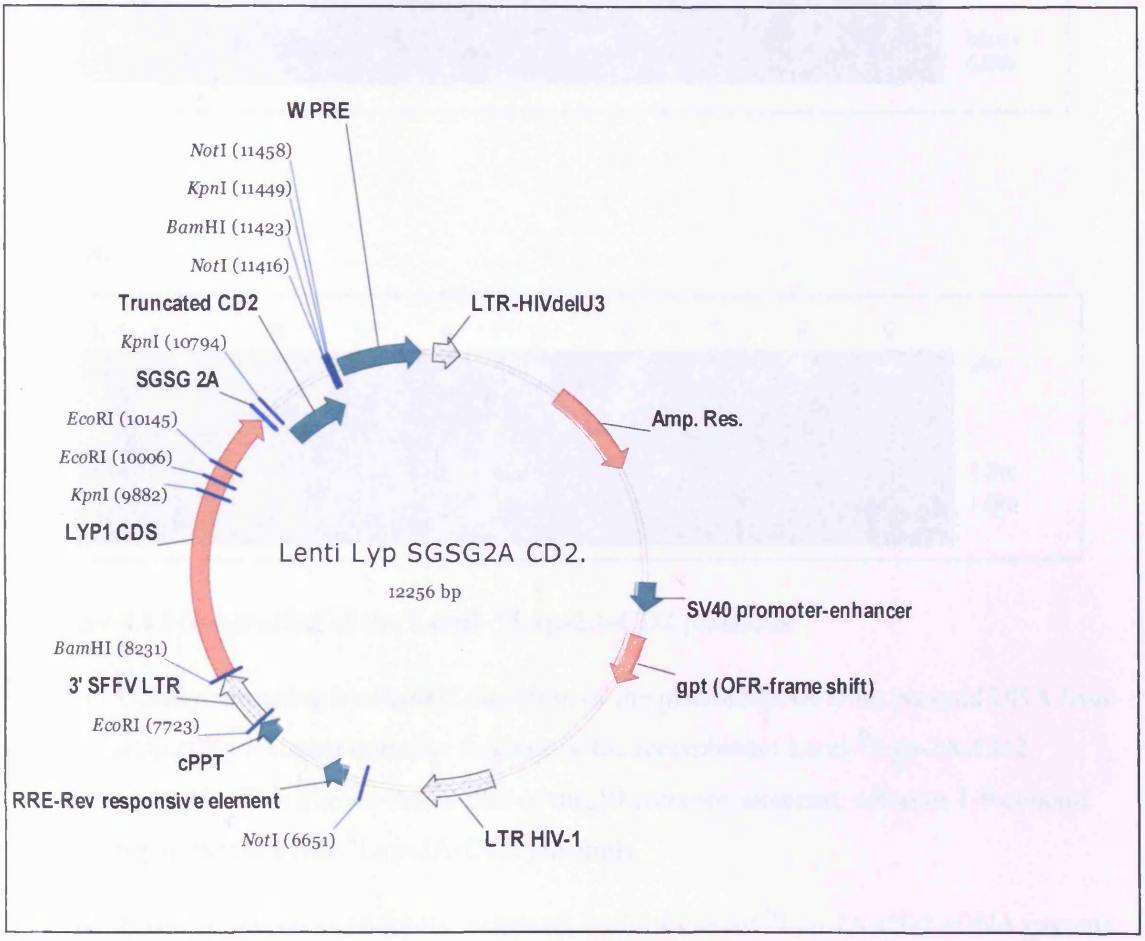
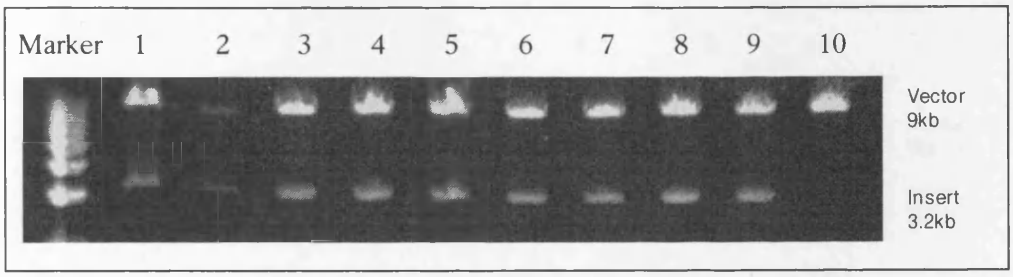


Figure 4.12 Generation of the Lenti-$^{R/W}$Lyp-2A-CD2 plasmids

Schematic representation of the Lenti-Lyp-2A-CD2 plasmid. The $^{R/W}$Lyp-2A-CD2 cDNA cassettes were extracted from the pUC $^{R/W}$Lyp-2A-CD2 recombinant plasmids by *BamHI* restriction digestion and cloned into the *BamHI* site of the lentivirus plasmid vector (Lenti SxW) to generate Lenti-$^{R/W}$Lyp-2A-CD2 plasmids. In this plamid vector, Spleen focus forming virus promoter (Sffv) facilitates the transgene, $^{R/W}$Lyp-2A-CD2, transcription and Woodchuck hepatitis regulatory element (WPRE) enhances the transcription levels of the transgene.

A.



B.

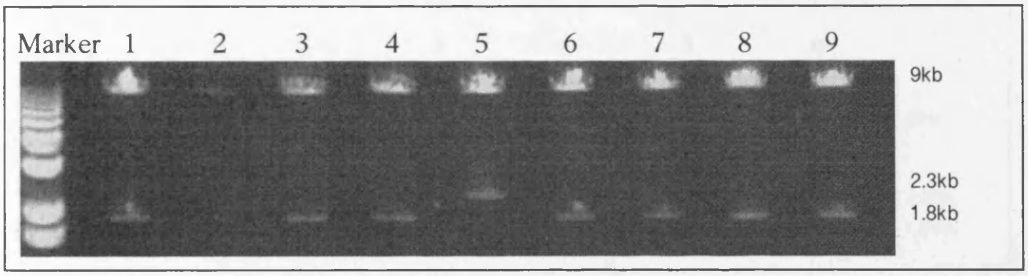
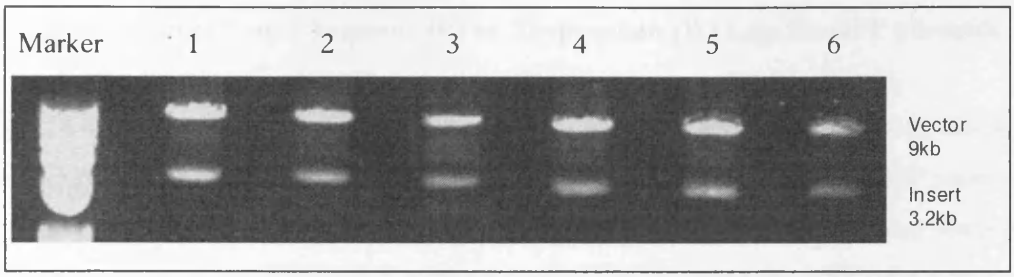


Figure 4.13 Generation of the Lenti- ^{R}Lyp-2A-CD2 plasmids

A. Colony screening by *BamHI* digestion of the plasmid DNA from plasmid DNA from ampicillin resistant colonies to identify the recombinant Lenti-^{R}Lyp-2A-CD2 plasmids. This Figure shows that of the 10 colonies screened, colonies 1-9 contain recombinant Lenti-^{R}Lyp-2A-CD2 plasmids.

B. *EcoRI* digestion to check the orientation of the cloned ^{R}Lyp-2A-CD2 cDNA cassette in the lentivirus vector. Cloning in the correct orientation produces an indicative DNA fragment at 2283bp whereas cloning in the incorrect orientation produces a DNA fragment at 1786bp. This Figure shows that only colony 5 contains plasmids with ^{R}Lyp-2A-CD2 cDNA cassette in the correct orientation.

A.



B.

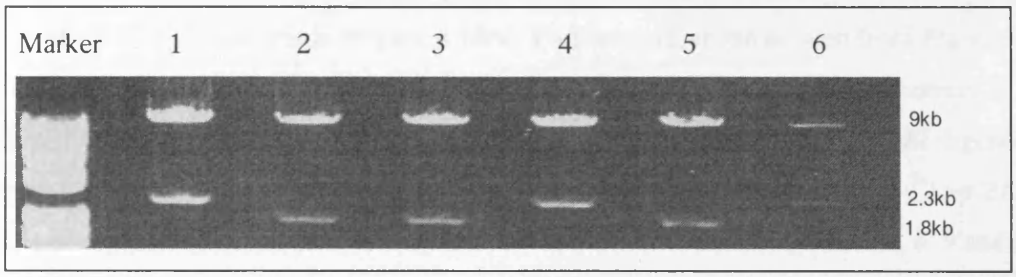


Figure 4.14 Generation of the Lenti-^{W}Lyp-2A-CD2 plasmids

A. Colony screening by *BamHI* digestion of the plasmid DNA from plasmid DNA from ampicillin resistant colonies to identify the recombinant Lenti-^{W}Lyp-2A-CD2 plasmids. This Figure shows that of the 6 colonies screened, all contain recombinant Lenti-^{W}Lyp-2A-CD2 plasmids.

B. *EcoRI* digestion to check the orientation of the cloned ^{W}Lyp-2A-CD2 cDNA cassette in the lentivirus vector. Cloning in the correct orientation produces an indicative DNA fragment at 2283bp whereas cloning in the incorrect orientation produces a DNA fragment at 1786bp. Above Figure shows that only colonies 1, 4 and 6 contain plasmids with ^{W}Lyp-2A-CD2 cDNA cassette in the correct orientation.

4.4.2 Generation of Lenti- Arginine (R) or Tryptophan (W) Lyp-2A-GFP plasmids

^{R}Lyp-2A-GFP and ^{W}Lyp-2A-GFP cDNA cassettes were extracted and purified by *BamHI*, *AseI* double digestion from the pUC19 ^{R}Lyp-2A-GFP and pUC19 ^{W}Lyp-2A-GFP plasmids respectively. The cDNA cassettes were cloned into *BamHI* digested, SAP treated lentivirus vector to generate Lenti $^{R/W}$Lyp-2A-GFP/CD2 plasmids (Figure 4.15). The DNA was transformed into DH5α competent cells and colonies screened using *BamHI* digestion to identify recombinant colonies and *EcoRI* digestion to check the orientation of the cloned insert. Of the 10 colonies screened using *BamHI* digestion, 2 to 10 contained recombinant Lenti-^{R}Lyp-2A-GFP plasmids (Figure 4.16A). Furthermore, it can be seen from Figure 4.16B that clones 4, 5, 9 and 10 contained plasmids with cDNA inserts cloned in the correct orientation as indicated by the release of a DNA fragment of 2283bp upon *EcoRI* digestion. Figure 4.17A shows that all 10 colonies screened contained recombinant Lenti-^{W}Lyp-2A-GFP plasmids and from Figure 4.17B it can be deduced that colonies 1, 3, 4, 7, 8, 9 and 10 contained plasmids with cDNA inserts cloned in the correct orientation.

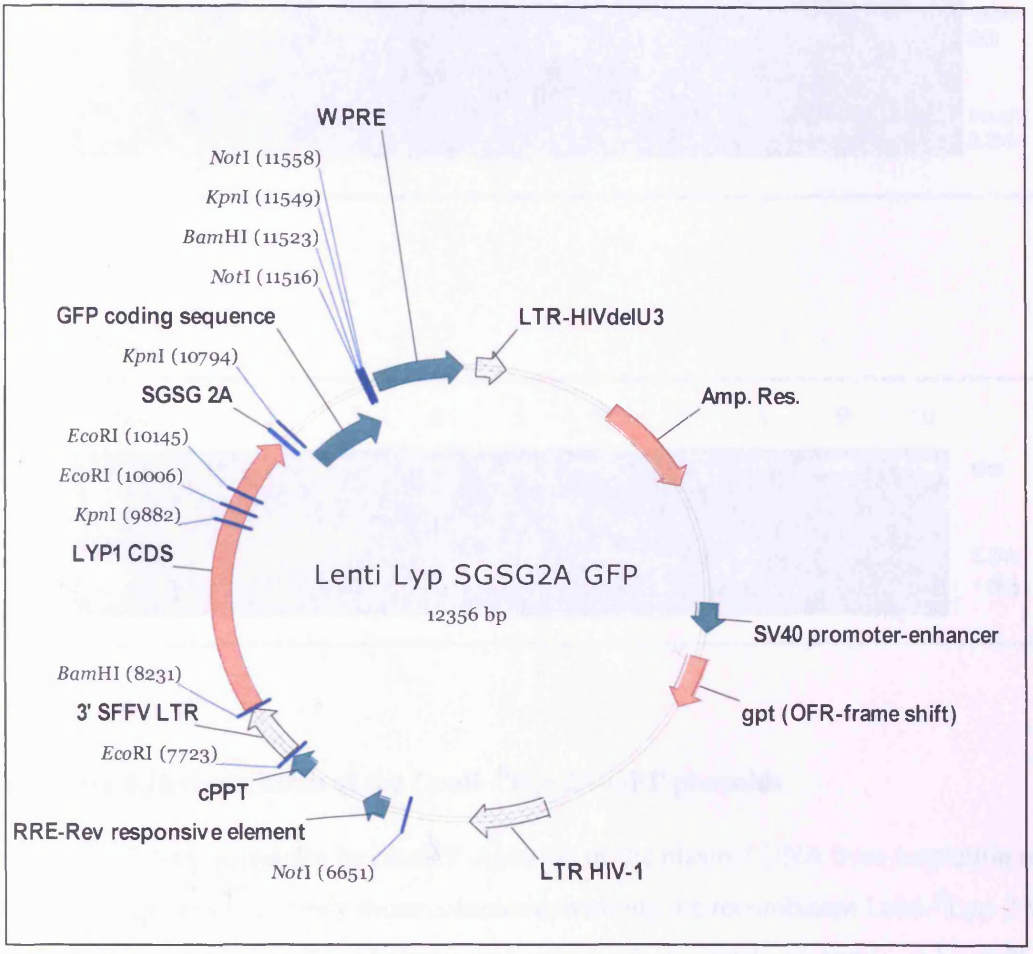
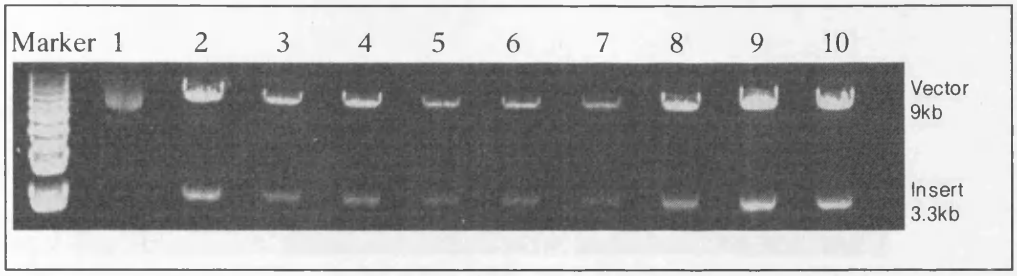


Figure 4.15 Schematic diagram showing the design of the Lentivirus $^{R/W}$Lyp-2A-GFP plasmid

The $^{R/W}$Lyp-2A-GFP cDNA cassettes were extracted from the pUC $^{R/W}$Lyp-2A-GFP recombinant plasmids by *BamHI* restriction digestion and cloned into the *BamHI* site of the lentivirus plasmid vector (Lenti SxW) to generate Lenti-$^{R/W}$Lyp-2A-GFP plasmids. In this plamid vector, Spleen focus forming virus promoter (Sffv) facilitates the transgene, $^{R/W}$Lyp-2A-GFP, transcription and Woodchuck hepatitis regulatory element (WPRE) enhances the transcription levels of the transgene.

A.



B.

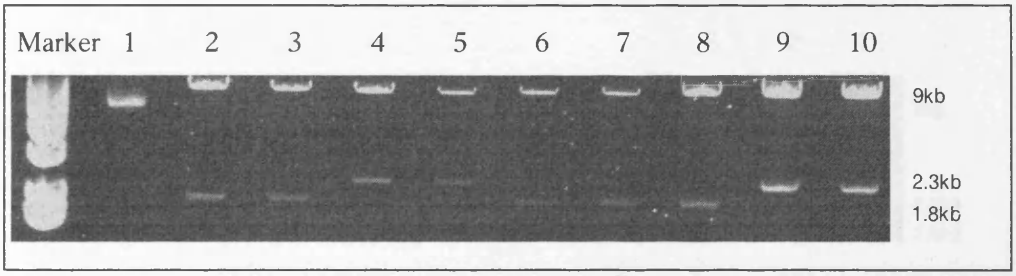
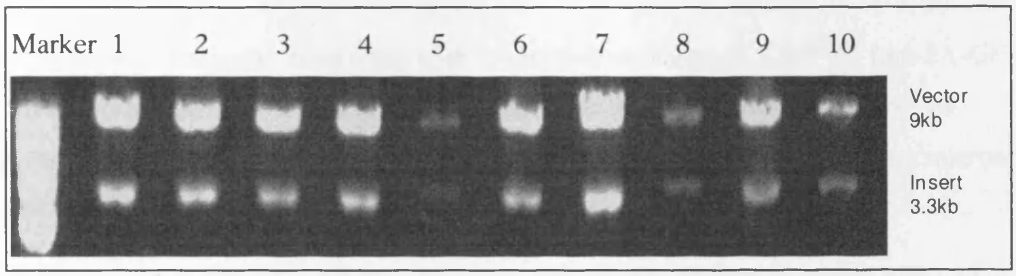


Figure 4.16 Generation of the Lenti-^{R}lyp-2A-GFP plasmids

A. Colony screening by *BamHI* digestion of the plasmid DNA from ampicillin resistant colonies to identify those colonies containing the recombinant Lenti-^{R}Lyp-2A-GFP plasmids. This Figure shows that colonies 2-10 contain recombinant Lenti-^{R}Lyp-2A-GFP plasmids.

B. *EcoRI* digestion to check the orientation of the cloned ^{R}Lyp-2A-GFP cDNA cassette in the lentivirus vector. Cloning in the correct orientation is indicated by the release of a DNA fragment of 2283bp. Above Figure shows that colonies 4, 5, 9, and 10 contain plasmids with ^{R}Lyp-2A-GFP cDNA cassette in the correct orientation.

A.



B.

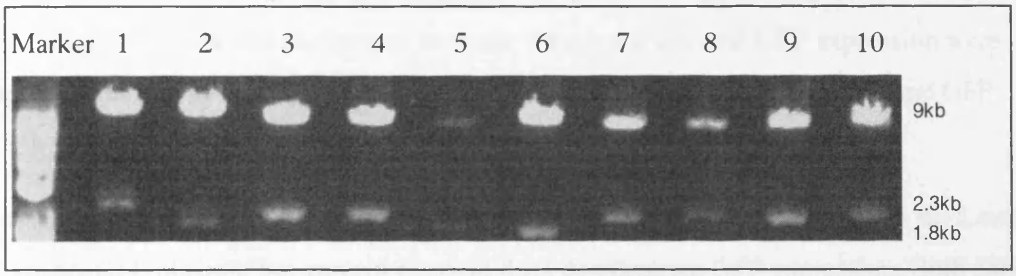


Figure 4.17 Generation of the Lenti-^{W}Lyp-2A-GFP cDNAs

A. Colony screening by *BamHI* digestion of the plasmid DNA from the plasmid DNA from ampicillin resistant colonies to identify those colonies containing recombinant Lenti-^{W}Lyp-2A-GFP plasmids. This Figure shows that all the colonies screened contain the recombinant Lenti-^{W}Lyp-2A-GFP plasmids.

B. *EcoRI* digestion to check the orientation of the cloned ^{W}Lyp-2A-GFP cDNA cassette in the lentivirus vector. Cloning in the correct orientation releases an indicative DNA fragment at 2283bp whereas cloning in the incorrect orientation releases a DNA fragment at 1786bp. From this Figure it can be seen that colonies 1, 3, 4, 7, 8, 9 and 10 contain plasmids with ^{W}Lyp-2A-GFP cDNA cassette in the correct orientation.

4.5 Testing of the Lenti Lyp-2A-GFP plasmids

293T cells were transient transfected with the lentivirus plasmids, Lenti $^{R/W}$ Lyp-2A-GFP, to confirm expression of $^{R/W}$Lyp and GFP. Transfection efficiency, as indicated by the expression of GFP, was checked by both visualising the cells using fluorescence microscopy and by flow cytometry.

There was a very low level of GFP expression observed in 293T cells transfected with the Lenti ^{W}Lyp-2A-GFP plasmid compared to the positive control (Lenti SeW) plasmid (Figure 4.18A & 4.18B). The positive control, Lenti SeW is the same lentivirus plasmid as the one used to generate the Lentivirus Lyp plasmids but with eGFP cloned into the *BamHI* site. Immunoblot analysis was performed to check if both the Lyp and GFP expression were impaired and/or whether cleavage was taking place between the 2A sequence and GFP (Figure 4.19).

Immunoblot blot data shows some Lyp expression and no GFP expression with the Lenti-^{W}Lyp-2A-GFP plasmid but control plasmid does demonstrate GFP expression. Both control Lenti-GFP and Lenti-Lyp-2A-GFP are driven by the same Spleen Focus Forming Virus (SFFV) promoter known to be efficient in driving expression in T cells and 293T cells. First interpretation of this result may be that Lyp is being expressed but not GFP but this discrepancy in results could be due to the affinity of the antibodies rather than differential expression of the two proteins. Therefore, expression of both the proteins was analaysed using an "*in vitro* coupled transcription translation" in collaboration with Prof Martin Ryan at the University of St Andrews. The pGEM 4Z Lyp-2A-GFP and Lyp-2A-CD2 plasmids were generated by myself and sent to Prof Martin Ryan, University of St Andrews where the "*in vitro* coupled transcription translation" analysis was carried out.

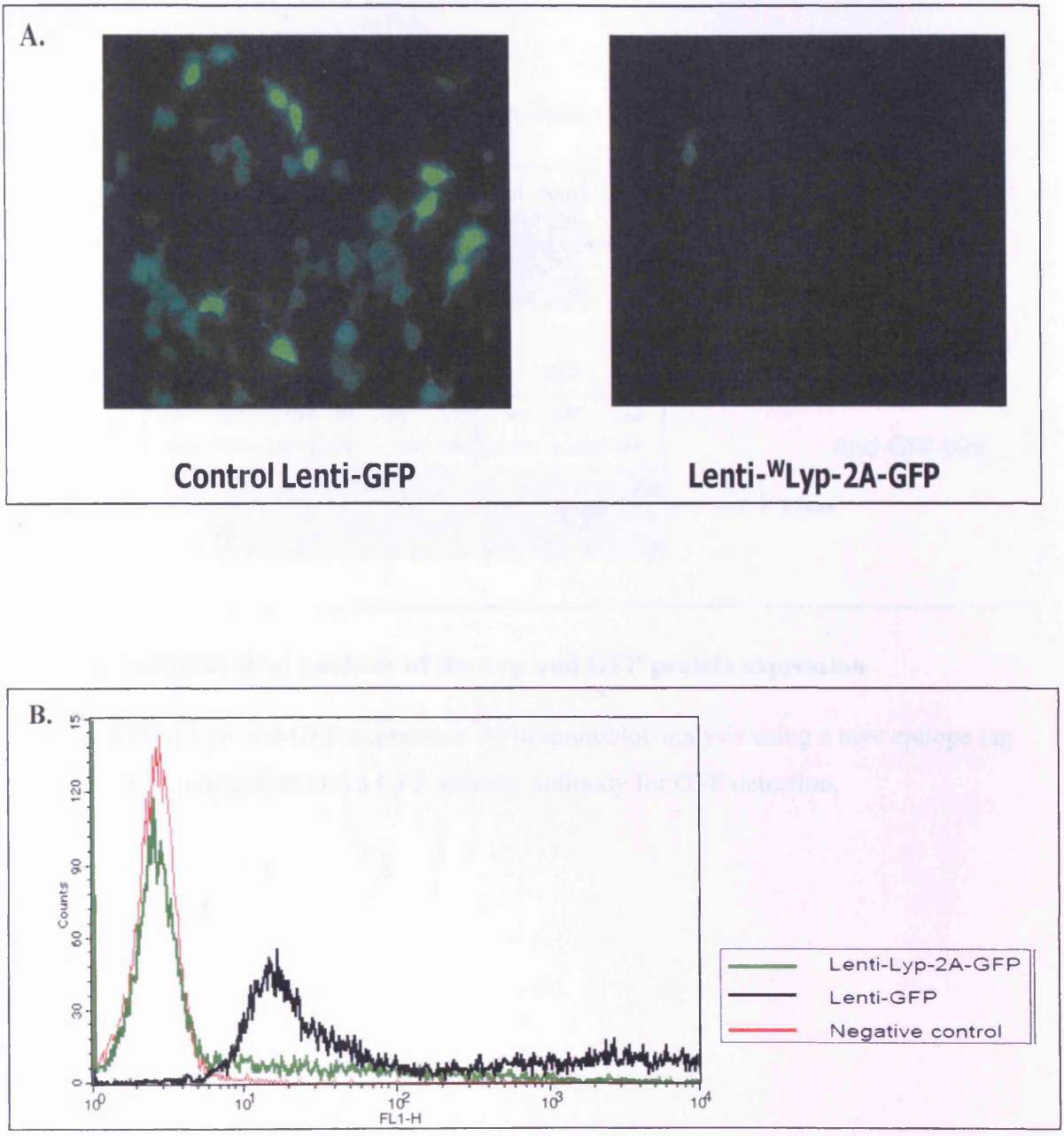


Figure 4.18 Testing of the Lenti Lyp -2A-GFP plasmids

A. Fluorescent microscopic analysis of GFP expression by 293 T cells following transient transfection with Lenti-^{W}Lyp-2A-GFP plasmid.

B. Flow cytometric analysis of GFP expression by 293T cells following transient transfection with Lenti-^{W}Lyp-2A-GFP plasmid.

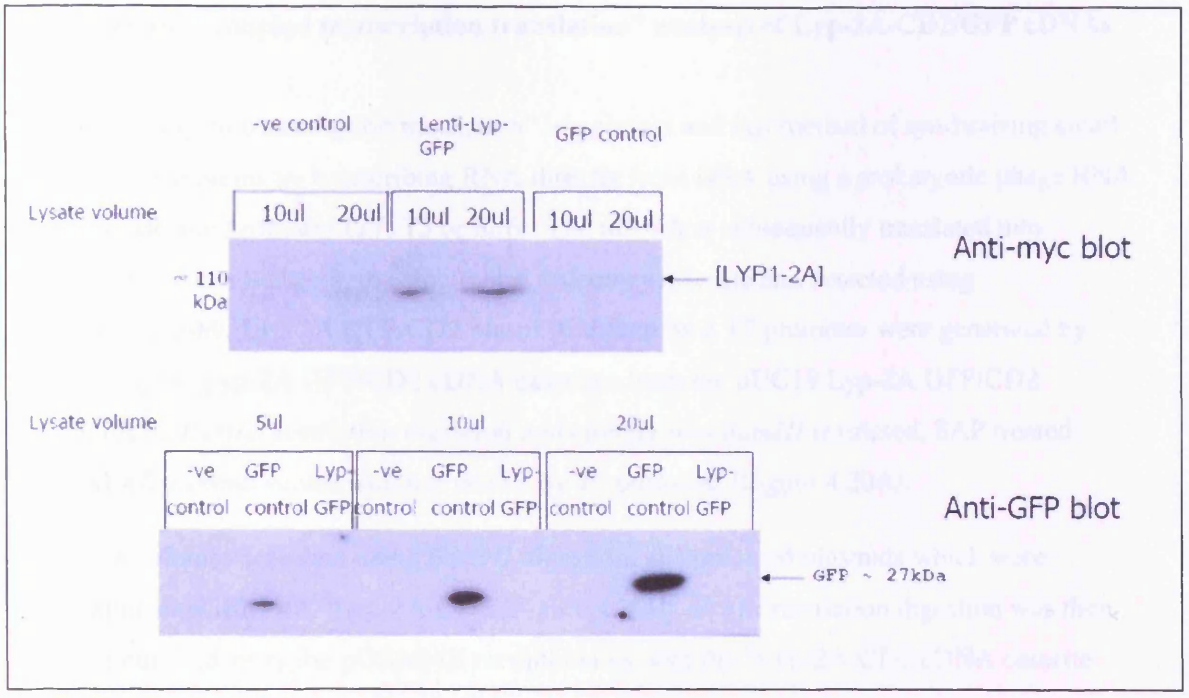


Figure 4.19 Immunoblot analysis of the Lyp and GFP protein expression

Confirmation of Lyp and GFP expression by immunoblot analysis using a myc epitope tag antibody for Lyp detection and a GFP specific antibody for GFP detection.

4.5.1 "*In vitro* coupled transcription translation" analysis of Lyp-2A-CD2/GFP cDNAs

"*In vitro* coupled transcription translation" is a simple and fast method of synthesizing small amounts of proteins by transcribing RNA directly from DNA using a prokaryotic phage RNA polymerase and promoter (T7, T3 or SP6). The mRNA is subsequently translated into radioactively labelled protein using rabbit reticulocyte lysate and detected using autoradiography. Lyp-2A GFP/CD2 plasmids driven by a T7 promoter were generated by extracting the Lyp-2A GFP/CD2 cDNA cassettes from the pUC19 Lyp-2A GFP/CD2 plasmids by *BamHI* restriction digestion and cloning into *BamHI* restricted, SAP treated pGEM-4Z plasmid vector which is driven by T7 promoter (Figure 4.20A).

Of the 8 colonies screened using *BamHI* digestion, all contained plasmids which were recombinant pGEM-4Z ^{R}Lyp-2A-CD2 (Figure 4.20B). *EcoRI* restriction digestion was then carried out to identify the pGEM-4Z recombinants with the ^{R}Lyp-2A-CD2 cDNA cassette cloned in the correct orientation. If the insert was cloned in the correct orientation DNA fragments of 139bp, 1386bp and 4513bp were expected whereas cloning in the incorrect orientation was expected to release DNA fragments of 139bp, 1796bp and 4103bp upon *EcoRI* digestion. It can be seen from Figure 4.20C that clones 1, 2 and 5 have plasmids with inserts that have been inserted in the correct orientation releasing a DNA fragment of 1386bp upon *EcoRI* digestion. Of the 10 colonies screened using *BamHI* digestion, 2, 4, 6 and 10 contained recombinant pGEM-4Z ^{W}Lyp-2A-GFP plasmids (Figure 4.21A). It can be seen from Figure 4.21B that clones 4, 6 and 10 have plasmids with the ^{W}Lyp-2A-GFP cDNA cassette inserted in the correct orientation as shown by the release of a DNA fragment of 1386bp upon *EcoRI* digestion.

"*In vitro* transcription translation" analysis showed no major differences in the expression levels of Lyp and GFP (Figure 4.22) suggesting that both Lyp and GFP were being expressed. There were also no Lyp-GFP/CD2 fusion protein detected suggesting an almost 100% self processing of the 2A peptide (Figure 4.22). However in addition there seemed to be several Lyp internal initiation products being synthesised indicating that the problem may not be with the cleavage between the two proteins, or continuation of translation after the 2A sequence but the initiation of translation at the 5' end of Lyp mRNA. It seems that the ribosome may not able to efficiently start translation at the first 'in frame' start codon suggesting that the Kozak consensus sequence that has been used may not be optimal for the purpose.

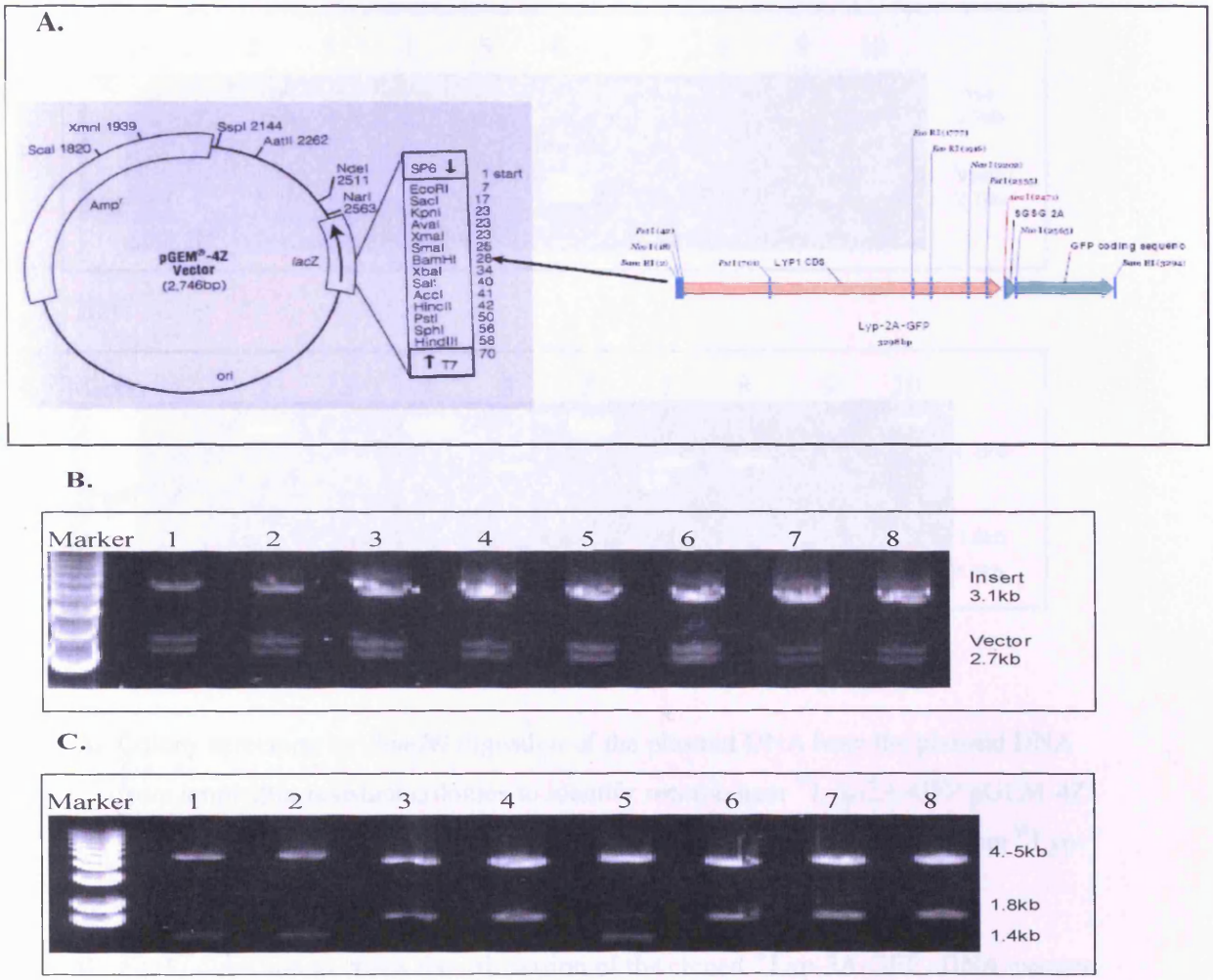


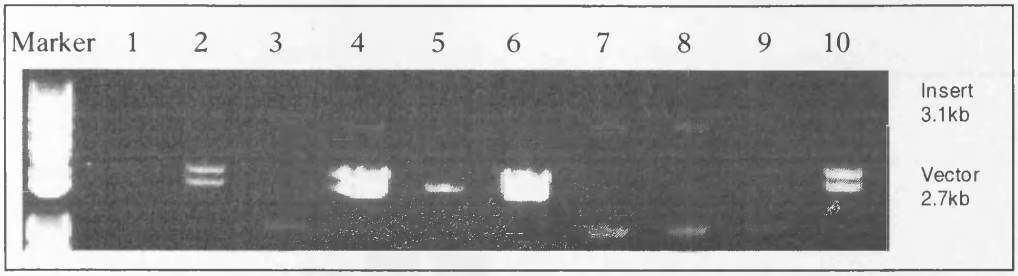
Figure 4.20 Generation of the pGEM-4Z Lyp-2A-GFP/CD2 plasmids

A. Schematic diagram showing the design of the Lyp- 2A-GFP pGEM-4Z plasmid. In this plasmid the transgene expression was driven by T7 promoter.

B. Colony screening by *BamHI* digestion of the plasmid DNA from ampicillin resistant colonies to identify theose colonies containing recombinant ^{R}Lyp-2A-CD2 pGEM-4Z plasmids. This Figure shows that all the colonies screened contain recombinant ^{R}Lyp-2A-CD2 pGEM-4Z plasmids.

C. *EcoRI* digestion to check the orientation of the cloned ^{R}Lyp-2A-CD2 cDNA cassette in the pGEM-4Z vector. Cloning in the correct orientation is indicated by release of a 1.4 kb DNA fragment. This Figure shows that colonies 1, 2 and 5 contain plasmids with ^{R}Lyp-2A-CD2 cDNA cassette in the correct orientation.

A.



B.

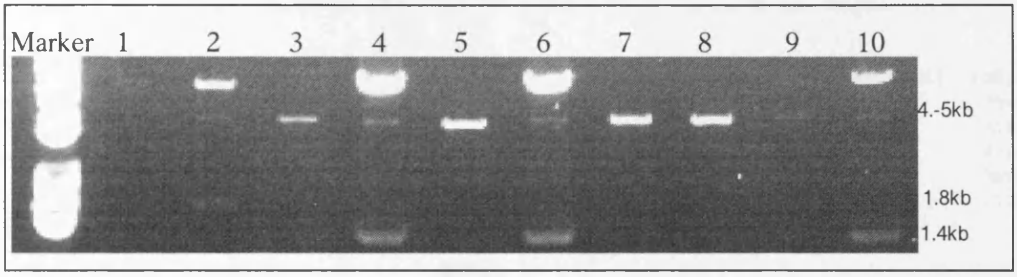


Figure 4.21 Generation of the pGEM-4Z ^{W}Lyp-2A-GFP/CD2 plasmids

A. Colony screening by *BamHI* digestion of the plasmid DNA from the plasmid DNA from ampicillin resistant colonies to identify recombinant ^{W}Lyp-2A-GFP pGEM-4Z plasmids. This Figure shows that colonies 2, 4, 6 and 10 contain recombinant ^{W}Lyp-2A-GFP pGEM-4Z plasmids

B. *EcoRI* digestion to check the orientation of the cloned ^{W}Lyp-2A-GFP cDNA cassette in the pGEM-4Z vector. Cloning in the correct orientation is indicated by release of a 1.4 kb DNA fragment. This Figure shows that colonies 4 and 6 contain plasmids with ^{W}Lyp-2A-CD2 cDNA cassette in the correct orientation.

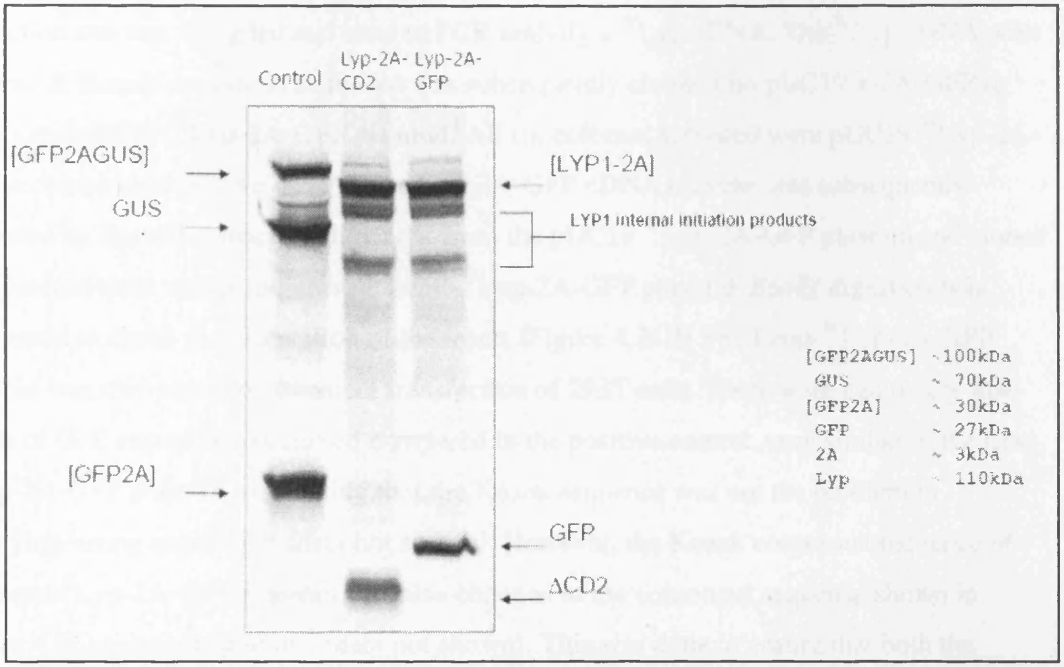


Figure 4.22 Evaluation of the self processing of the 2A peptide

"*In vitro* coupled transcription translation" was used to evaluate the self processing of the 2A peptide and to verify if any Lyp-GFP or Lyp-CD2 fusion was being expressed. This experiment was performed and the data provided by the group of Prof. Martin Ryan, University of St Andrews.

4.6 Changing the Kozak consensus sequence

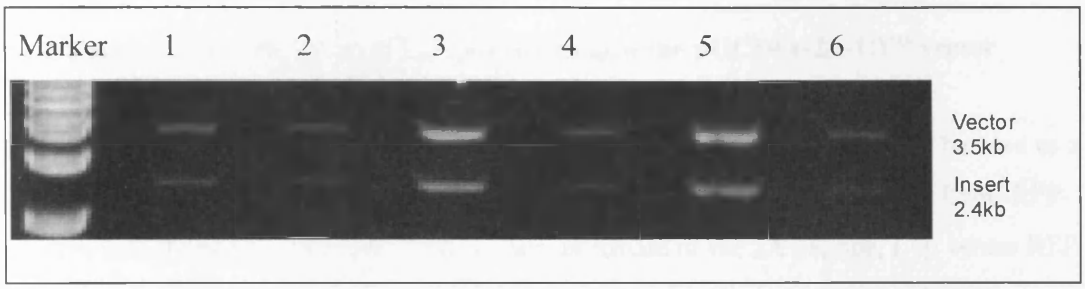
A 5' oligo incorporating a different, longer consensus sequence (Figure 4.23) and an *XbaI* restriction site was designed and used to PCR amplify a ^{W}Lyp cDNA. The ^{W}Lyp cDNA with the new 5' Kozak consensus sequence was subsequently cloned into pUC19 x-2A-GFP to generate a pUC19 ^{W}Lyp-2A-GFP plasmid. All six colonies screened were pUC19 ^{W}Lyp-2A-GFP recombinants (Figure 4.24A).The Lyp-2A-GFP cDNA cassette was subsequently extracted by *BamHI* restriction digestion from the pUC19 ^{W}Lyp-2A-GFP plasmid and cloned into the lentivirus vector to generate Lenti-^{W}Lyp-2A-GFP plasmid. *EcoRI* digestion was performed to check the orientation of the insert (Figure 4.24B) The Lenti-^{W}Lyp-2A-GFP plasmid was then tested by transient transfection of 293T cells. There were again very low levels of GFP expression observed compared to the positive control, very similar to the first ^{W}Lyp-2A-GFP plasmid, suggesting that the Kozak sequence was not the problem in achieving strong expression (data not shown). However, the Kozak consensus sequence of the Lenti-^{R}Lyp-2A-GFP plasmid was also changed to the consensus sequence shown in Figure 4.22 as described above (data not shown). This was done to ensure that both the sequence of the Lenti-^{R}Lyp-2A-GFP and Lenti-^{W}Lyp-2A-GFP recombinant plasmids had no other difference other than the two isoforms of Lyp.

As the Kozak sequence did not appear to be the reason for the difficulty experienced with the expression of the Lenti-$^{R/W}$Lyp-2A-GFP recombinant plasmids, it was decided to generate Lenti- RFP-2A-GFP recombinant plasmid as a positive control using the same Lenti SxW vector backbone and strategy of cloning as used in the generation of Lenti-$^{R/W}$Lyp-2A-GFP recombinant plasmids.

Figure 4.23 Kozak consensus sequences

Figure showing the sequence of the original Kozak consensus sequence used and the new Kozak concensus sequence that was used to replace it.

A.



B.

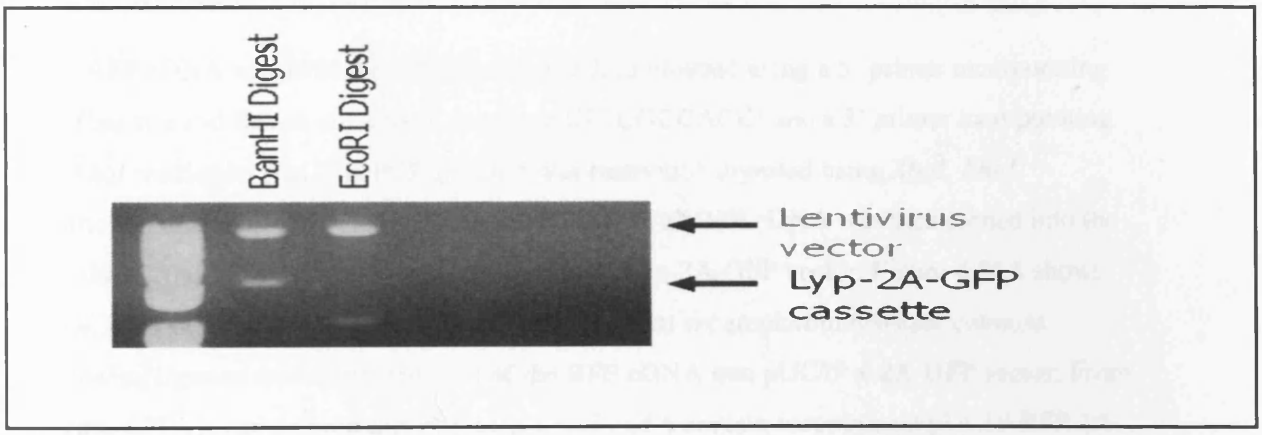


Figure 4.24 Generation of the Lenti ^{W}Lyp-2A-GFP plasmids with a new Kozak sequence

A. Colony screening by *XbaI*, *XhoI* digestion of the plasmid DNA from plasmid DNA from ampicillin resistant colonies to identify the colonies containing recombinant pUC19-^{W}Lyp-2A-GFP plasmids. This Figure shows that all the colonies screened contain pUC19-^{W}Lyp-2A-GFP plasmids.

B. Colony screening by *BamHI* digestion of the plasmid DNA from ampicillin resistant colonies to identify the recombinant Lenti-^{W}Lyp-2A-GFP plasmids followed by *EcoRI* digestion to check the orientation of the cloned ^{W}Lyp-2A-GFP cDNA cassette in the lentivirus vector.

4.7 Generation of Lenti- RFP-2A-GFP plasmid as a positive control

4.7.1 Cloning of red fluorescent protein (RFP) into the pUC19 x-2A-GFP vector

The generation of a recombinant Lenti-RFP-2A-GFP plasmid would allow it to be used as a positive control for the Lenti x-2A-GFP vector. As the only difference between Lenti-RFP-2A-GFP and Lenti-Lyp-2A-GFP is the protein upstream of the 2A peptide, Lyp versus RFP, the expression of the Lenti-RFP-2A-GFP plasmid would delineate where problems lie in expression of Lyp. With the RFP-2A-GFP cDNA cassette insert, cells expressing both the upstream and downstream proteins, RFP and GFP respectively, should fluoresce green when stimulated with light of blue wavelength and fluoresce red when stimulated with light of green wavelength.

The RFP cDNA was PCR amplified from a DsRed plasmid using a 5' primer incorporating an *XbaI* site and Kozak consensus sequence (GCCGCCACC) and a 3' primer incorporating an *XhoI* restriction site. The PCR product was restriction digested using *XbaI*, *XhoI* restriction enzymes and purified by gel extraction. The RFP cDNA was then cloned into the 'x' site of *XbaI*, *XhoI* digested, SAP treated pUC19 x-2A-GFP vector. Figure 4.25A shows *XbaI*, *XhoI* digestion screening of plasmid DNA from six ampicillin resistant colonies following ligation and transformation of the RFP cDNA into pUC19 x-2A-GFP vector. From Figure 4.25A it can be seen that colonies 3, 4, 5 and 6 contain recombinant pUC19 RFP-2A-GFP plasmids.

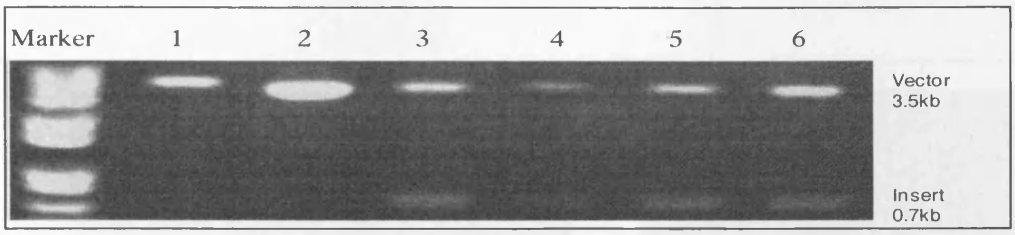
To generate recombinant lentivirus plasmid, the RFP-2A-GFP cDNA cassette was extracted and purified by *BamHI*, *AseI* double digestion from pUC19 RFP-2A-GFP plasmid. The cDNA cassette was then ligated into *BamHI* digested, SAP treated lentivirus vector. The DNA was transformed into DH5α competent cells and colonies were screened using *BamHI* digestion to identify recombinants and a *BsaI* digestion was performed to determine the orientation of the cloned insert. The size of the RFP-2A-GFP cDNA cassette and the lentivirus vector are 1516bp and 9kb respectively. Of the 7 colonies screened using BamH1 digestion, all contained recombinant Lenti-RFP-2A-GFP plasmids (Figure 4.25B). *BsaI* digestion was further performed to check the orientation of the cloned insert in the vector. If the insert had been cloned in the correct orientation DNA fragments of sizes 1830bp, 1985bp, 2746bp and 4010bp would have been expected whereas cloning in the incorrect orientation would produce DNA fragments of 1454bp, 1830bp, 3757bp and 4010bp. From Figure 4.25C

it can be seen that colonies 2, 3, 5, 6, 7, 8 have insert in the correct orientation as they release a DNA fragment at 2746bp upon *BsaI* digestion.

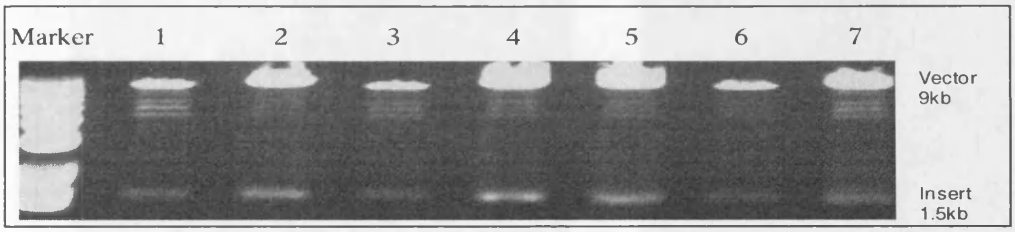
Following transient transfection of 293T cells with the Lenti-RFP-2A-GFP plasmid, a high percentage of coincident red and green cells were observed. This result suggested that the problems with expression originated with Lyp and the rest of the vector was functioning correctly (Figure 4.26A). The Lenti- RFP-2A-GFP plasmid demonstrated the efficiency of cleavage with the 2A peptide as no fusion protein could be detected. Immunoblot using anti-GFP antibody on the lysates of 293 T cells transfected with Lenti-RFP-2A-GFP indicated a 100% self processing efficiency as no migrating protein band corresponding to a RFP-2A-GFP fusion protein was observed (Figure 4.26B).

The 5' and 3' ends of Lyp and the 2A-GFP sequence had been checked by repeat DNA sequencing but the entire 2.4kb Lyp sequence had never been sequenced. At this stage, it was thought necessary to check the entire Lyp cDNA sequence in a further attempt to identify the problem experienced with the expression of Lenti- Lyp -2A-GFP recombinant plasmids.

A.



B.



C.

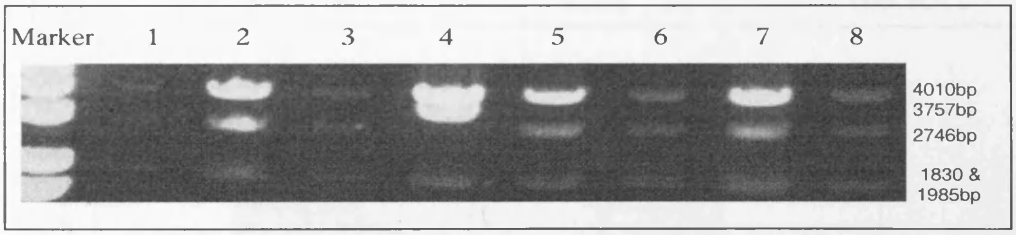


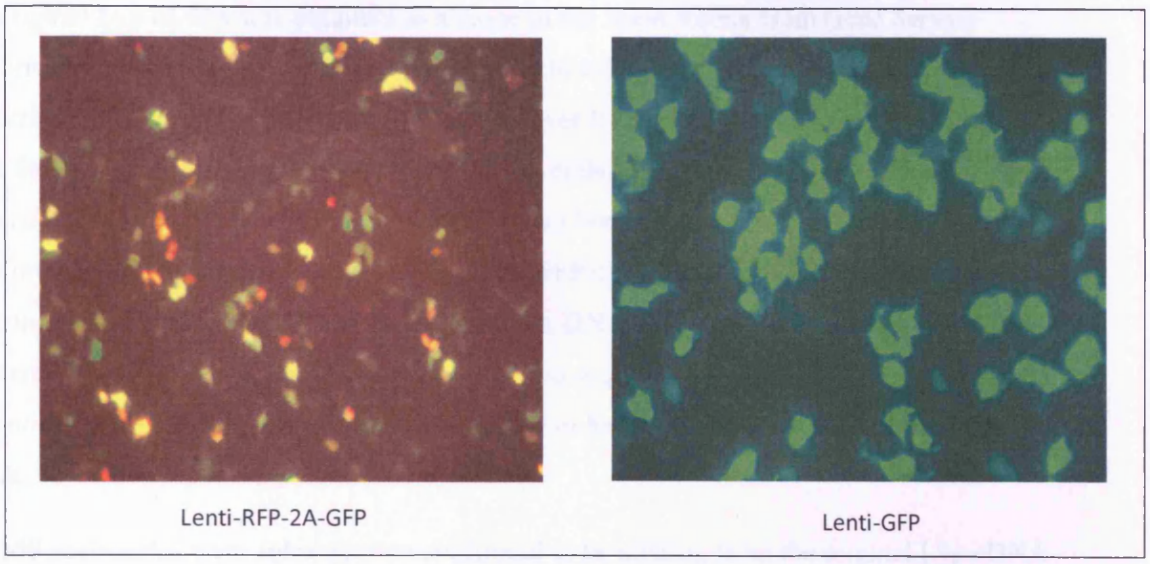
Figure 4.25 Generation of the Lenti-RFP-2A-GFP plasmid

A. Colony screening by *XbaI*, *XhoI* digestion of the plasmid DNA from ampicillin resistant colonies to identify those colonies containing recombinant pUC19- RFP-2A-GFP plasmids. This Figure shows that colonies 3, 4, 5 and 6 contain recombinant pUC19- RFP-2A-GFP plasmids.

B. Colony screening by *BamHI* digestion of the plasmid DNA from ampicillin resistant colonies to identify colonies containing recombinant Lenti-RFP-2A-GFP plasmids. This Figure shoes that all the colonies screened contain recombinat Lenti-RFP-2A-GFP plasmids.

C. *BsaI* digestion to check the orientation of the cloned RFP-2A-GFP cDNA cassette in the lentivirus vector. Cloning of the insert in the correct orientation is indicated by the presence of a DNA fragment at 2746 bp and an absence of a DNA fragment at 3757 bp. This Figure shows that colonies 1, 2, 3, 5, 6, 7 and 8 contain plasmids with RFP-2A-GFP cDNA cassette in the correct orientation.

A.



B.

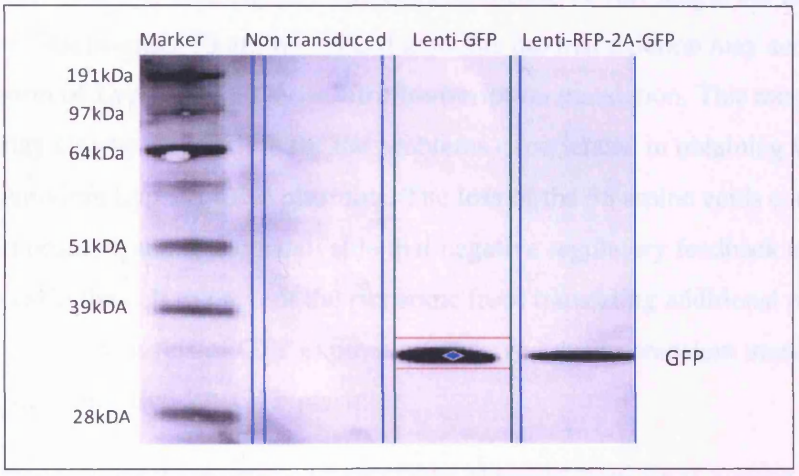


Figure 4.26 Testing the Lenti-RFP-2A-GFP plasmid

A. RFP and GFP expression by 293Tcells following transient transfection of 293T cells with Lenti-RFP-2A-GFP plasmid (left) and Lenti-GFP plasmid (right).

B. Immuno blot data showing cleavage efficiency of the 2A peptide. No RFP-2A-GFP fusion protein was detected when immunoblotted with an anti-GFP antibody.

4.8 Modifying/Correcting Lyp cDNA

The original Lyp cDNA was obtained as a clone in the Sport Vector from Gene Service Cambridge and advertised as a full length cDNA clone from the NIH Mammalian Gene Collection repository of full length cDNAs. However it emerged from discussion with the Gene Service, Cambridge that whilst the 5' and 3' ends of the Lyp had been checked by DNA sequencing the entire 2.4kb Lyp sequence had never been sequenced. At this stage, it was therefore decided to sequence the entire Lyp-2A-GFP cDNA cassette in the pUC19 vector to confirm that the Lyp sequence was correct. Indeed, DNA sequencing revealed that there was an internal deletion of 165 nucleotides from the Lyp sequence (Figure 4.27). The missing 165 nucleotides corresponded to two exons that appear to have been spliced out from the Lyp cDNA.

The 165 nucleotides were subsequently confirmed to be missing from the original Lyp cDNA from Gene Service. The Lyp cDNA had been assumed to be full-length but unfortunately was not the case. The missing 55 amino acids due to this internal deletion may account for the truncated form of Lyp observed by *in vitro* transcription translation. This missing Lyp sequence may also be responsible for the problems experienced in obtaining GFP expression with the Lentivirus Lyp-2A-GFP plasmids. The loss of the 55 amino acids could make the Lyp protein unstable and it is conceivable that negative regulatory feedback mechanisms might operate in the cell to prevent the ribosome from translating additional protein. This may explain the low levels of GFP expression observed during transient transfection of 293T cells using the Lenti-Lyp-2A-GFP plasmid.

It was decided to correct the original Lyp cDNA obtained from Gene Services using a second full length Lyp cDNA. However, in parallel another Lenti- ^{R}Lyp-2A-GFP plasmid was also generated using the second full length Lyp cDNA in the event that there were further problems associated with the original Lyp cDNA.

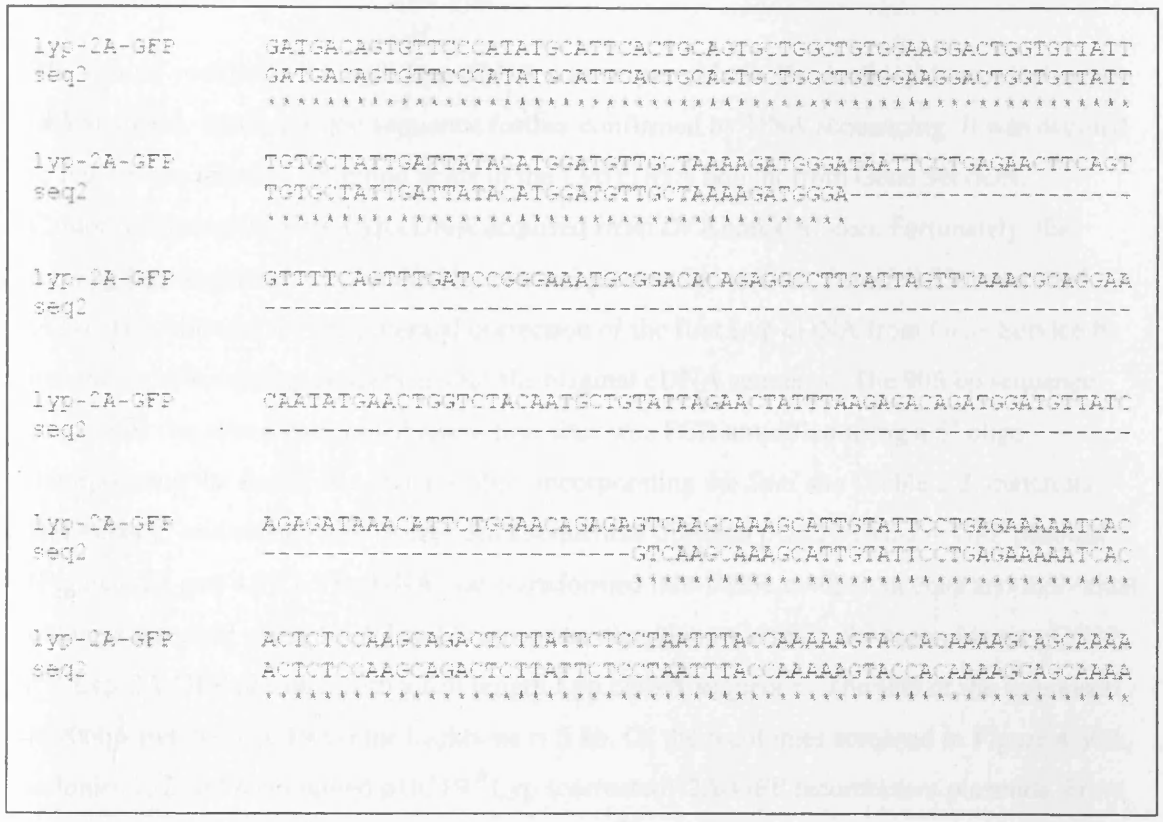


Figure 4.27 Sequence alignment of Lyp showing the missing exons

Top sequence is the reference sequence of full length Lyp cDNA, Bottom is the cDNA sequence of Lyp obtained from Gene Service, Cambridge.

4.8.1 Strategy for correcting the internal deletion in the original Lyp cDNA

The second verified full length Lyp cDNA was acquired from Dr. Andres Alonso, University of Valladolid, Spain and the sequence further confirmed by DNA sequencing. It was decided to replace the missing 55 amino acids in the Lyp cDNA bought from Gene Services, Cambridge using the new Lyp cDNA acquired from Dr Andres Alonso. Fortunately, the missing Lyp sequence was flanked by two unique restriction sites, *BsaAI* (1530) and *SacI* (624). This allowed for the potential correction of the first Lyp cDNA from Gene Service by insertion of the missing sequences into the original cDNA sequence. The 906 bp sequence flanked by the above mentioned restriction sites was PCR amplified using a 5' oligo incorporating the *BsaAI* site and a 3'oligo incorporating the *SacI* site (Table 2.2, materials and method) and cloned into *BsaAI, SacI* sequential digested pUC19 Lyp-2A-GFP plasmid (Figure 4.28 and 4.29). The DNA was transformed into DH5α competent cells and individual colonies screened using *SacI, BsaAI* restriction digestion to identify the recombinant pUC19 $^{R/W}$ Lyp-2A-GFP plasmid with a full length Lyp cDNA sequence. The size of the Lyp insert is 906bp and the pUC19 vector backbone is 5 kb. Of the 6 colonies screened in Figure 4.30A, colonies 1, 2 and 4 contained pUC19 ^{R}Lyp (corrected)-2A-GFP recombinant plasmids. From the 10 colonies screened for recombinant pUC19 ^{W}Lyp-2A-GFP, colonies 2, 4, 5, 6, 7 and 9 contained the correct recombinant plasmids as they have both the vector and insert DNA fragment present (Figure 4.30B).

To generate lentivirus plasmids, ^{R}Lyp-2A-GFP and ^{W}Lyp-2A-GFP cDNA cassettes were extracted and purified by *BamHI, AseI* double digestion from pUC19 ^{R}Lyp-2A-GFP and pUC19 ^{W}Lyp-2A-GFP plasmids respectively. The cDNA cassettes were subsequently cloned into *BamHI* digested, SAP treated lentivirus vector. The DNA was transformed into DH5a competent cells and colonies screened using a *BamHI* digestion to identify colonies containing recombinant plasmids and *EcoRI* digestion was performed to determine the orientation of the cloned insert. The size of the $^{R/W}$Lyp-2A-GFP cDNA cassette and the lentivirus vector were 3.3kb and 9kb respectively. Of the 10 colonies screened using *BamHI* digestion, 1, 5 and 10 contained recombinant Lenti-^{R}Lyp-2A-GFP plasmids (Figure 4.31A). *EcoRI* digestion was performed to check the orientation of the cloned cDNA insert in the vector. For inserts cloned in the correct orientation DNA fragments of sizes 139bp, 2283bp and 9326bp were expected whereas cloning in the incorrect orientation generated DNA

fragments of 139bp, 1786bp and 9823bp. From Figure 4.31B, it can be seen that only colony 1 has the insert in the correct orientation as it is the only clone that releases a DNA fragment at 2283bp upon *EcoRI* digestion. Figure 4.32A shows that colonies 1, 2, 3, 5, 6, 7, 8 and 9 contained recombinant Lenti- ^{W}Lyp-2A-GFP plasmids and from Figure 4.32B it can be deduced that colonies 7 and 8 contain plasmid DNA whereby the cDNA cassette is inserted in the correct orientation. The corrected $^{R/W}$Lyp-2A-GFP cDNA cassettes were checked by DNA sequencing and found to be correct.

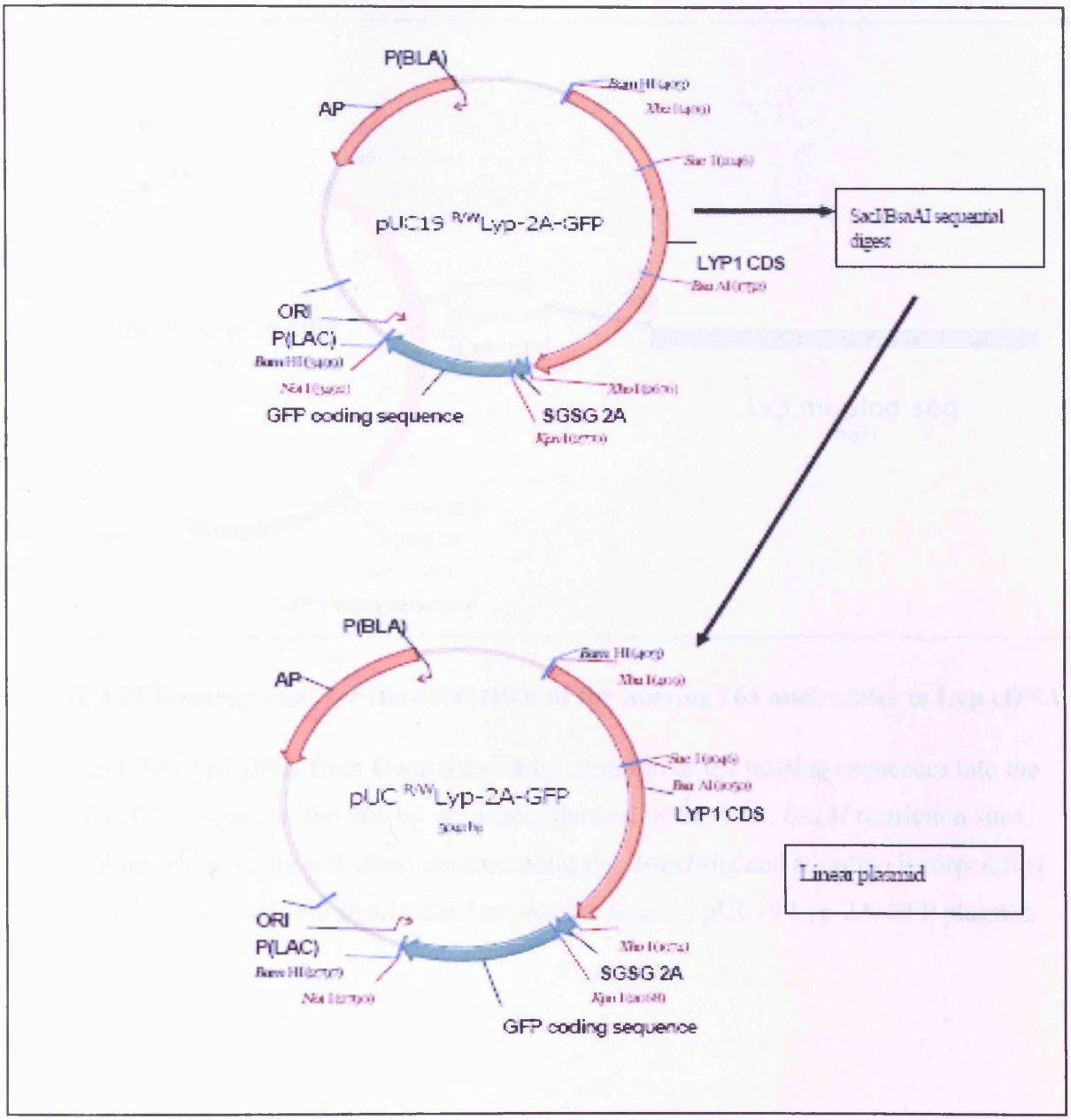


Figure 4.28 Schematic diagram showing the strategy used to correct the missing 165 nucleotides in Lyp cDNA

The missing 165 nucleotides from the Lyp cDNA bought from Gene Service was corrected by insertion of the missing sequences flanked by SacI/ BsaAI restriction sites into the original cDNA sequence. Therefore, the pUC R/W Lyp-2A-GFP plasmid vector was prepared by *BsaAI*, *SacI* sequential digestion for the cloning of the missing sequence.

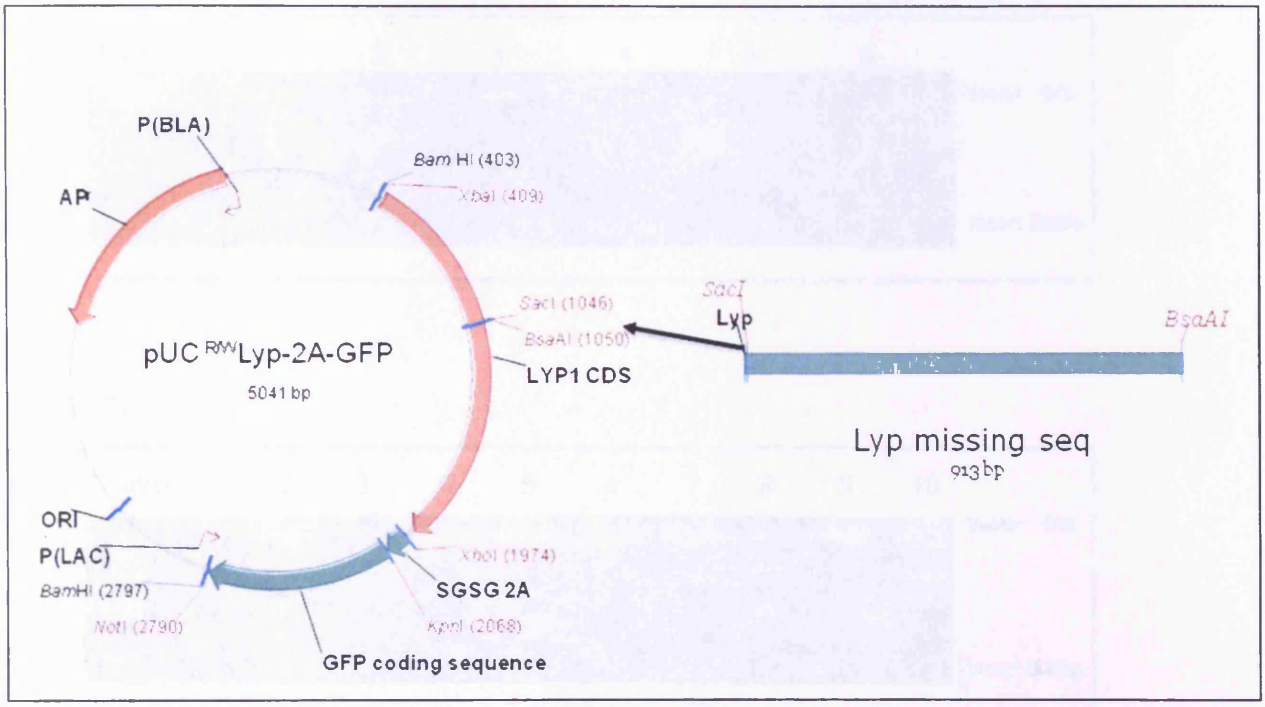
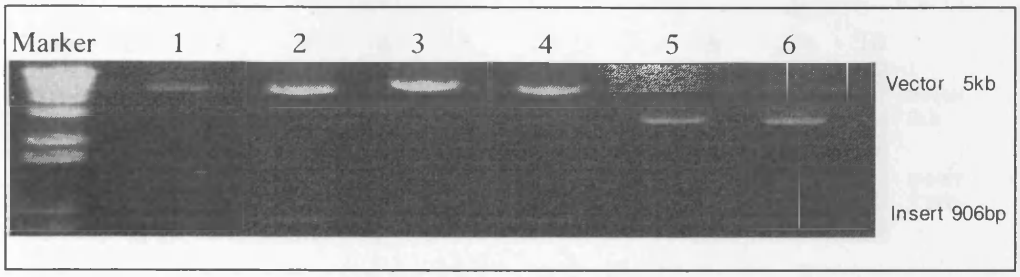


Figure 4.29 Strategy used for the correction of the missing 165 nucleotides in Lyp cDNA

To correct the Lyp cDNA from Gene Service by insertion of the missing sequences into the original cDNA sequence, the 906 bp sequence, flanked by the *SacI*, *BsaAI* restriction sites, was PCR amplified using a 5' oligo incorporating the *BsaAI* site and a 3'oligo incorporating the *SacI* site and cloned into *BsaAI*, *SacI* sequential digested pUC19 Lyp-2A-GFP plasmid.

A.



B.

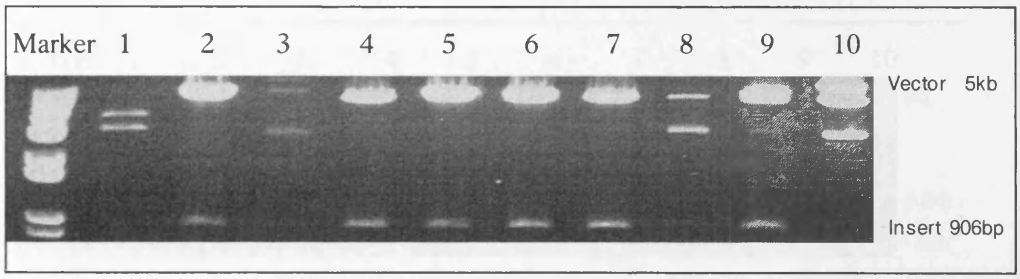
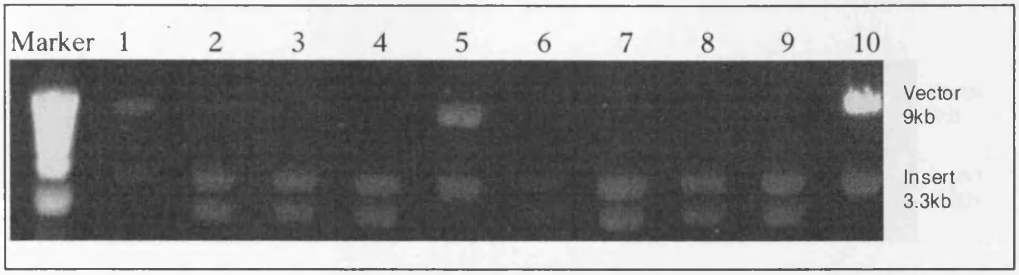


Figure 4.30 Generation of the corrected pUC $^{R/W}$Lyp-2A-GFP plasmids

A. Colony screening by *SacI, BsaAI* restriction digestion of the plasmid DNA from ampicillin resistant colonies to identify colonies containing recombinant pUC19-^{R}Lyp-2A-GFP plasmids. From this Figure it can be seen that colonies 1, 2 and 4 contain recombinant pUC19-^{R}Lyp-2A-GFP plasmids.

B. Colony screening by *SacI, BsaAI* restriction digestion of the plasmid DNA from ampicillin resistant colonies to identify the colonies containing the recombinant pUC19-^{W}Lyp-2A-GFP plasmids. This Figure shows that colonies 2, 4, 5, 6, 7 and 9 contain recombinant pUC19 ^{W}Lyp-2A-GFP plasmids.

A.



B.

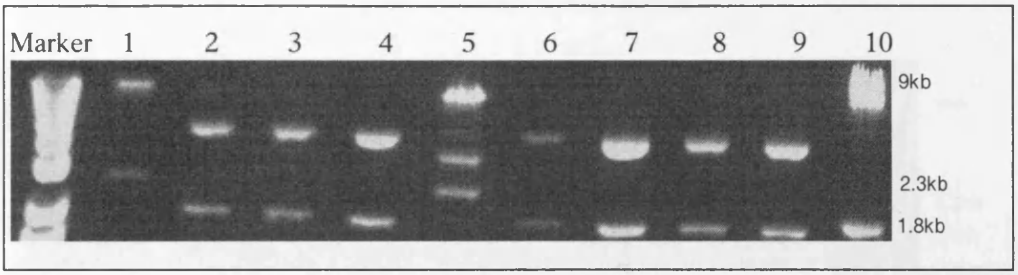
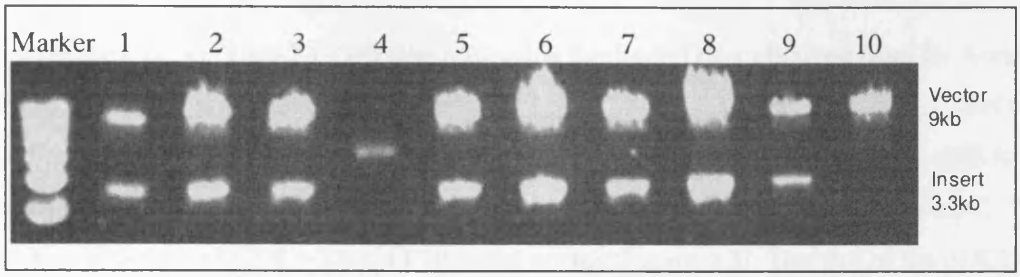


Figure 4.31 Generation of the corrected Lenti ^{R}Lyp-2A-GFP plasmids

A. Colony screening by *BamHI* digestion of the plasmid DNA from ampicillin resistant colonies to identify the colonies containing recombinant Lenti-^{R}Lyp-2A-GFP plasmids. From this Figure it can be seen that colonies 1, 5 and 10 contain recombinant Lenti-^{R}Lyp-2A-GFP plasmids.

B. *EcoRI* digestion to check the orientation of the cloned ^{R}Lyp-2A-GFP cDNA cassette in the lentivirus vector. Cloning in the correct orientation releases an indicative DNA fragment at 2283bp whereas cloning in an incorrect orientation releases a DNA fragment at 1786bp. This Figure shows that only colony 1contains plasmids with ^{R}Lyp-2A-GFPcassette in the correct orientation.

A.



B.

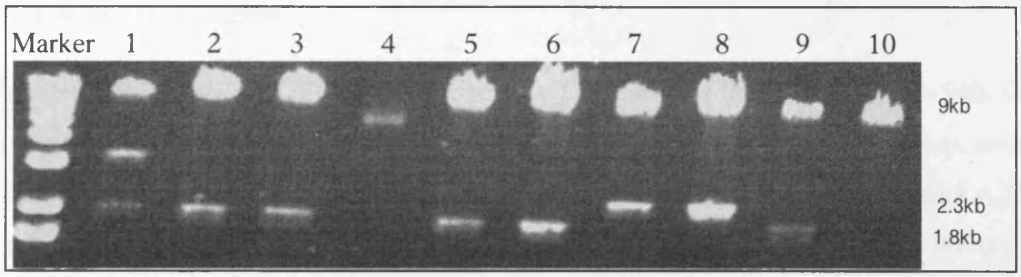


Figure 4.32 Generation of the corrected Lenti ^{W}Lyp-2A-GFP plasmids

A. Colony screening by *BamHI* digestion of the plasmid DNA from ampicillin resistant colonies to identify the colonies containing recombinant Lenti- ^{W}Lyp-2A-GFP plasmids. From this Figure it can be seen that colonies 1, 2, 3, 5, 6, 7, 8 and 9 contain recombinant Lenti- ^{W}Lyp-2A-GFP plasmids.

B. *EcoRI* digestion to check the orientation of the cloned ^{W}Lyp-2A-GFP cDNA cassette in the lentivirus vector. Cloning in the correct orientation releases an indicative DNA fragment at 2283bp whereas cloning in an incorrect orientation releases a DNA fragment at 1786bp. This Figure shows that colonies 7 and 8 contain plasmids with ^{W}Lyp-2A-GFPcassette cloned in the correct orientation.

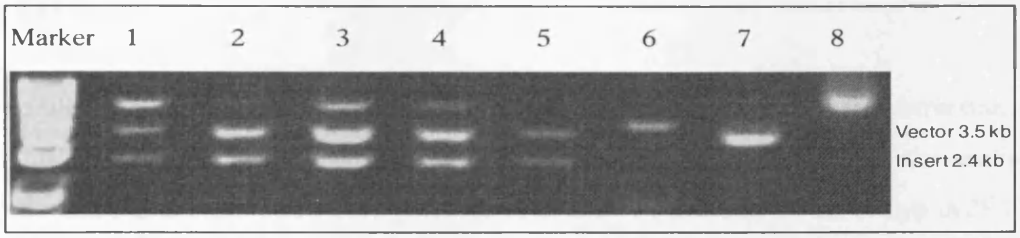
4.9 Generation of Lenti-Lyp-2A-GFP plasmids using full length ^{R}Lyp cDNA

To generate a Lenti ^{R}Lyp-2A-GFP plasmid using the Lyp cDNA obtained from Dr Andreas Alonso in parallel with correcting the original Lenti $^{R/W}$Lyp-2A-GFP, the internal *XbaI* site was first removed from the Lyp cDNA using site directed mutagenesis. The Lyp cDNA was subsequently PCR amplified, *XbaI*, *XhoI* double digested and cloned into SAP treated, *XbaI*, *and XhoI* digested pUC19 x-2A-GFP plasmid vector (Figure 4.3). The size of the pUC19 x-2A-GFP vector and Lyp insert are 3.3kb and 2.5kb respectively. From Figure 4.33A it can be seen that colonies 1 to 5 contain recombinant plasmids. The ^{R}Lyp-2A-GFP cDNA cassette was then extracted by *BamHI* restriction digestion and cloned into SAP treated, *BamHI* digested lentivirus vector.

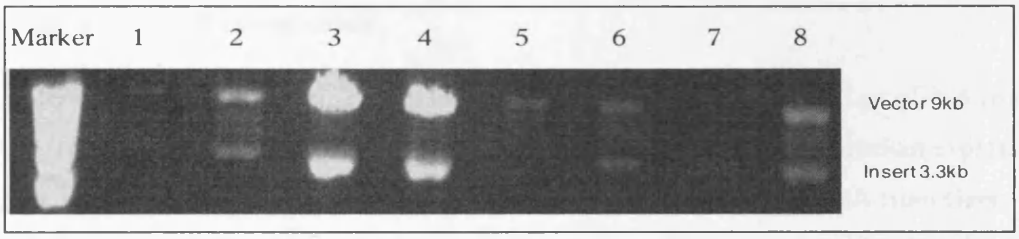
The size of the ^{R}Lyp-2A-GFP cDNA cassette is 3.3kb and the lentivirus vector is 9kb. Of the 8 plasmid DNA from ampicillin resistant colonies screened using *BamHI* digestion, colonies 1,2,3,4,6 and 8 contained recombinant Lenti-^{R}Lyp(Spain)-2A-GFP colonies (Figure 4.33B). *EcoRI* digestion was performed to check the orientation of the insert cloned into the vector. If the insert had been cloned in the correct orientation, DNA fragments of sizes 139bp, 2283bp and 9326bp would have been expected whereas cloning in the incorrect orientation would have given DNA fragments of 139bp, 1786bp and 9823bp. From Figure 4.33C it can be seen that only colony 4 contained recombinant plasmid DNA in the correct orientation as recombinant plasmid DNA from this colony released a DNA fragment of 2283bp upon *EcoRI* digestion.

In order to check that there were no further problems associated with achieving Lyp expression using the corrected ^{W}Lyp and the full length ^{R}Lyp from Spain without the complication of the 2A-GFP reporter genes Lyp- pCS2 plasmids were also generated in parallel to the generation of corrected Lenti $^{R/W}$Lyp-2A-GFP and the Lenti ^{R}Lyp (Spain)-2A-GFP plasmids.

A.



B.



C.

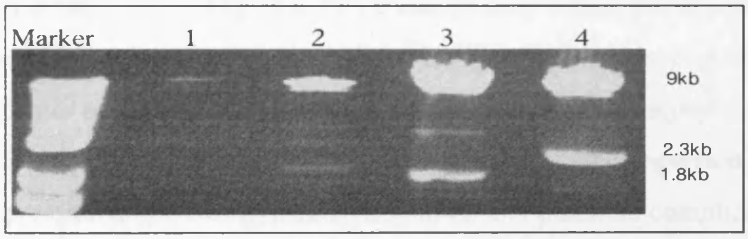


Figure 4.33 Generation of Lenti-^{R}Lyp (Spain)-2A-GFP plasmids

A. Colony screening by *XbaI*, *XhoI* digestion of the plasmid DNA from ampicillin resistant colonies to identify the colonies containing the recombinant pUC19-^{R}Lyp (Spain)-2A-GFP plasmids. This Figure shows that colonies 1-5 contain recombinant pUC19-^{R}Lyp (Spain)-2A-GFP plasmids.

B. Colony screening by *BamHI* digestion of the plasmid DNA from ampicillin resistant colonies to identify the colonies containing the recombinant Lenti-^{R}Lyp (Spain)-2A-GFP plasmids. This Figure shows that colonies 1, 2, 3, 4, 6 and 8 contain recombinant Lenti-^{R}Lyp (Spain)-2A-GFP plasmids.

C. *EcoRI* digestion to check the orientation of the cloned ^{R}Lyp (Spain)-2A-GFP cDNA cassette in the Lentivirus vector. Cloning in the correct orientation releases an indicative DNA fragment at 2283bp whereas cloning in an incorrect orientation releases a DNA fragment at 1786bp. This Figure shows that only colony 4 contain plasmids with ^{R}Lyp (Spain)-2A-GFPcassette cloned in the correct orientation.

4.10 Generation of the Lyp-pCS2 plasmids for parallel comparison of the two Lyp cDNAs

In parallel to the generation of Lenti- Lyp (Spain)-2A-GFP plasmids and the correction of the original $^{R/W}$Lyp-2A-GFP, Lyp-pCS2 plasmids were also generated. pCS2 is a mammalian expression plasmid driven by a T3 promoter that allows quick expression of Lyp in 293 T cells in order to confirm that Lyp expression could be achieved (using both constructs) without the 2A-GFP complication.

In order to test the "corrected" Lyp cDNA in parallel with the full length Lyp cDNA from Spain, two further Lyp plasmids were generated in the conventional mammalian expression vector, pCS2, using a "Spanish" Lyp cDNA and the "corrected" Lyp cDNA from Gene Services (Figure 4.34). The sizes of the Lyp inserts and pCS2 vector were 2.4kb and 4.66kb respectively. From Figure 4.35A it can be seen that of the 10 colonies screened, 1-6, 9 and 10 contained recombinant "corrected" ^{W}Lyp pCS2 plasmids. Figure 4.35B shows that of the 10 colonies screened, 2 and 7 are the only ones to contain recombinant ^{R}Lyp (spain)- pCS2 plasmids. The pCS2 plasmids allowed simultaneous comparison in mammalian cells of the expression of the two Lyp cDNA, without any potential complications relating to the "2A-GFP" reporter gene.

Transient transfection of 293T cells with the two pCS2 plasmids (^{R}Lyp (Spain) and ^{W} Lyp (corrected) was then carried out, cell lysates prepared following successful transient transfection and Immuno blot analysis performed on the transfected cell lysates. From the immunoblot data (Figure 4.36) it can be seen that both the Lyp cDNAs (Corrected ^{W}Lyp cDNA and the ^{R}Lyp cDNA from Spain) could be expressed in 293 T cells using the pCS2 expression vector. This result suggested that if there were further problems encountered in getting Lyp expression using a lentivirus plasmid then the problem may lie with the lentivirus plasmid, either the assembly of the Lyp-2A-GFP cDNA cassette or simply the combination of Lyp cDNA and the lentivirus promoter.

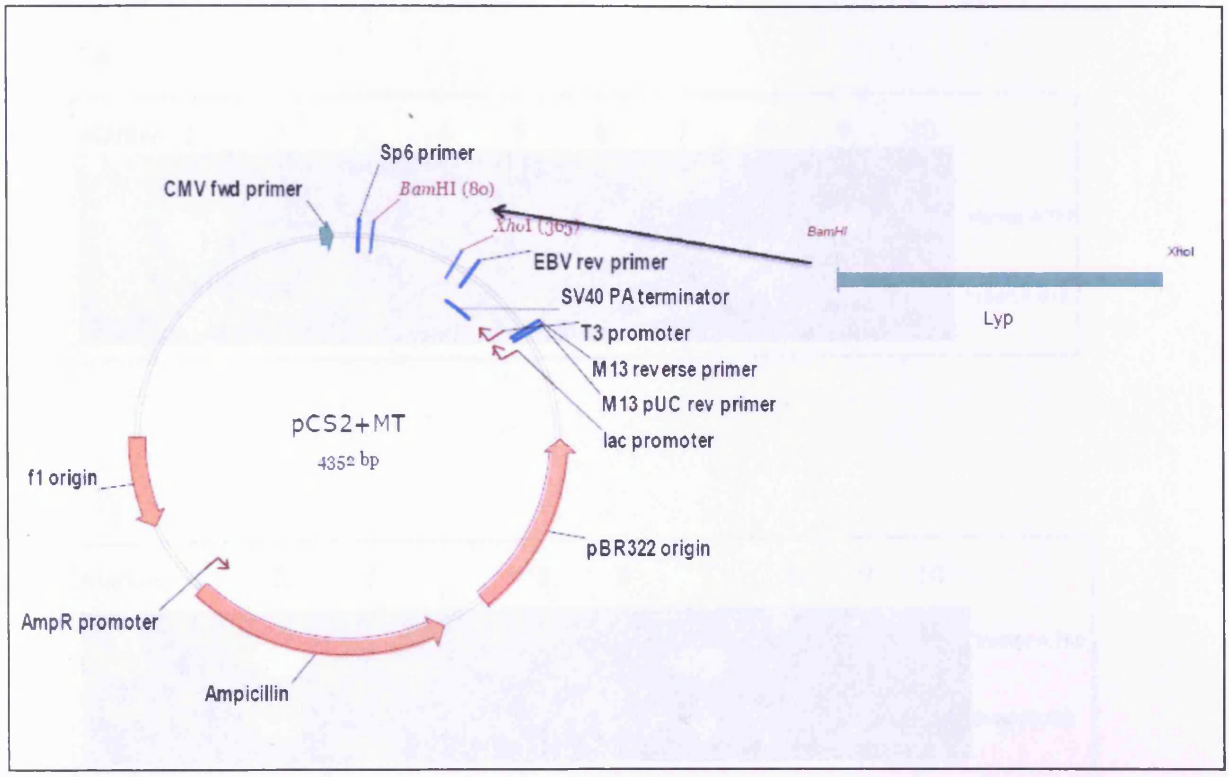
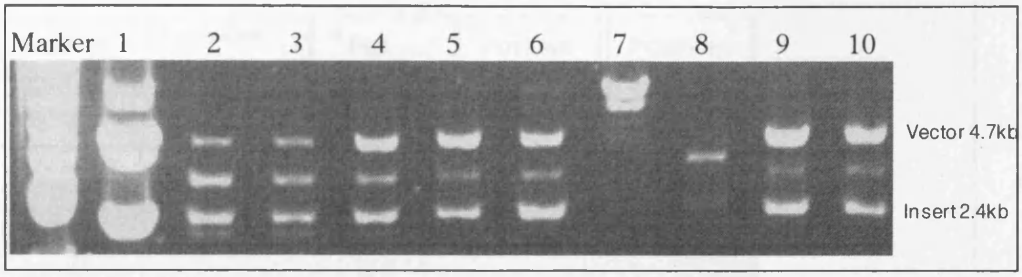


Figure 4.34 Generation of the pCS2 $^{R/W}$Lyp plasmids

Schematic diagram showing the strategic generation of the two pCS2 $^{R/W}$Lyp plasmids. Both the "Corrected" and the "Spanish" Lyp cDNAs were PCR amplified incorporating the *BamHI* and *XhoI* restriction sites and cloned into the *BamHI/XhoI* site of the pCS2 plasmid.

A.



B.

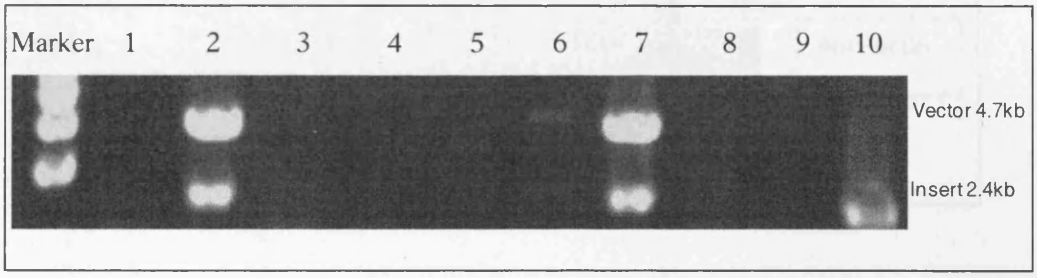


Figure 4.35 Generation of the $^{R/W}$Lyp pCS2 plasmids

A. Colony screen of the plasmid DNA from ampicillin resistant colonies by *BamHI*, *XhoI* double digestion to identify the colonies containing recombinant ^{W}Lyp (corrected) pCS2 plasmids. This Figure shows that colonies 1-6, 9 and 10 contain recombinant ^{W}Lyp (corrected) pCS2 plasmids.

B. Colony screen of the plasmid DNA from ampicillin resistant colonies by *BamHI*, *XhoI* double digestion to identify the colonies containing the recombinant ^{R}Lyp (Spain) pCS2 plasmids. This Figure shows that colonies 2 and 7 contain recombinant ^{R}Lyp (corrected) pCS2 plasmids.

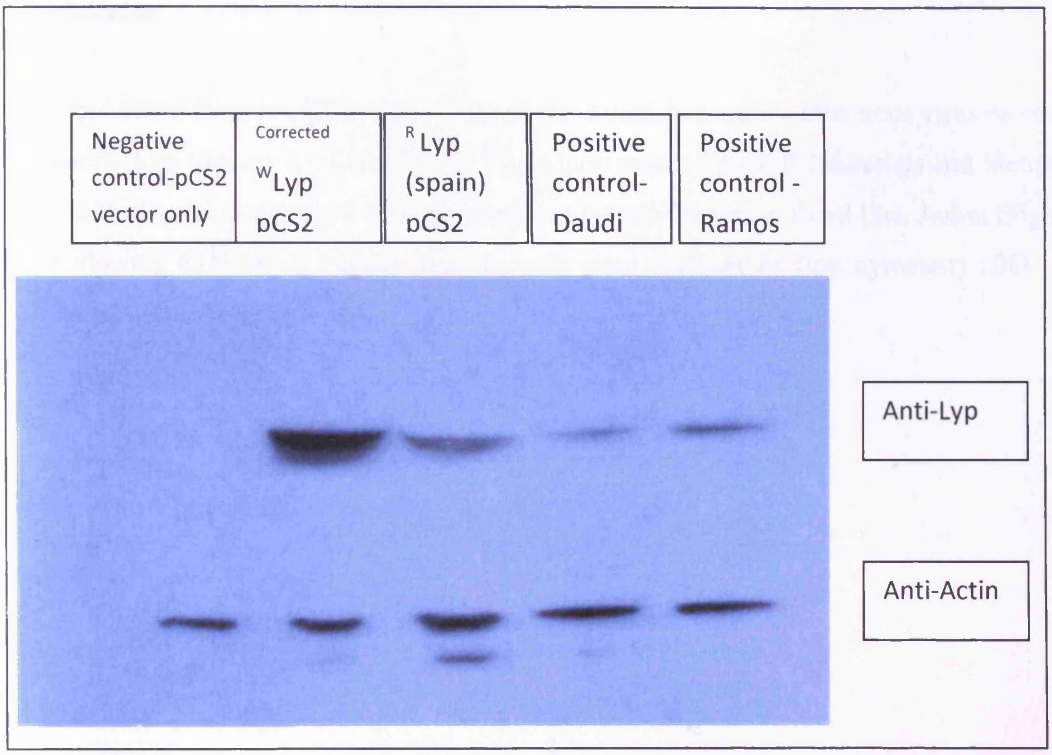


Figure 4.36 Analysis of the Lyp protein expression using the pCS2 plasmids

Immunoblot analysis of the expression levels of Lyp following transfection of 293T cells with either ^{W} Lyp (corrected) pCS2 or ^{R} Lyp (Spain) pCS2 plasmids. This figure shows that the Lyp protein is being overexpressed by 293T cells expressing the exogenous Lyp.

4.10.1 Testing the Lenti-^{R}Lyp (Spain) 2A-GFP and Lenti- ^{W}Lyp (corrected) 2A-GFP plasmids

Lipid based reagents (Effectine, Qiagen) were used to produce infectious virus particles of Lenti-^{R}Lyp (Spain)-2A-GFP; Lenti- ^{W}Lyp (corrected)-2A-GFP (Materials and Method). These virus particles were used to transduce human leukemic T cell line, Jurkat (Figure 4.37). Following 48 hours of transduction the cells were analysed by flow cytometry (BD FacsCalibur) (Figure 4.38).

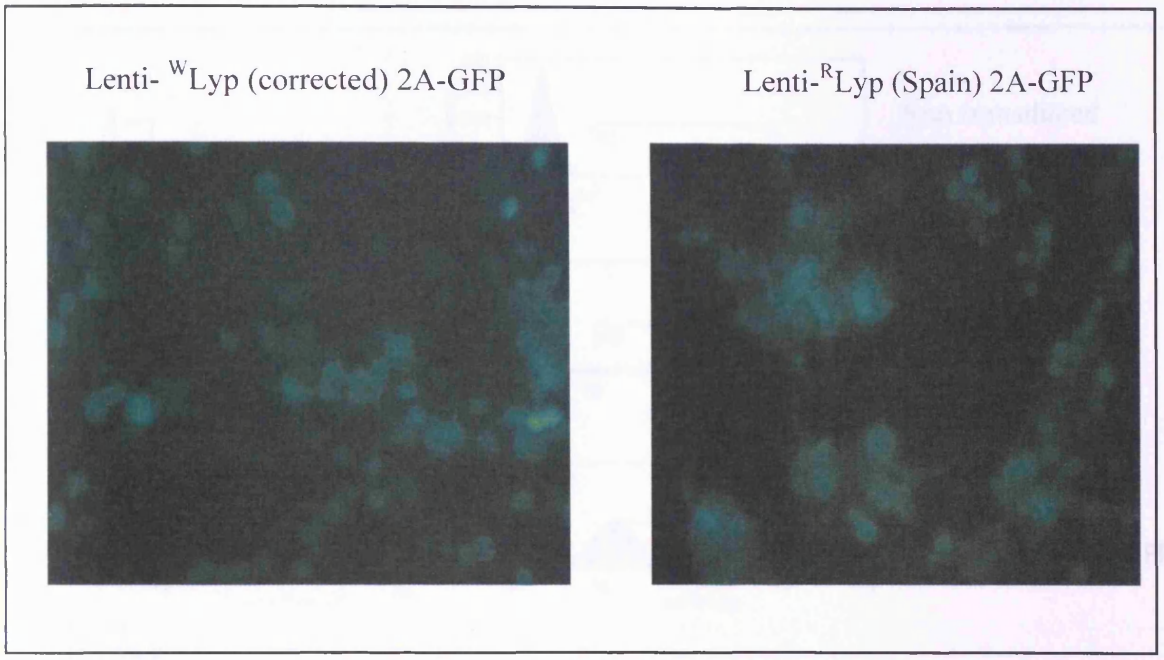


Figure 4.37 Testing the Lenti- Lyp-2A- GFP plasmids

Fluorescent image showing transduced Jurkat cells in green with the Lenti- ^{W}Lyp (corrected) 2A-GFP and Lenti-^{R}Lyp^{spain} 2A-GFP plasmids.

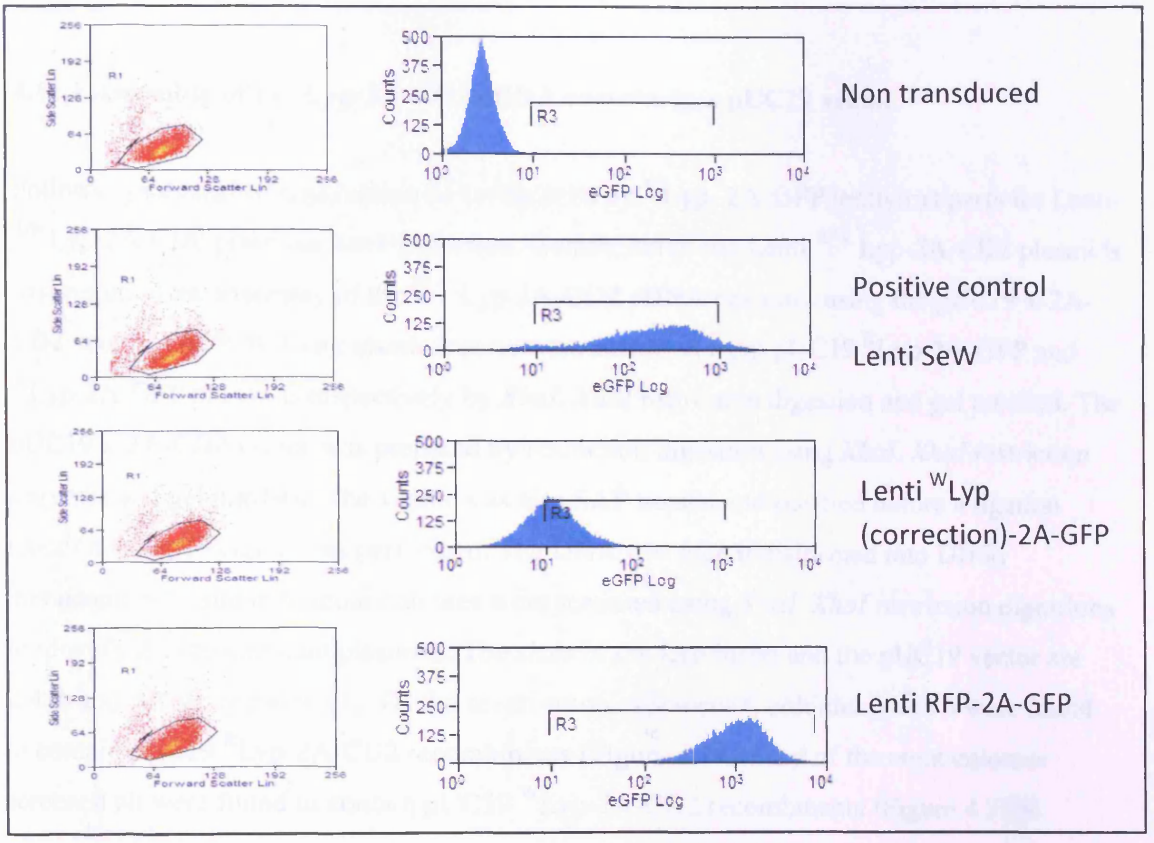


Figure 4.38 Flow cytometric analysis of the Lenti ^{W}Lyp-2A-GFP plasmid

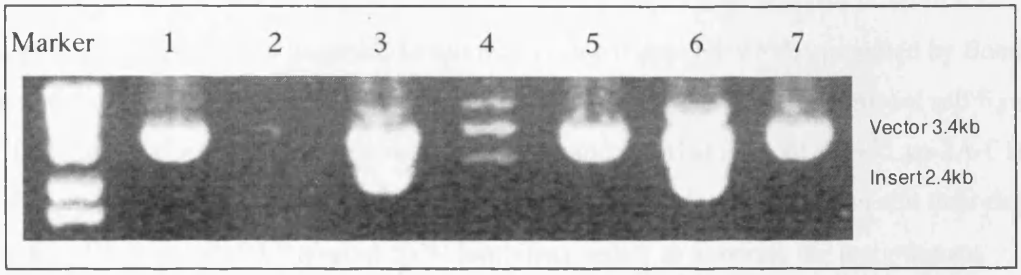
Flow cytometry data showing successful transduction of Jurkat cells by the Lenti-^{W}Lyp-2A-GFP viruses. Expression was assessed using GFP expression.

4.11 Generation of Lenti $^{R/W}$Lyp-2A-CD2 plasmids

4.11.1 Assembly of the Lyp-2A-CD2 cDNA cassette in a pUC19 vector

Following successful transduction of Jurkat cells by ^{W}Lyp- 2A-GFP lentivirus particles Lenti-$^{R/W}$Lyp-2A-CD2 plasmids were generated. Generation of the Lenti $^{R/W}$ Lyp-2A-CD2 plasmids first required the assembly of the $^{R/W}$ Lyp-2A-CD2 cDNA cassettes using the pUC19 x-2A-CD2 vector. The R/W Lyp cassette inserts were extracted from pUC19 ^{R}Lyp-2A-GFP and ^{W}Lyp-2A-GFP plasmids respectively by *XbaI, XhoI* restriction digestion and gel purified. The pUC19 x-2A-CD2 vector was prepared by restriction digestion using *XbaI, XhoI* restriction enzymes and gel purified. The vector was also SAP treated and purified before a ligation reaction using T4 ligase was performed. The DNA was then transformed into DH5α competent cells and individual colonies were screened using *XbaI, XhoI* restriction digestions to identify the recombinant plasmids. The sizes of the Lyp insert and the pUC19 vector are 2.4kb and 3.4 kb, respectively. Of the seven colonies screened, colonies 3 and 6 were found to contain pUC19 ^{R}Lyp-2A-CD2 recombinants (Figure 4.39A) and of the eight colonies screened all were found to contain pUC19 ^{W}Lyp-2A-CD2 recombinants (Figure 4.39B).

A.



B.

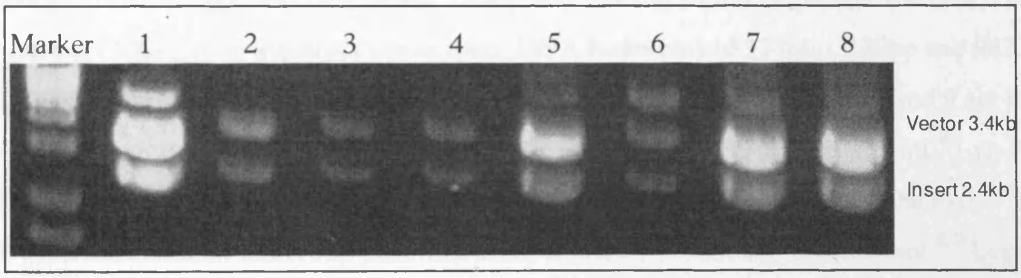


Figure 4.39 Generation of Lenti $^{R/W}$Lyp-2A-CD2 plasmids

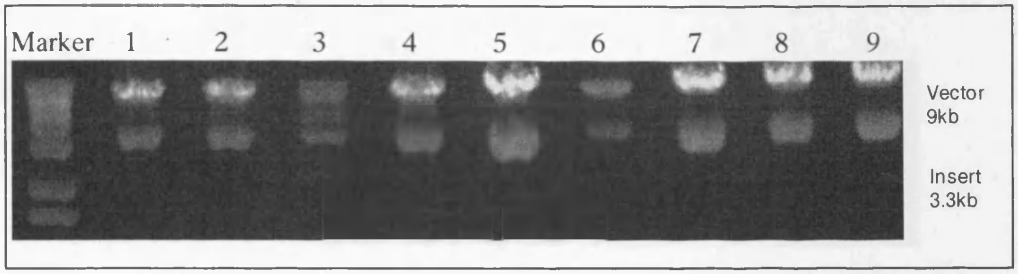
A. Colony screen by *XbaI*, *XhoI* restriction digestion of plasmid DNA to identify the colonies containing recombinant pUC19-^{R}Lyp-2A-CD2 plasmids. This Figure shows that of the seven colonies screened only colonies 3 and 6 contain recombinant pUC19-^{R}Lyp-2A-CD2 plasmids.

B. Colony screen by *XbaI*, *XhoI* restriction digestion of plasmid DNA to identify the colonies containing recombinant pUC19-^{W}Lyp-2A-CD2 plasmids. This Figure shows that of the eight colonies screened all contain recombinant pUC19-^{W}Lyp-2A-CD2 plasmids.

4.11.2 Generating Lenti $^{R/W}$ Lyp 2A CD2 plasmids

To generate the lentivirus plasmid, Lentivirus vector (Lenti-SxW) was prepared by *BamHI* restriction digestion to generate the appropriate ends and SAP treated to prevent self ligation. $^{W/R}$Lyp-2A- CD2 cDNA cassettes were extracted and purified from pUC19 ^{R}Lyp-2A-CD2 and ^{W}Lyp-2A-CD2 plasmids respectively by *BamHI*, *AseI* double digestion and then cloned into *BamHI* digested, SAP treated SxW lentivirus vector to generate the recombinant lentivirus plasmids. From Figure 4.40A it can be seen that all the clones screened were recombinant Lenti ^{R}Lyp-2A-CD2 plasmids. The orientation of the insert cloned was checked by *EcoRI* restriction digestion. If the ^{R}Lyp-2A-CD2 cDNA cassette was cloned in the correct orientation DNA fragments of 2283bp, 139bp and 9326bp would be present whereas if the insert was cloned in an incorrect orientation, DNA fragments of 1786bp, 139bp and 9823bp would be observed. From Figure 4.40B it can be seen that clones 3, 4, 5, 6, 8 and 9 are in correct orientation. From Figure 4.41A it can be seen that only clone 2 is a Lenti ^{W}Lyp-2A-CD2 recombinant and Figure 4.41B shows that it is in a correct orientation. The two $^{R/W}$Lyp -2A-CD2 recombinant lentivirus plasmids were tested by producing recombinant $^{R/W}$Lyp -2A-CD2 lentivirus particles and transducing Jurkat T cell lines. The $^{R/W}$Lyp -2A- CD2 lentivirus particles were observed to successfully transduce Jurkat T cells when analysed by flow cytometry of the rat CD2 PE staining (Figure 4.42).

A.



B.

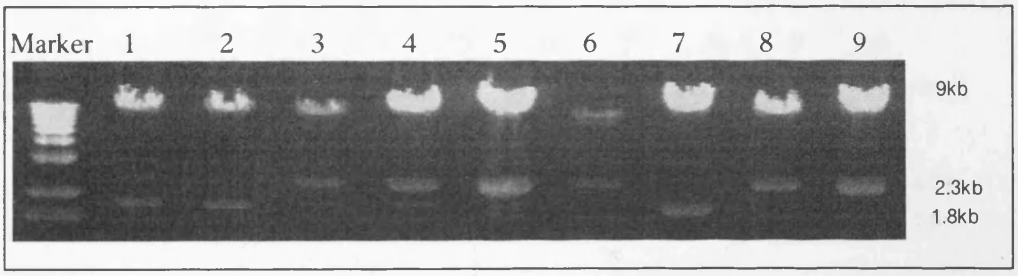
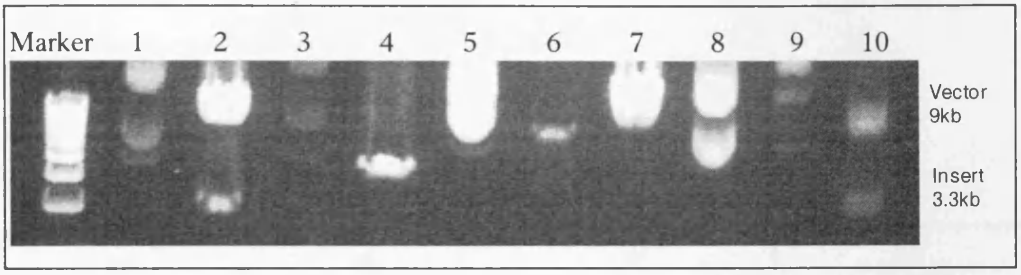


Figure 4.40 Generation of the Lenti-^{R}Lyp-2A-CD2 plasmid

A. Colony screen by *BamHI* restriction digestion of plasmid DNA to identify the colonies containing recombinant Lenti-^{R}Lyp-2A-CD2 plasmids. This Figure shows that all nine colonies screened contain recombinant Lenti-^{R}Lyp-2A-CD2 plasmids.

B. *EcoRI* digestion of plasmid DNA to identify the colonies containing recombinant plasmid with ^{R}Lyp-2A-CD2 cDNA cassette cloned in the correct orientation. Cloning in the correct orientation is indicated by the release of DNA fragments of 2283bp whereas cloning in an incorrect orientation releases DNA fragments of 1786bp. From this Figure it can be seen that clones 3, 4, 5, 6, 8 and 9 contain plasmids with ^{R}Lyp-2A-CD2 cDNA cassette cloned in correct orientation.

A.



B.

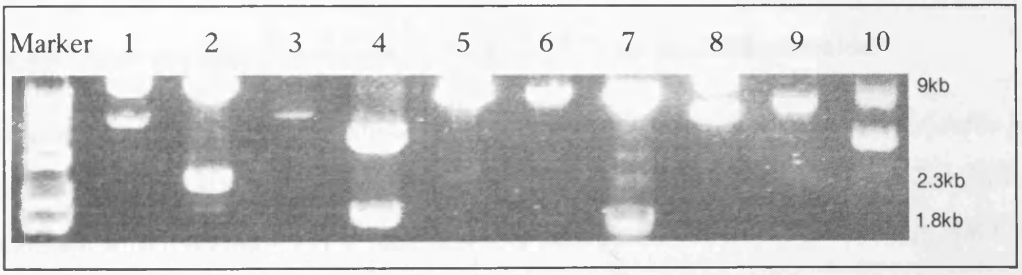


Figure 4.41 Generation of the Lenti-^{W}Lyp-2A-CD2 plasmids

A. Colony screen by *BamHI* digestion of the plasmid DNA to identify colonies containing recombinant Lenti-^{W}Lyp-2A-CD2 plasmids. This Figure shows that only clonies 2 and 10 contain recombinant Lenti-^{W}Lyp-2A-CD2 plasmids.

B. *EcoRI* digestion of plasmid DNA to identify colonies containing the plasmids with the ^{W}Lyp-2A-CD2 cDNA cassette cloned in the correct orientation. Cloning in the correct orientation is indicated by the release of DNA fragments of 2283bp whereas cloning in an incorrect orientation releases DNA fragments of 1786bp. From this Figure it can be seen that only clone 2 contains plasmid with ^{W}Lyp-2A-CD2 cDNA cassette cloned in correct orientation.

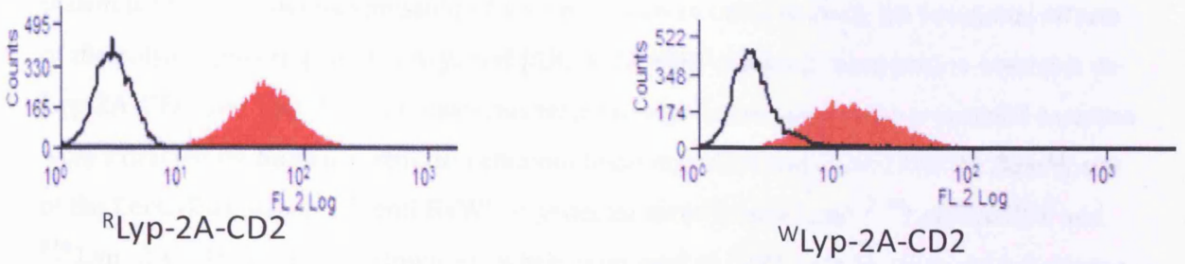


Figure 4.42 Flow cytometric analysis of the Lenti $^{R/W}$Lyp-2A-CD2 plasmids

Flow cytometry data showing a successful transduction of Jurkat cells by the Lenti- Lyp-2A-GFP virus particles. Jurkat T cells were lentivirally transduced with $^{R/W}$Lyp-2A-CD2 recombinant viruses. Expression was assessed using anti-rat CD2 PE staining. Rat CD2 staining of the parental H9^{gag+} cell line not transduced with rat CD2 was used as a negative control and is shown in black.

4.12 Discussion

This chapter describes the generation of recombinant R and W Lyp expressing lentivirus plasmids for use in over-expression of Lyp in T cells in order to study the functional effects of the polymorphism. pUC x-2A-y, and pUC x-2A-GFP plasmids were used to assemble the Lyp-2A-CD2 and Lyp-2A-GFP cassettes respectively. Subsequently, the assembled cassettes were extracted by *BamHI* restriction endonuclease digestion and cloned into the *BamHI* site of the Lentivirus plasmid (Lenti SxW) to generate recombinant Lenti $^{R/W}$Lyp-2A-GFP and $^{R/W}$Lyp -2A-CD2 plasmids. However, when expressed in 293T cells by transient transfection very low levels of GFP expression were observed with the recombinant Lentivirus Lyp-2A-GFP plasmids when compared to the positive control Lenti-GFP (Lenti SeW) plasmid.

Initially, it was thought that the low level reporter gene GFP expression observed in 293T cells may be due to some problems with the self-processing of the 2A peptide. At the initial stage it was not clear if the problem was with the expression of GFP only allowing the upstream protein Lyp to be expressed. Immunoblot analysis was performed to check if both the Lyp and GFP expression were impaired and/or whether cleavage was taking place between the 2A sequence and GFP. Immunoblot data showed some Lyp expression but no GFP expression with the Lenti-^{W}Lyp-2A-GFP plasmid but control plasmid did demonstrate GFP expression. This result suggested that Lyp was being expressed but not GFP but this discrepancy in results could be due to the affinity of the antibodies rather than differential expression of the two proteins. Therefore, the expression of both the proteins was analysed using an "*in vitro* coupled transcription translation" in collaboration with Prof Martin Ryan at the University of St Andrews. "*In vitro* coupled transcription/translation" showed no major differences in the expression levels of Lyp and GFP and there were also no Lyp-GFP/CD2 fusion protein detected suggesting an almost 100% self processing of the 2A peptide. Therefore it was thought that the low level expression of GFP with Lenti-Lyp-2A-GFP may be due to defective transcription initiation of the Lyp sequence. The Kozak consensus sequence used was thought to be sub optimal and was changed to a more widely used Kozak sequence (GCCGCCATG). However, despite this change in the design of the vector, the level of GFP expression with the Lenti Lyp-2A-GFP plasmids was not improved. Lenti-RFP-2A-GFP plasmid was therefore generated as a positive to control to ensure that the problem with GFP expression downstream of Lyp was due to the Lyp-2A-GFP cassette and not due to any intrinsic problems with the lentivirus plasmid vector (Lenti SxW). The Lenti RFP-2A-GFP

plasmid gave successful expression of both the upstream protein RFP and the downstream protein GFP ensuring that the problems experienced in expression of Lenti Lyp-2A-GFP were due to Lyp and not the lentivirus vector.

At this stage, the entire Lyp-2A-GFP cDNA cassette, which had never before been sequenced, was sequenced to ensure that the Lyp sequence was correct. DNA sequencing revealed that there was an internal deletion of 165 nucleotides from the Lyp sequence and the missing 165 nucleotides that have been spliced out of the Lyp cDNA was thought to be the reason for the observed problems with GFP expression using the Lenti-Lyp-2A-GFP plasmids. It was plausible that the lack of 55 amino acids from the Lyp cDNA could make the Lyp protein unstable and there may exist a negative regulatory mechanism in the cell that was preventing the ribosome from translating additional Lyp protein.

The missing 55 amino acids were replaced into the original ^{W}Lyp cDNA using a full length ^{R}Lyp cDNA obtained from Dr Andreas Alonso, University of Valladolid, Spain. Additionally recombinant Lenti- ^{R}Lyp-2A-GFP plasmids were generated in parallel using the ^{R}Lyp cDNA obtained from Dr Andreas Alonso. In parallel to the generation of Lenti- Lyp (Spain)-2A-GFP plasmid, Lyp-pCS2 plasmids were also generated in order to test the "corrected" Lyp cDNA in comparison with the full length Lyp cDNA from Spain, without the complication of the 2A-GFP and outside the context of the lentivirus vector. Transient transfection of 293T cells with the two pCS2 plasmids (^{R}Lyp (Spain) and ^{W} Lyp (corrected) pCS2) followed by immunoblotting showed a successful expression of Lyp using both the recombinant plasmids. The immunoblotting data also confirmed that Lyp could be successfully expressed using a pCS2 plasmid.

Following successful generation of the Lenti-^{R}Lyp^{spain}-2A-GFP, Lenti- ^{W}Lyp ^{corrected}-2A-GFP recombinant plasmids, they were tested by production of recombinant lenvirus particles and used to infect human leukemic Jurkat T cell line. The recombinant lentiviruses expressing both the ^{R}Lyp (Spain) 2A-GFP and the ^{W}Lyp (corrected) 2A-GFP were observed to successfully infect the Jurkat T cells. Following successful infection of Jurkat T cells by ^{W}Lyp- 2A-GFP lentivirus particles, Lenti- $^{R/W}$Lyp-2A-**CD2** recombinant plasmids were also successfully generated and tested.

In summary, in this chapter recombinant $^{R/W}$Lyp -2A- GFP lentivirus plasmids were successfully generated and used to successfully transduce Jurkat T cells despite the initial set-back due to the missing 55 amino acids in Lyp cDNA sequence. In this chapter the self

processing ability of the 2A peptide was also assessed and found to be extremely efficient by "*in vitro* coupled transcription/translation" experiment performed in collaboration with Prof. Martin Ryan and by immunoblotting of GFP in RFP-2A-GFP expressing 293T cell lysates. Following the successful generation of the GFP reporter gene expressing lentivirus plasmids ($^{R/W}$Lyp -2A-GFP), $^{R/W}$Lyp -2A- **CD2** lentivirus plasmids expressing truncated rat CD2 as a reporter gene were also successfully generated. The rationale for generating recombinant lentivirus plasmids with the rat CD2 reporter gene was that it would allow economic and convenient means of sorting transduced cells. The $^{R/W}$Lyp -2A- **CD2** lentivirus plasmids were tested by generation of $^{R/W}$Lyp -2A- **CD2** lentivirus particles, which were observed to successfully transduce Jurkat T cells. Collectively, the data presented in this chapter demonstrate that the ^{R}Lyp-2A-CD2, ^{W}Lyp-2A-CD2 lentivirus plasmids were successfully generated and were capable of expressing Lyp in T cells.

Chapter 5

Generation of Lyp T cell lines over-expressing Lyp

5.1 Introduction

In Chapter 4, Lenti $^{R/W}$Lyp-2A-CD2 recombinant lentivirus constructs were generated for the purpose of over-expressing the R and W isoforms of Lyp in T cells. This part of the study was focussed on generating these T cell lines. Leukaemic T cell lines were used as these are the basic model system which has been extensively used in the study of T cell signalling. The advantages of using the leukaemic T cell lines over primary T cells is their relative ease to culture, requiring no cytokine supplements and their monoclonal origin which means that they act in a uniform manner. The use of T cell lines also eliminates the main disadvantage associated with the use of primary T cells, namely a potential variation in the composition of T cell sub-populations, which may make interpretation of results difficult. The major disadvantages of using T cell lines are that as a single clonal population, observations made in these cells may not necessarily demonstrate what happens in other T-cell lineages. Furthermore, as these cells are transformed, mutations may exist in important cell signalling intermediates. T cell clones which have also been widely used for cell signalling studies were also a good potential source of T cells for studying Lyp functions. T cell clones of different specificities were potentially available, however, they are technically more demanding and time consuming to generate and maintain. As time was a major limiting factor, it was thought best to use the readily available leukaemic T cell lines, E6.1 (Jurkat) and H9 (Hut78), in this study.

Two established T cell lines, E6.1^{gag+} and H9^{gag+}, generated by Dr John Bridgeman (Cardiff University) were used to generate T cell lines permanently over-expressing $^{R/W}$Lyp. E6.1^{gag+} and H9^{gag+} cell lines are E6.1 (Jurkat) and H9 (Hut78) cell lines respectively expressing an exogenous TCR that recognises the HIV gag peptide sequence, SLYNTVATL (SLY), as a result of transduction of the E6.1 and H9 cells with a pSLY-TCRalpha.T2A.TCRbeta recombinant lentivirus. The exogenous gag TCR expression by the T cells allows them to be activated by HLA-A2 specific SLY peptide pulsed APCs.

$^{R/W}$Lyp-2A-CD2 and CD2 lentivirus particles generated using a three plasmid system were used to transduce E6.1 $^{gag+}$ and H9 $^{gag+}$ cells to generate, E6.1$^{gag+\ R}$Lyp-2A-CD2, E6.1^{gag+}

^{W}Lyp-2A-CD2 and E6.1^{gag+} CD2 and H9^{gag+} ^{R}Lyp-2A-CD2, H9^{gag+} ^{W}Lyp-2A-CD2, H9^{gag+} CD2 cell lines respectively.

5.2 Genotyping of the E6.1 $^{gag+}$ and H9 $^{gag+}$ cells

The E6.1 $^{gag+}$ and H9 $^{gag+}$ cell lines were genotyped to ensure that they were homozygous for the PTPN22 C1858 allele. This was necessary because the aim was to introduce exogenous ^{W}Lyp into homozygote wild type (^{R}Lyp/^{R}Lyp) T cell populations. As ^{W}Lyp has been observed to function in a dominant manner it was thought plausible that the introduction of ^{W}Lyp into homozygote wild type (^{R}Lyp/^{R}Lyp) T cell populations would partially mimic the situation in heterozygote individuals and allow examination of the effect of the R620W polymorphism. PCR restriction fragment length polymorphism analysis was performed on each cell line as previously described by (Bottini et al. 2004). Briefly, genomic DNA was extracted from the E6.1 $^{gag+}$ and H9 $^{gag+}$ cells and a PCR reaction was performed out as described in Materials and Methods using the forward and reverse primers shown in Table 2.3. The amplified 215bp fragment of the PTPN22 gene sequence encompassing the C1858T polymorphism was genotyped by *XcmI* restriction endonuclease digestion. *XcmI* recognizes its target sequence only when the PTPN22 1858T allele is present. Figure 5.1 shows that both the E6.1 and H9 cell lines only release a 215bp DNA fragment upon *XcmI* restriction endonuclease digestion. Therefore both cell lines are homozygous for the PTPN22 C1858 allele. A control reaction was also set up using pUC WLyp-2A-GFP to ensure that the *XcmI* restriction endonuclease was recognizing its target sequence (data not shown).

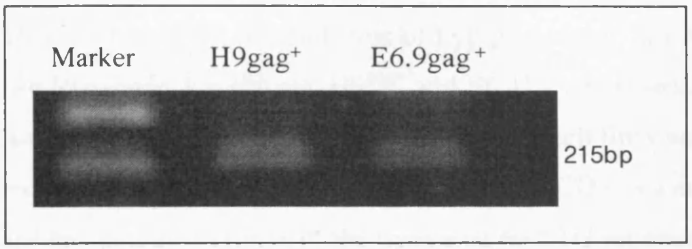


Figure 5.1 Genotyping of the T cell lines

The E6.1 $^{gag+}$ and H9 $^{gag+}$ cell lines were genotyped by a restriction fragment length polymorphism-PCR based assay. The C to T change at codon 620 creates a restriction site for *XcmI* in the *T allele. The polymorphism was identified by *XcmI* restriction endonuclease digestion of the PCR amplified fragment. The digestion was resolved on a 1% agarose gel. Following electrophoresis, the gel was stained with ethidium bromide and the fragments were visualized by U.V.

5.3 Generation of the panel of Lyp modified leukaemic T cell lines

To test whether the two isoforms of Lyp give rise to any functional differences in T cells, the two leukaemic T cell lines, $H9^{gag+}$ and $E6.1^{gag+}$, were lentivirally transduced with the two isoforms of Lyp. The two $H9^{gag+}$ and $E6.1^{gag+}$ cell lines were also transduced with a recombinant lentivirus encoding truncated rat CD2 as a control for the lentiviral integration and any potential effects of the truncated rat CD2 reporter gene used in the Lyp-2A-CD2 recombinant plasmids.

Generation of Lyp expressing $H9^{gag+}$ derived leukaemic T cell lines

Recombinant Lenti Lyp-2A-CD2 plasmids generated in Chapter 4 and recombinant Lenti CD2 plasmid, a kind gift from Mr Laurence Pearce, were used for the production of recombinant lentivirus particles using a three plasmid system consisting of a plasmid encoding the gene of interest under the control of an SFFV promoter, a pCMVdelta8.91 plasmid (coding for the *gag/pol* proteins under the control of a CMV promoter) and a pMGD2 plasmid (coding for the VSVG *env* protein) required for lentiviral replication and packaging, and viral entry into the cell respectively, under the control of the CMV promoter. A lipid based transfection method as described in Materials and Methods, was used to generate the recombinant lentivirus particles using this system.

$H9^{gag+}$ cells were transduced with either recombinant ^{R}Lyp-2A-CD2 or ^{W}Lyp-2A-CD2 or CD2 lentiviruses to generate the $H9^{gag+\ R}$Lyp-2A-CD2 or $H9^{gag+\ W}$Lyp-2A-CD2 or $H9^{gag+}$ CD2 cell lines, respectively. Following expansion the cells were assessed by flow cytometry for truncated rat CD2 reporter gene expression using an anti-rat CD2 PE antibody (Figure 5.2). To obtain populations of cells with high rat CD2 expression the $H9^{gag+}$ T cells were stained and positive cells sorted using MACs anti-PE magnetic sorting. Following sorting, cells were again stained to demonstrate the success of the sorting process, expanded and sorted further. Transduction efficiencies with all three lentiviruses, ^{R}Lyp-2A-CD2, ^{W}Lyp-2A-CD2 and CD2, were low with a maximum transduction efficiency of 37% (Figure 5.2). However, the percentage of cells staining positive for truncated rat CD2 was increased to 99% for $H9^{gag+\ R}$Lyp-2A-CD2 and $H9^{gag+\ W}$Lyp-2A-CD2 (Figure 5.2) by three subsequent rounds of cell sorting of the rat CD2 PE positive cells using anti PE magnetic beads as described in Materials and Methods. The cells were expanded after each sort before being

sorted again. The percentage of truncated rat CD2 expressing cells was increased to 97% for $H9^{gag+}$ CD2 (Figure 5.3) after two anti-PE magnetic sorts.

Generation of Lyp expressing $E6.1^{gag+}$ derived cell lines

$E6.1^{gag+}$ cells were transduced with either recombinant ^{R}Lyp-2A-CD2 or ^{W}Lyp-2A-CD2 or CD2 lentiviruses to generate $E6.1^{gag+}$ ^{R}Lyp-2A-CD2 or $E6.1^{gag+}$ ^{W}Lyp-2A-CD2 or $E6.1^{gag+}$ CD2 cell lines, respectively. Transduction efficiencies with ^{R}Lyp-2A-CD2 and ^{W}Lyp-2A-CD2 lentiviruses were comparable with over 90% of cells expressing rat CD2 as assessed by staining with anti-ratCD2 antibody (Figure 5.4). Transduction efficiency with CD2 lentiviruses was very high with over 99% of the cells expressing rat CD2 (Figure 5.4). Therefore, no cell sorting was performed.

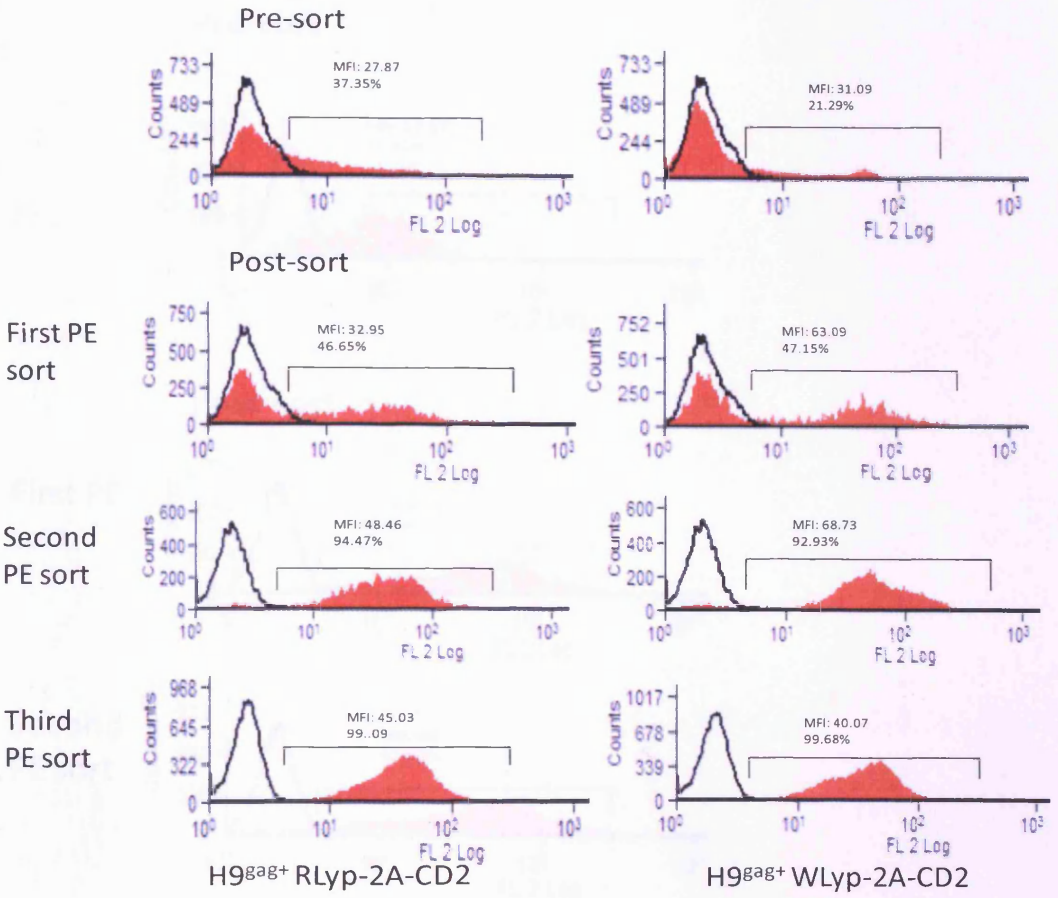


Figure 5.2 CD2 expression in H9^{gag+} cells pre- and post- sorting.

H9^{gag+} cells were lentivirally transduced with $^{R/W}$Lyp-2A-CD2 or CD2 recombinant viruses. Expression was assessed using anti-ratCD2 PE staining. Rat CD2 staining of the parental H9^{gag+} cell line not transduced with rat CD2 was used as a negative control and is shown in black. The cells were expanded after each sort and then sorted again three further times to get a final percentage of rat CD2 transduced cells of 99%.

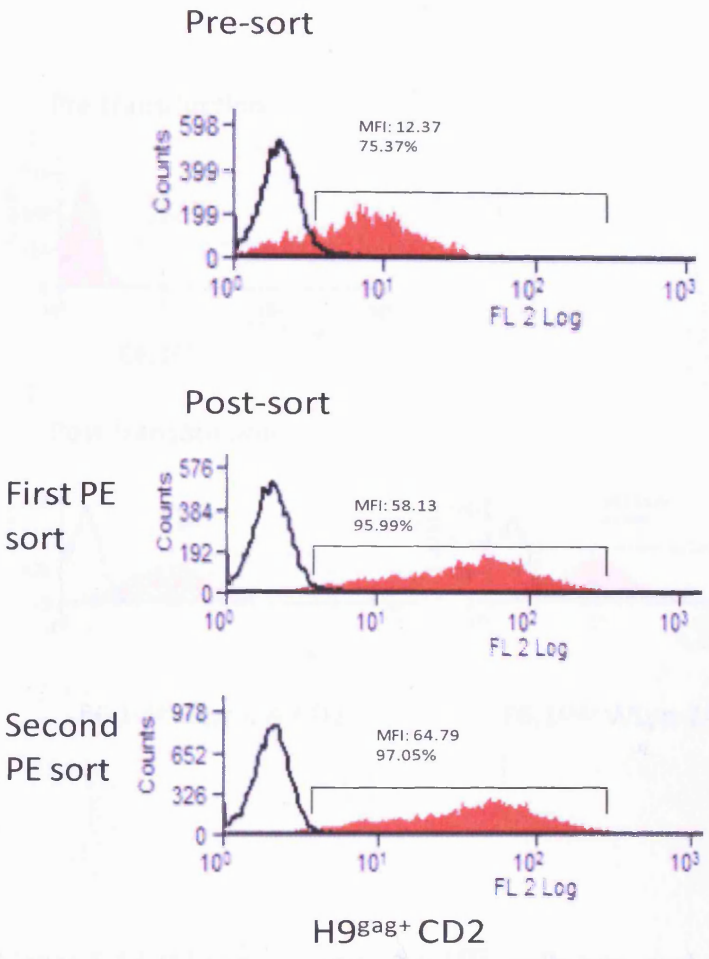


Figure 5.3 CD2 expression in H9^{gag+} cells pre- and post- sorting.

H9^{gag+} cells were lentivirally transduced with truncated rat CD2. Expression was assessed using anti-ratCD2 PE staining. Rat CD2 staining of the parental H9^{gag+} cell line not transduced with rat CD2 was used as a negative control and is shown in black. The cells were expanded after each anti-PE magnetic sort and then sorted again to get final percentage of rat CD2 expressing cells of over 97%.

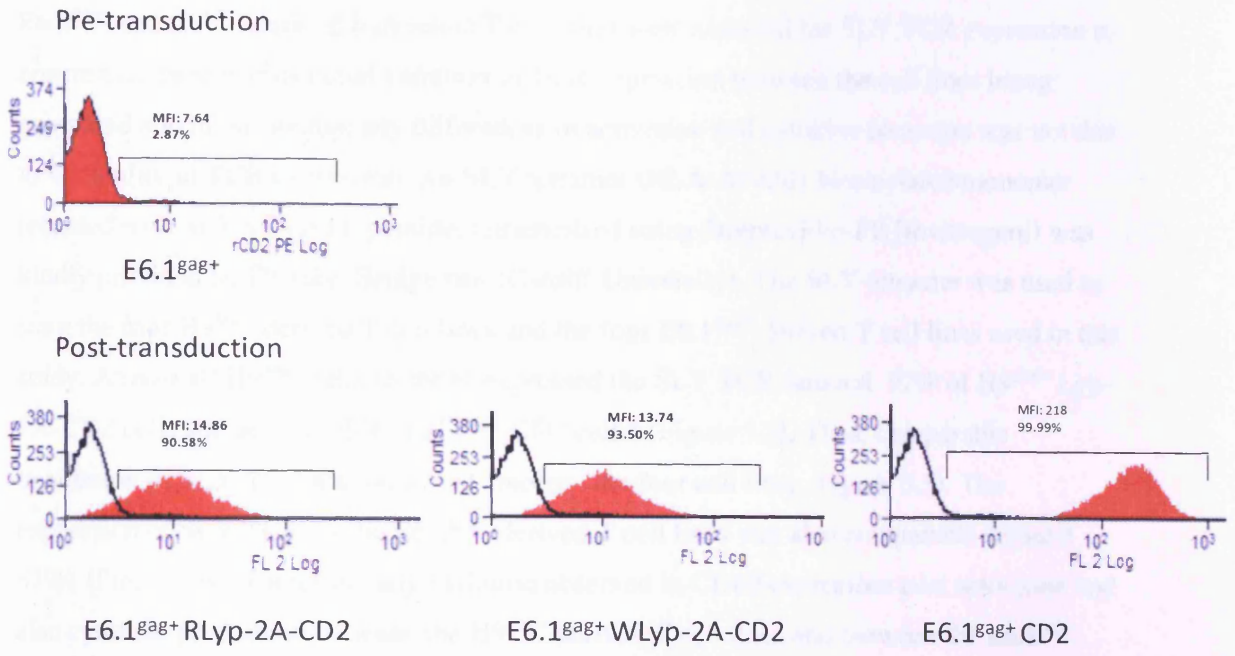


Figure 5.4 CD2 expression in E6.1^{gag+} cells pre- and post- transduction.

E6.1^{gag+} cells were lentivirally transduced with $^{R/W}$Lyp-2A-CD2 or CD2 recombinant viruses. Lyp expression was assessed using anti-ratCD2 PE staining of the truncated rat CD2 reporter gene. Rat CD2 staining of the parental E6.1^{gag+} cell line not transduced with rat CD2 was used as a negative control and is shown in black.

5.4 Assessment of the SLY TCR expression by the modified T cell lines

E6.1^{gag+} and H9^{gag+} derived leukaemic T cell lines were assessed for SLY TCR expression to ensure that there was minimal variation in TCR expression between the cell lines being compared and to ensure that any differences in activation and cytokine secretion was not due to variability in TCR expression. An SLY tetramer (HLA-A*0201 biotinylated monomer refolded with SLYNTVATL peptide, tetramerised using Streptavidin-PE [Invitrogen]) was kindly provided by Dr John Bridgeman (Cardiff University). The SLY tetramer was used to stain the four H9^{gag+} derived T cell lines and the four E6.1^{gag+} derived T cell lines used in this study. Almost all H9^{gag+} cells (> 99%) expressed the SLY TCR (around 97% of H9^{gag+} Lyp-2A-CD2 cells and around 95% of H9^{gag+} CD2 cells) (Figure 5.5). Thus, comparable expression of SLY TCR was observed amongst the four cell lines (Figure 5.5). The expression of SLY TCR by the E6.1^{gag+} derived T cell lines was also comparable (around 87%) (Figure 5.6). Therefore, any variation observed in CD69 expression post activation and also cytokine production between the H9^{gag+} derived T cell lines and between the E6.1^{gag+} derived cell lines should not be due to variation in TCR expression between the different cell lines.

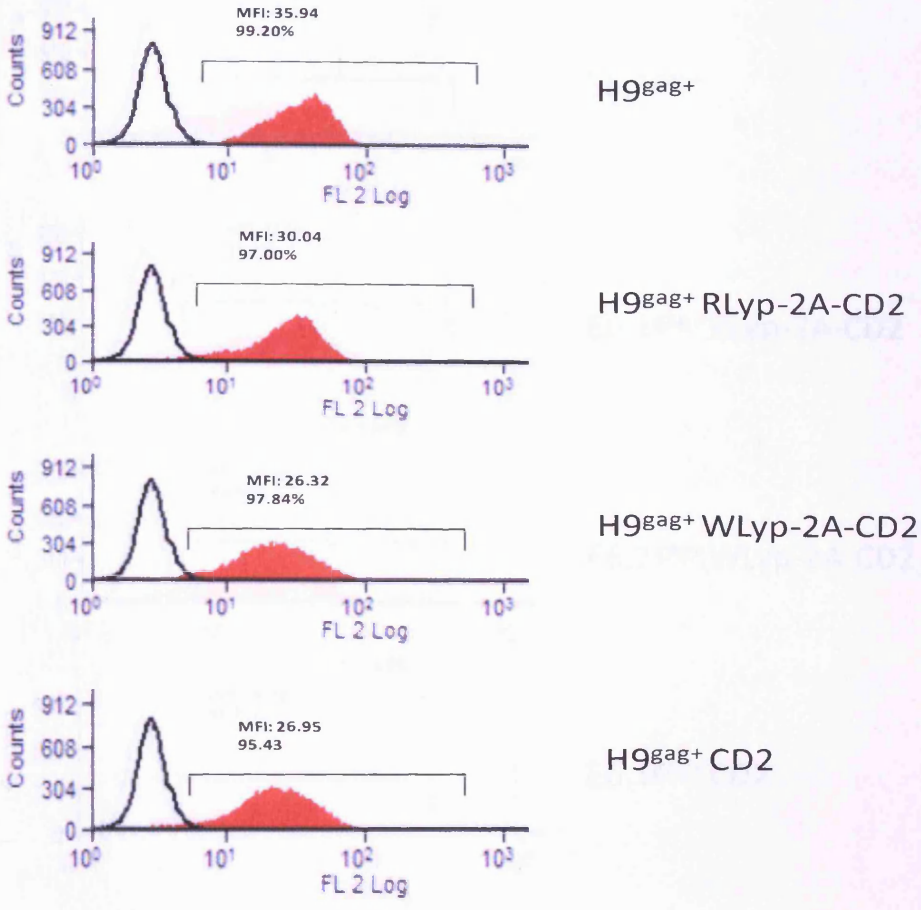
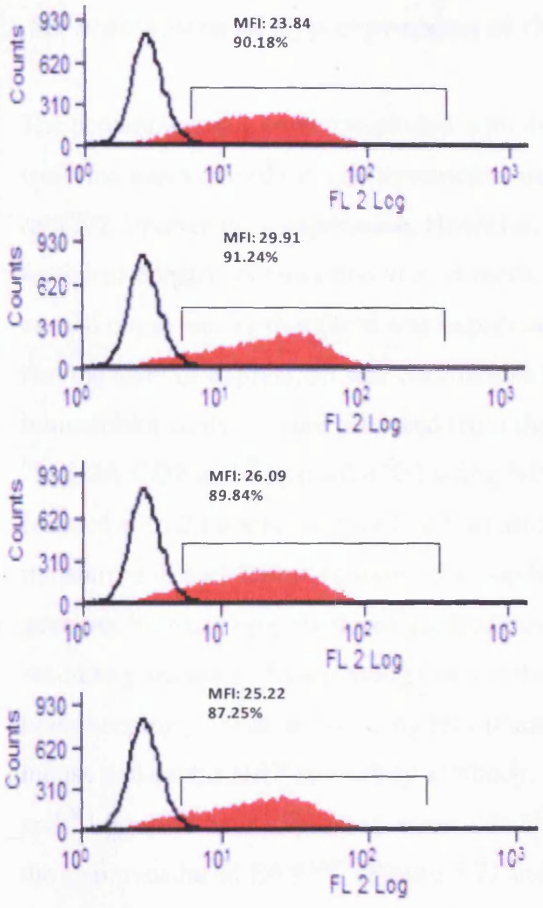


Figure 5.5 Assessment of the SLY TCR expression by the four H9gag$^+$ T cell lines.

SLY TCR expression (red) was assessed using a PE conjugated SLY tetramer. 1 x 10^6 cells were stained using a saturating concentration of a anti-SLY tetramer PE conjugate. A PE conjugated non-specific tetramer (black) was used as a negative control.



E6.1^{gag+}

E6.1^{gag+} RLyp-2A-CD2

E6.1^{gag+} WLyp-2A-CD2

E6.1^{gag+} CD2

Figure 5.6 Assessment of the SLY TCR expression by the four E6.1^{gag+} T cell lines

SLY TCR expression (red) was assessed using a PE conjugated SLY tetramer. 1 x 10^6 cells were stained using a saturating concentration of a anti-SLY tetramer PE conjugate. A PE conjugated non-specific tetramer (black) was used as a negative control.

5.5 Assessment of Lyp expression of the T cell lines by immunoblotting

The percentage of T cells transduced with the recombinant lentiviruses for each of the Lyp isoforms was verified by flow cytometric analysis for expression of the downstream truncated rat CD2 reporter gene expression. However, the upstream Lyp expression within the lentivirus constructs remained to be directly assessed. Therefore, immunoblotting was carried out to ensure that there was expression of the Lyp isoforms and not just rat CD2 and that the level of expression was comparable between the two isoforms of Lyp. Lysates for immunoblot analysis were prepared from the E6.9 $^{gag+}$ and H9^{gag+} T cells transduced with ^{R}Lyp-2A-CD2 and ^{W}Lyp-2A-CD2 using NP-40 as a membrane detergent. Lysates were reduced with β-mercaptoethanol and separated on a 10% polyacrylamide gel before being transferred to a PVDF membrane. The top half of the PVDF membrane was probed with a goat anti-human Lyp polyclonal antibody in conjunction with a mouse anti-goat HRP secondary antibody. As a loading control the bottom half of the membrane was probed for the housekeeping protein, actin, using rabbit anti-Actin polyclonal antisera in conjunction with a mouse anti-rabbit HRP secondary antibody. Immunoblot data demonstrated that both ^{R}Lyp and ^{W}Lyp were over-expressed as revealed by the stronger band that resolves at 110kDa in the Lyp transduced E6.9 $^{gag+}$ (Figure 5.7) and H9^{gag+} lysates (Figure 5.8) when compared to the non-transduced and CD2 transduced E6.9 $^{gag+}$ and H9^{gag+} T cell lines, respectively. The levels of over-expression between the R and W isoforms of Lyp were also found to be comparable in both the E6.9 $^{gag+}$ and H9^{gag+} T cell lines (Figures 5.7 and 5.8).

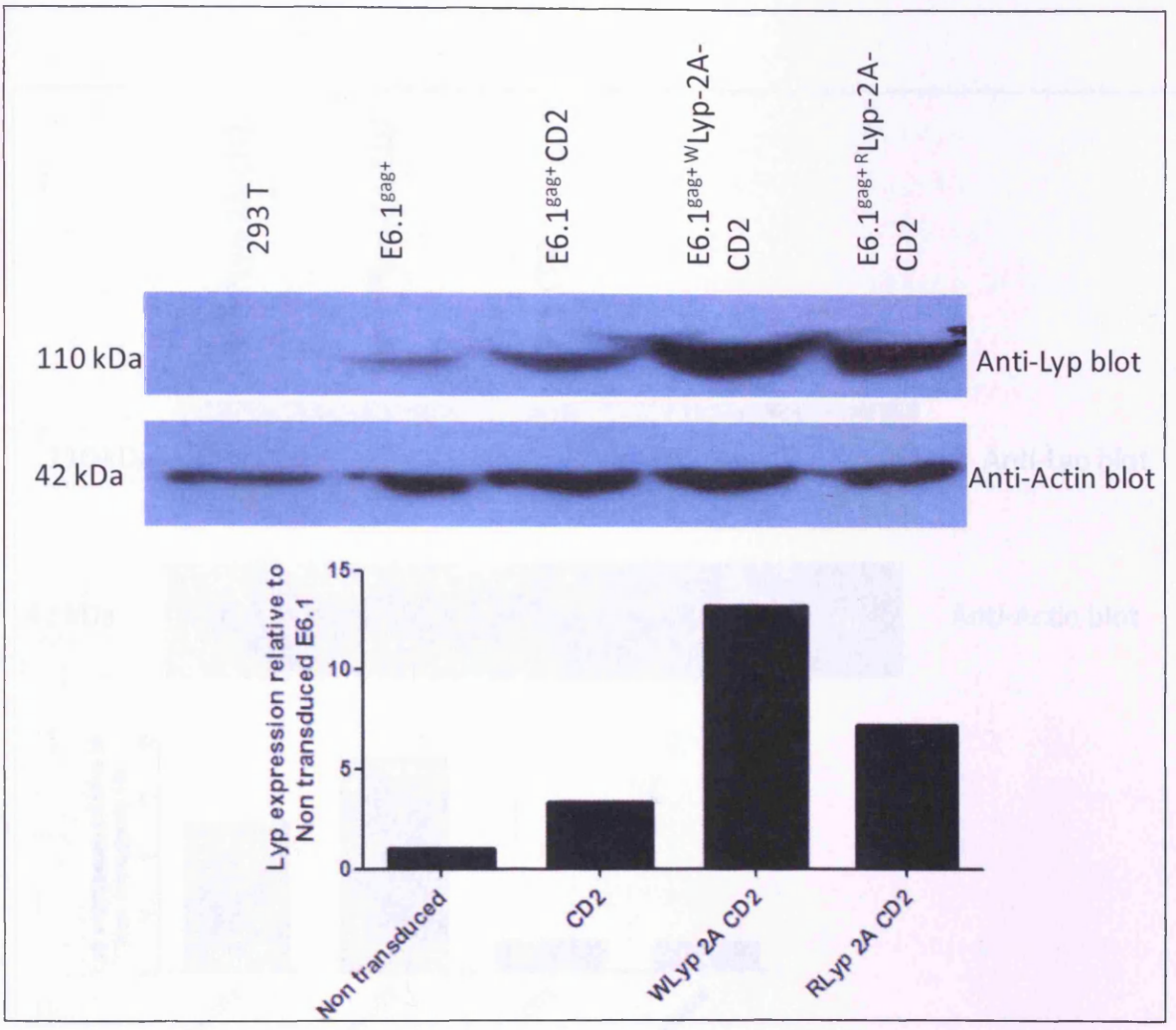


Figure 5.7 Immunoblot analysis of Lyp protein expression by the panel of E6.1^{gag+} T cell lines.

Transduced and non-transduced cells were lysed in NP-40 lysis buffer, reduced and equivalent numbers of cells loaded into each lane of a 10% SDS-PAGE gel. The PVDF membrane was either probed with a goat anti-human Lyp polyclonal antibody and mouse anti-goat HRP secondary antibody (top) or with rabbit anti-Actin polyclonal antibody and mouse anti-rabbit HRP secondary antibody (bottom). The 293T (Human Embryonic Kidney) cells were used as a negative control for Lyp expression. The densities of Lyp protein bands relative to Lyp protein band density in Non-transduced control cells are represented as bar graph (Bottom).

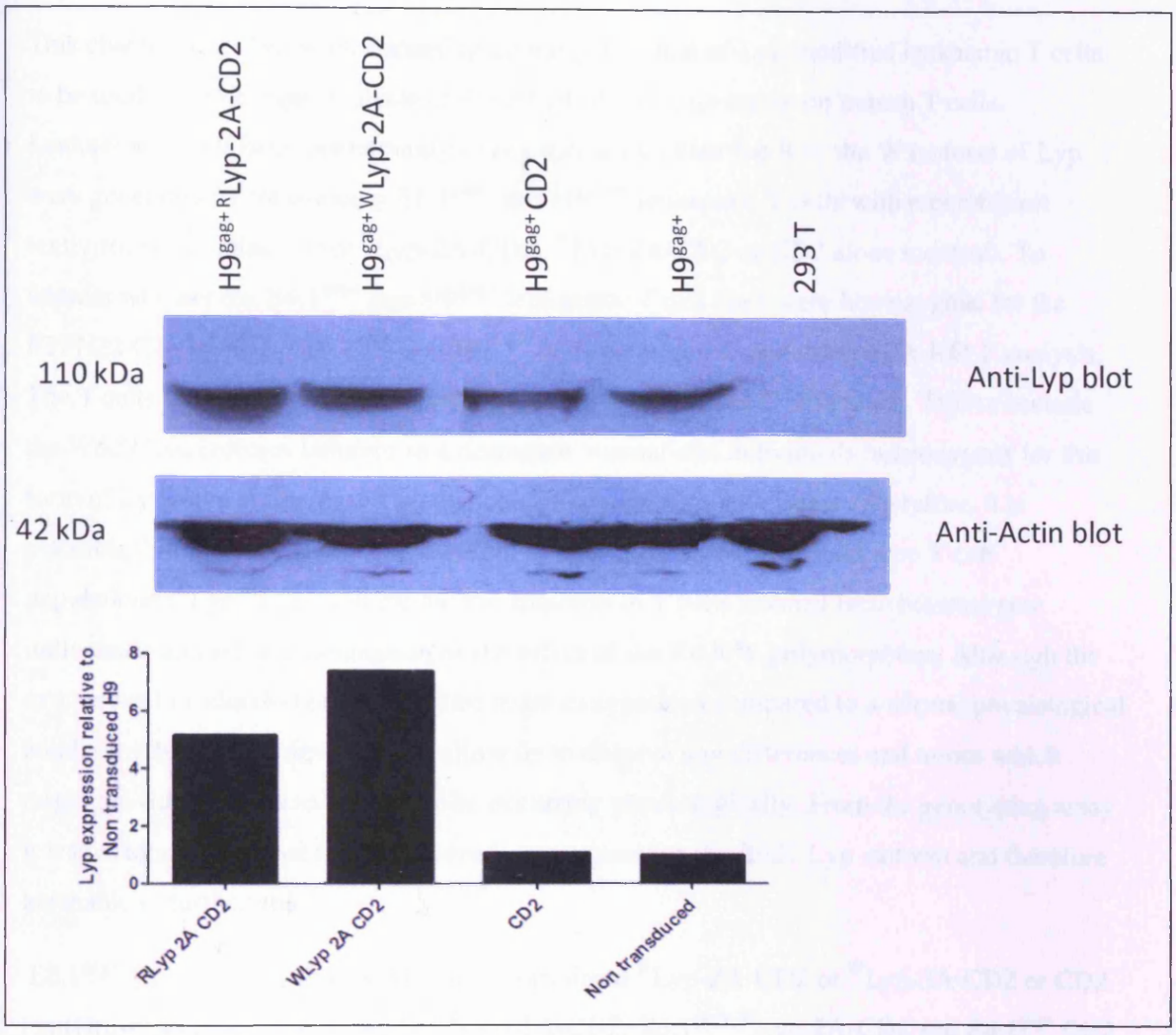


Figure 5.7 Immunoblot analysis of Lyp protein expression by the panel of H9^{gag+} T cell lines.

Transduced and non-transduced cells were lysed in NP-40 lysis buffer, reduced and equivalent numbers of cells loaded into each lane of a 10% SDS-PAGE gel. The PVDF membrane was either probed with a goat anti-human Lyp polyclonal antibody and mouse anti-goat HRP secondary antibody (top) or with rabbit anti-Actin polyclonal antibody and mouse anti-rabbit HRP secondary antibody (bottom). The 293T (Human Embryonic Kidney) cells were used as a negative control for Lyp expression. The densities of Lyp protein bands relative to Lyp protein band density in Non-transduced control cells are represented as bar graph (Bottom).

5.6 Discussion

This chapter describes work focussing on the generation of Lyp modified leukaemic T cells to be used for functional analysis of the effect of Lyp expression on human T cells. Leukaemic T cell lines permanently over expressing either the R or the W isoform of Lyp were generated by transducing E6.1^{gag+} and H9^{gag+} leukaemic T cells with recombinant lentiviruses encoding either ^{R}Lyp-2A-CD2, ^{W}Lyp-2A-CD2 or CD2 alone (control). To address whether the E6.1^{gag+} and H9^{gag+} leukaemic T cell lines were homozygous for the PTPN22 C1858 allele, E6.1^{gag+} and H9^{gag+} cells were genotyped using PCR-RFLP analysis. The T cells were required to be homozygous for the PTPN22 C1858 allele. This is because the W620 Lyp isoform behaves in a dominant manner and individuals heterozygous for this form of Lyp have an increased predisposition to autoimmune disease. Therefore, it is plausible that introducing exogenous Lyp W620 into homozygote wild type T-cell populations (^{R}Lyp/^{R}Lyp) will mimic the situation in T cells isolated from heterozygote individuals and allow examination of the effect of the R620W polymorphism. Although the exogenous introduction of Lyp may be more exaggerated compared to a normal physiological condition, this should nevertheless allow us to observe any differences and trends which might provide clues as to what may be occurring physiologically. From the genotyping assay it was demonstrated that the cells were homozygous for the R620 Lyp isoform and therefore amenable to further study.

E6.1^{gag+} T cells were transduced with recombinant ^{R}Lyp-2A-CD2 or ^{W}Lyp-2A-CD2 or CD2 lentiviruses to generate the E6.1^{gag+} ^{R}Lyp-2A-CD2, E6.1^{gag+} ^{W}Lyp-2A-CD2 and E6.1^{gag+} CD2 cell lines respectively. E6.1^{gag+} cells are derived from the Jurkat T cell line and are easily transducible. In this chapter, it was observed that E6.1^{gag+} T cells are very susceptible to viral entry as expected and gave transduction efficiencies of over 91% when transduced using the recombinant ^{R}Lyp-2A-CD2 or ^{W}Lyp-2A-CD2 or CD2 lentiviruses. The transduction efficiency using recombinant CD2 lentivirus was much higher at almost 100% compared to around 90% with the ^{R}Lyp-2A-CD2 or ^{W}Lyp-2A-CD2 recombinant lentiviruses. This is in line with the greater infectivity observed with the CD2 alone lentivirus compared to the others. It is not clear why this should be the case. It may be that the size of the inserted gene affects the generation of infectious virus particle and infectivity in that the larger the size of the gene insert the lower the virulence of the generated lentiviruses. Nevertheless, since all E6.1 derived cell lines had a transduction efficiency of greater than 90% and the R and W

isoforms of Lyp expressing E6.1 cells had comparable transduction efficiencies, it was concluded that any functional difference observed in the cells expressing the two isoforms of Lyp could be attributed to the effect of isoforms of Lyp on the cell thus the cell lines were considered ready for further analysis.

$H9^{gag+}$ T cells were also transduced with recombinant ^{R}Lyp-2A-CD2, ^{W}Lyp-2A-CD2 and CD2 lentiviruses to generate the $H9^{gag+\ R}$Lyp-2A-CD2, $H9^{gag+\ W}$Lyp-2A-CD2 and $H9^{gag+}$CD2 T cell lines, respectively. $H9^{gag+}$ T cells were more difficult to transduce compared to E6.1 T cells giving transduction efficiencies of 37% and 22% when transduced with recombinant ^{R}Lyp-2A-CD2 and ^{W}Lyp-2A-CD2, respectively. However, transduction with the recombinant CD2 alone lentiviruses gave a higher transduction efficiency of 75% but this was still lower than those observed with $E6.1^{gag+}$ T cells. The much higher transduction efficiencies seen with recombinant CD2 alone lentivirus compared to the recombinant Lyp encoding lentivirus is once again similar to that seen with $E6.1^{gag+}$ T cells and could be due to similar issues relating to insert size in the vector. The transduced rat CD2 expressing $H9^{gag+}$ cells were magnetically sorted multiple times on rat CD2 PE increasing the percentage of rat CD2 expressing cells to > 99% and 97% for $H9^{gag+\ R}$Lyp-2A-CD2, $H9^{gag+\ W}$Lyp-2A-CD2 and $H9^{gag+}$CD2 T cell lines, respectively. Since all $H9^{gag+}$ derived cell lines had a transduction efficiency of higher than 97% and the R and W isoforms of Lyp expressing $H9^{gag+}$ cells had comparable transduction efficiency at 99.09% and 99.68% respectively, it was concluded that any functional difference observed in the cells expressing the two isoforms of Lyp could be attributed to the effect of isoforms of Lyp on the cell and therefore, the cell lines were considered ready for further analysis.

In this chapter it was also demonstrated that SLY TCR expression was comparable between the different cell lines. Therefore, any variation observed in functional analysis between the $H9^{gag+}$ derived T cell lines expressing Lyp and those not expressing Lyp and between the $E6.1^{gag+}$ derived cell lines expressing Lyp and those not expressing Lyp could be attributed to the affect of Lyp expression on these cells and not due to any variation in TCR expression. Furthermore, the expression of Lyp protein in the cell lines was also directly assessed using immunoblotting where it was demonstrated that the expression of Lyp protein was up-regulated in the $E6.1^{gag+}$ and $H9^{gag+}$ T cells expressing the two isoforms of Lyp. It was also observed that the expression of the Lyp isoforms was comparable between the ^{R}Lyp and the ^{W}Lyp expressing $E6.1^{gag+}$ and $H9^{gag+}$ T cells. Therefore, any observed difference in the functional analysis between the cells expressing either ^{R}Lyp or ^{W}Lyp would be as a result of

the expression of that particular isoform of Lyp in the cells and not due to variations in expression of Lyp between the cells expressing the two different Lyp isoforms.

In summary, in this chapter E6.1 and H9 T cells expressing either the R or W isoforms of Lyp or CD2 alone were generated and phenotypic analysis of the generated T cell lines was performed. Firstly, it was demonstrated that the E6.1^{gag+} and H9^{gag+} T cells used to generate Lyp expressing cells were homozygous for the R620 Lyp isoform. It was also demonstrated that the E6.1^{gag+} and H9^{gag+} T cells expressing either of the two isoforms of Lyp or CD2 alone had a comparable percentage of cells expressing the rat CD2 reporter gene. Thirdly, the expression of exogenous SLY TCR was demonstrated to be comparable between the generated E6.1^{gag+} T cell lines and between the generated H9^{gag+} T cell lines. Lastly, a direct assessment of Lyp protein expression was carried out between the ^{R}Lyp and ^{W}Lyp expressing E6.1^{gag+} and H9^{gag+} T cell lines and it was demonstrated that there was comparable levels of Lyp expression in cells expressing either the R or the W isoform of Lyp. Collectively, the data presented in this chapter demonstrated that the ^{R}Lyp-2A-CD2, ^{W}Lyp-2A-CD2 or CD2 alone expressing E6.1^{gag+} and H9^{gag+} cell lines generated are comparable and ready to be used for functional analysis of Lyp isoform expression on T cells.

Chapter 6

The effect of over-expression of Lyp isoforms on cytokine production by human leukaemic T Cell lines

6.1 Introduction

Lyp is regarded to be a suppressor of TCR signalling and the Lyp R620W variant has been further shown to be a "gain of function" polymorphism (Aarnisalo et al. 2008; Rieck et al. 2007; Vang et al. 2005). Previous studies have also investigated the effect of the R620W polymorphism on cytokine production by T cells. Vang et al (2005) was the first to observe that T cells (involving no purification of phenotypic lineages such as $CD4^+$ or $CD8^+$) from Type 1 Diabetes (T1D) patients who were heterozygous for the R620W polymorphism (genotype PTPN22 $^{1858C/1858T}$) secrete less IL-2 than T cells from T1D patients homozygous for the arginine isoform of Lyp (genotype PTPN22 $^{1858C/1858C}$). Aarnisalo et al (2008) also observed a reduction in IL-2 production by $CD4^+$ T cells from T1D patients heterozygous for the R620W polymorphism compared to $CD4^+$ T cells from T1D patients homozygous for the R isoform of Lyp. Rieck et al (2007) investigated the functional effect of the Lyp R620W polymorphism on $CD4^+$ T cells from healthy individuals by examining the secretion of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α and reported a significant reduction in IL-10 production by PTPN22 $^{1858C/1858T}$ expressing $CD4^+$ T cells relative to PTPN22 $^{1858C/1858C}$ expressing $CD4^+$ T cells. In addition they also noted a decrease in IL-2 and IL-4 secretion, which did not reach statistical significance and no difference in IFN-γ, and TNF-α secretion in $^{1858C/1858T}$ $CD4^+$ T cells relative to PTPN22 $^{1858C/1858C}$ expressing $CD4^+$ T cells (Rieck et al. 2007). Rieck et al also observed a decrease in CD25 activation marker expression by $^{1858C/1858T}$ expressing $CD4^+$ T cells relative to $^{1858C/1858C}$ expressing $CD4^+$ T cells. These results are striking and highlight the potential importance of Lyp in T cell biology. However, from both of these studies it cannot be conclusively determined that the differences seen in cytokine production and CD25 expression are due to an intrinsic effect of Lyp isoform expression on T cell function following TCR triggering. The above studies do not take into account possible skewing of T cell subsets as a result of Lyp isoform expression. For example, a possible decrease in IL-10 producing cells in genotype $^{1858C/1858T}$ individuals may account for the decreased IL-10 secretion that was observed and therefore there may be no intrinsic effect of the Lyp R620W polymorphism on TCR triggered cytokine production. Vang et al (2005)

reported that there was no skewing of $CD4^+$ T cell, $CD8^+$ T cell and memory T cell subset observed in the patient samples. However these heterogeneous polyclonal populations may contain subpopulations of memory and effector phenotypes that may be skewed as a result of Lyp isoform expression.

In addition, the T cells isolated from patient samples and $CD4^+$ cells isolated from healthy individuals used in the above studies (Rieck et al. 2007; Vang et al. 2005) may have distinct TCR signalling thresholds influenced by the products of multiple genes which would potentially obscure interpretation of the observed variations in cytokine secretion.

In the study by Vang et al (2005) it was also observed that primary human T cells nucleofected with ^{R}Lyp secrete less IL-2 than those nucleofected with ^{W}Lyp and that T cells transfected with either forms of Lyp produced less IL-2 that the control T cells. There is however an ambiguity about this study with the nucleofection efficiency stated at between 60-80%. It is not clear if the T cell populations transfected with the two Lyp isoforms have a comparable transfection efficiency. If the percentage of T cells transfected with ^{R}Lyp was 60% and the population transfected with ^{W}Lyp was 80%, the difference in IL-2 production between the two isoforms of Lyp could be simply due to more Lyp being expressed in the ^{W}Lyp transfected population rather than due to differences in the two Lyp isoforms. Vang et al (2005) also nucleofected Jurkat T cells with either ^{W}Lyp or ^{W}Lyp and a luciferase reporter gene driven by the nuclear factor of activated T cells (NFAT) with the two isoforms of Lyp. In this study it was observed that when stimulated via their TCR, Jurkat T cells expressing varying amount of Lyp show reduced luciferase activity in a dose dependent manner and that Jurkat T cells expressing ^{W}Lyp always exhibited lower luciferase activity when compared to ^{R}Lyp at each level of Lyp expression.

A comprehensive analysis of activation marker expression and cytokine production by T cells over-expressing either the R or W isoform of Lyp has not been performed. Therefore work described in this chapter sought to address whether expression of CD69, an early activation marker, is altered in cells over-expressing one or other of the two isoforms of Lyp. In addition, cytokine production by a homogenous population of T cells over-expressing either the R or the W isoform of Lyp was investigated using cells lines derived from two distinct human T cell leukaemias, E6.1 (Jurkat) and H9 (Hut78). The monoclonality of the T cells in each cell line meant that any observed effects due to Lyp isoform expression are intrinsic to that particular subset of T cells and are not due to skewing of the T cell subsets or other

genetic effects on the signalling thresholds. This focusing on a single homogenous population of T cells should allow further investigation and dissection of the pathways that might be affected by Lyp.

6.2 Analysis of CD69 expression by the panel of E6.1 $^{gag+}$ cells

E6.1$^{gag+\ R}$Lyp-2A-CD2, E6.1$^{gag+\ W}$Lyp-2A-CD2, E6.1^{gag+}CD2 and E6.1^{gag+} non transduced control cells were stimulated for 24 hours with APCs pulsed with varying concentrations of the SLY peptide before staining with PE conjugated anti-human CD69 antibodies and analysing by flow cytometry. Figure 6.2 shows the cellular response to SLY peptide stimulation with induced CD69 expression indicating the degree of T-cell activation (Figure 6.2). All T cells demonstrated a response to SLY peptide stimulation with a positive correlation of CD69 expression with peptide concentration. The lowest levels of CD69 expression were observed in response to the lowest concentrations of SLY peptide. Approximately 22% of all T cells express CD69 when left unstimulated which increases to approximately 43% when the cells are stimulated with the highest concentration of peptide pulsed APCs (Figure 6.2). All four cell lines exhibited comparable CD69 upregulation upon peptide stimulation. The result from the E6.1^{gag+} T cells suggest that over-expression of Lyp does not result in a down-regulation in the expression of CD69 (or inhibition of up-regulation) in Lyp expressing cells when compared to control cells (non-transduced and CD2 transduced) as might have been expected. In addition, there was no difference in CD69 expression between the cells transduced with the two isoforms of Lyp. However, it cannot be formally concluded from these data that there are no differences in the functional capacity of the Lyp transduced and control cells.

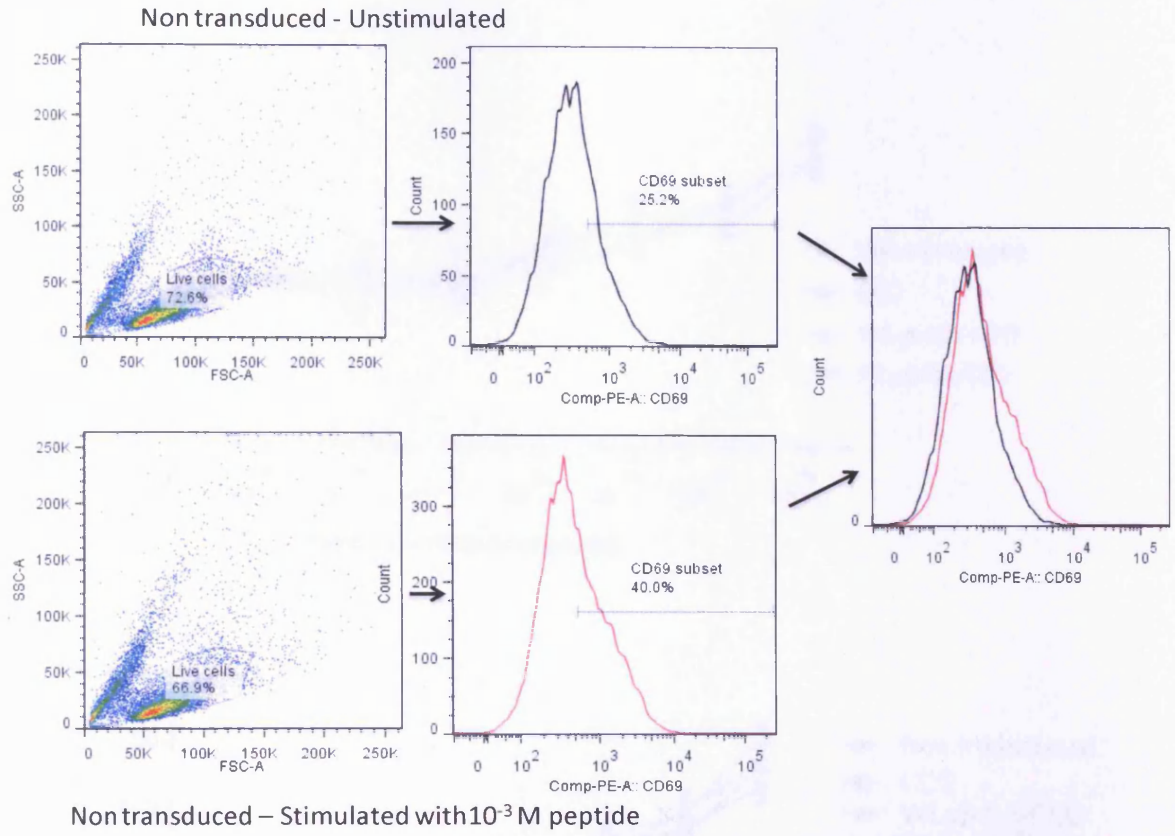


Figure 6.1 Analysis of flow cytometric data from T cell activation assays.

Figure showing the method used to analyse flow cytometric data from T cell activation assays. Non-transduced E6.1^{gag+} T cells, which were either left unstimulated (top, black line) or incubated in the presence 10^{-3} M concentration of SLY peptide (bottom, red line) for 24 hours were analysed using a flow cytometer following staining with PE-conjugated anti-CD69 antibody. FACS dot plot showing gating of live T cell population (left). The gated live T cell population was further analysed for CD69 PE expression using a histogram. The gating for separating CD69^{-} and CD69^{+} T cell population is indicated in the histogram (middle). Shown on the right is an overlay showing the CD69 expression in an unstimulated and 10^{-3} M peptide stimulated T cells.

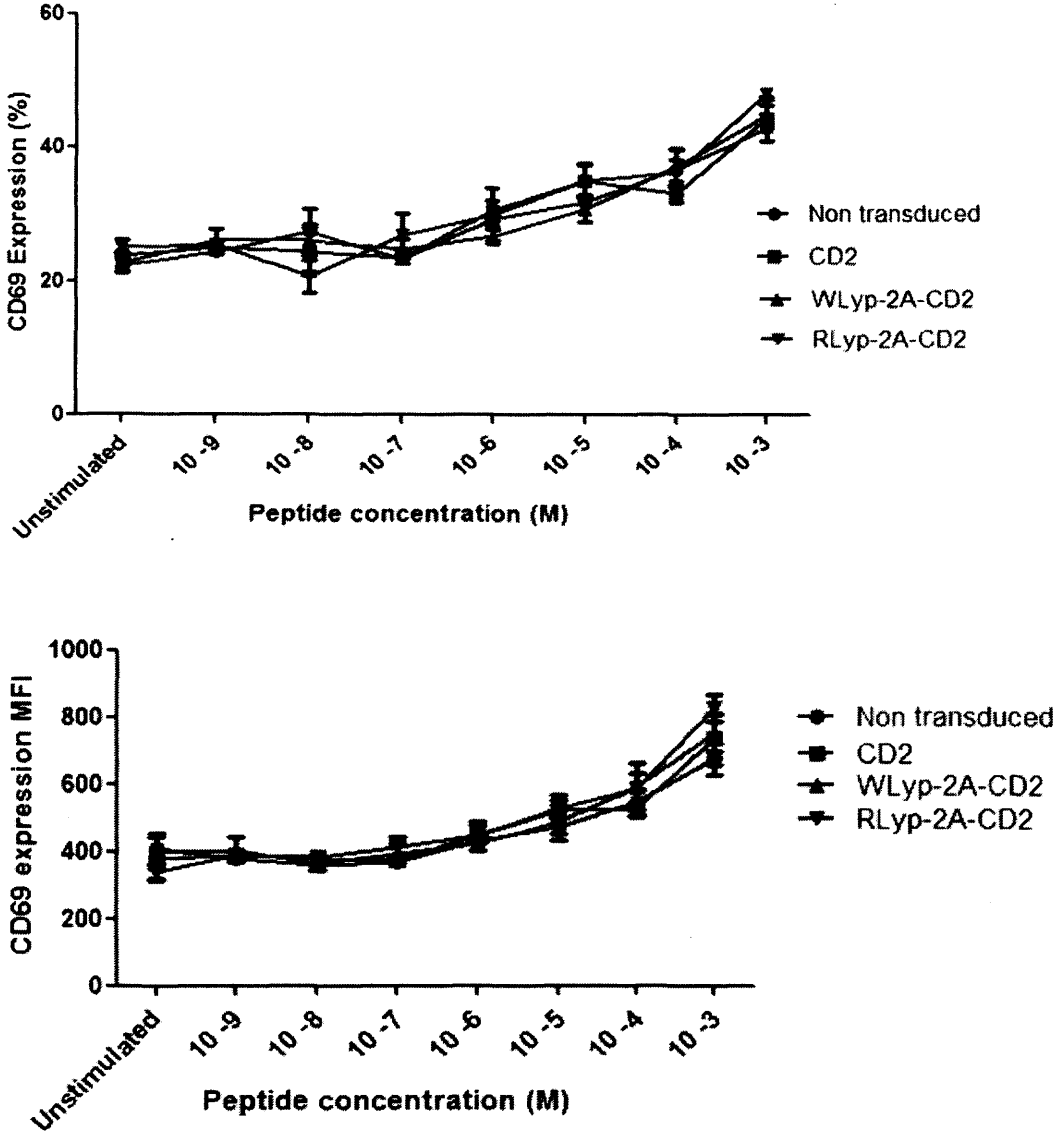


Figure 6.2 Activation profiles of E6.1^{gag+} T cell lines expressing the two isoforms of Lyp.

Non-transduced E6.1^{gag+} T cells, E6.1^{gag+} T cells expressing rat CD2 only or E6.1^{gag+} T cells expressing the indicated form of Lyp were incubated in the presence of varying concentrations of SL9 peptide for 24 hours. Following incubation T cells were stained with PE-conjugated anti-CD69 antibody and analysed using a flow cytometer. Results are representative of four separate experiments. Top figure shows percentage of CD69 expressing cells whereas bottom figure shows the Mean flurosence intensity (MFI) of the CD69 PE expression. The data are presented as mean ± SD.

6.3 Analysis of CD69 expression by the panel of H9 $^{gag+}$ cells

$H9^{gag+\ R}$Lyp-2A-CD2, $H9^{gag+\ W}$Lyp-2A-CD2, $H9^{gag+}$ CD2 and $H9^{gag+}$ non transduced control cells were stimulated for 24 hours with APCs pulsed with varying concentrations of SLY peptide before staining with PE conjugated anti-human CD69 antibodies and analysing by flow cytometry. Figure 6.3 shows the cellular response to SLY peptide stimulation with CD69 expression indicating the degree of T-cell activation. All cells showed a response to SLY peptide stimulation with a positive correlation between CD69 expression and peptide concentration. The lowest levels of CD69 expression were observed in response to no SLY peptide stimulation. Approximately 22% of all T cells expressed CD69 when unstimulated which increases to a maximum of 67% of all T cells when the cells are stimulated with the highest concentration of peptide pulsed APCs (Figure 6.3). All four T cell lines show a comparable ability to upregulate CD69 upon peptide stimulation. The result from the $H9^{gag+}$ T cell experiments suggest that over-expression of Lyp does not result in lower expression of CD69 when compared to control cells (non-transduced and CD2 transduced) as might have been expected. In addition, there is no difference in CD69 expression when comparing the cells transduced with the two isoforms of Lyp. However, as above, it cannot be formally concluded from these data that there are no differences in the functional capacity of the Lyp transduced and control H9 cells.

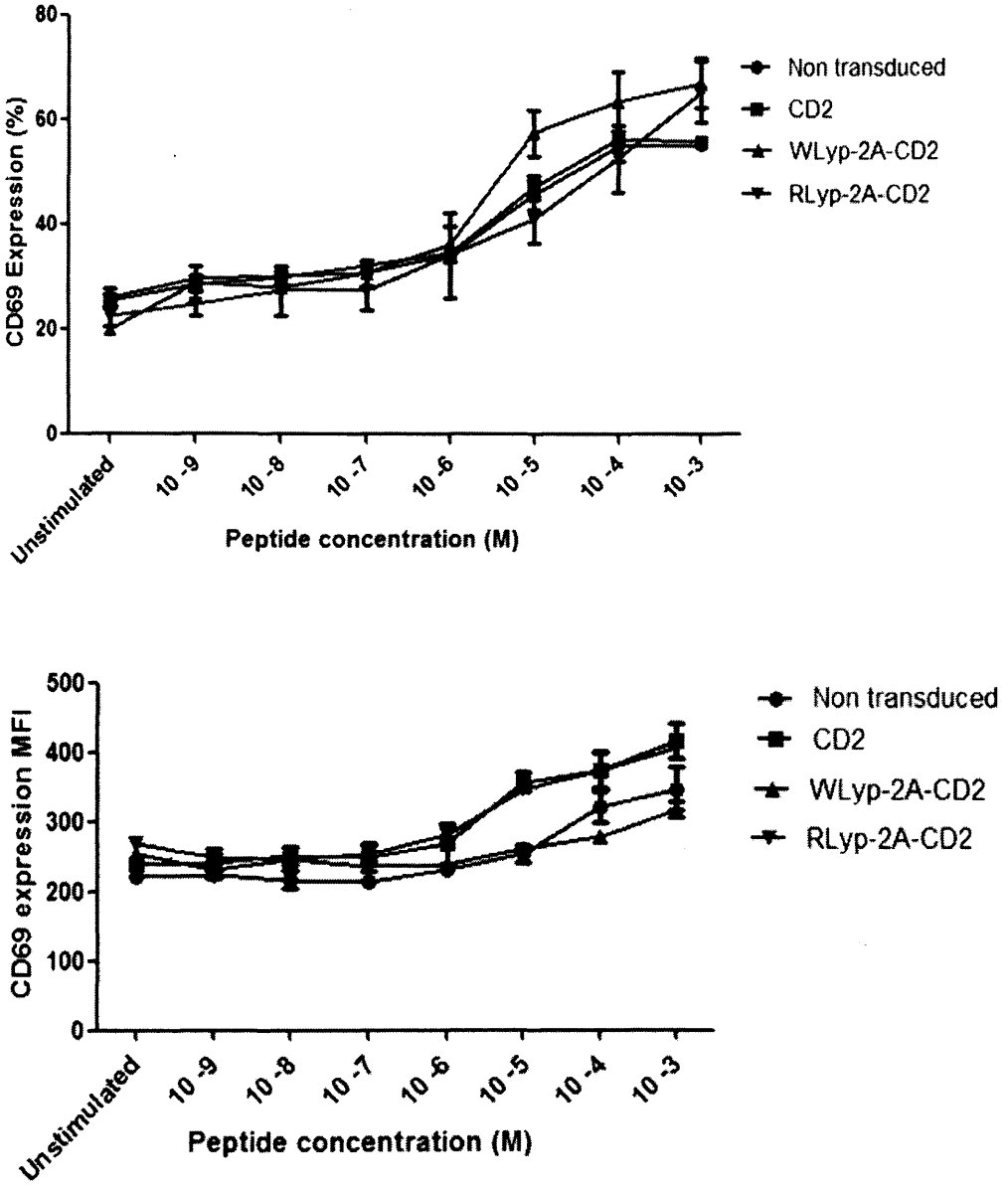


Figure 6.3 Activation profiles of H9^{gag+} T cell lines expressing the two isoforms of Lyp.

Non-transduced H9^{gag+}T cells, H9^{gag+} T cells expressing rat CD2 only or H9^{gag+} T cells expressing the indicated form of Lyp were incubated in the presence of varying concentrations of SLY peptide presented by APCs for 24 hours. Following incubation cells were stained with PE-conjugated anti-CD69 antibody and analysed using a flow cytometer. Results are representative of four separate experiments. Top figure shows percentage of CD69 expressing cells whereas bottom figure shows the Mean flurosence intensity (MFI) of the CD69 PE expression.The data are presented as mean ± SD.

6.4 Functional analysis of cytokine production by the panel of Lyp modified E6.1^{gag+} leukaemic T cells.

In order to determine whether significant experimental differences could be detected in the activation of the Lyp modified T cells, in this section, cytokine secretion by Lyp transduced and control cells (both non-transduced and truncated rat CD2 alone transduced) was investigated. E6.1^{gag+} T cells transduced and expanded as described in the previous chapter were tested for IL-2 and MIP-1β cytokine production in response to SLY peptide stimulation in the presence of APCs. E6.1^{gag+} T cells were left untransduced, transduced with truncated rat CD2 or transduced with either the R or W isoforms of Lyp. Following *in vitro* expansion, cells were stimulated for 24 hours with SLY peptide pulsed APCs and IL-2 and MIP-1β release in culture supernatants was measured (Figures 6.4-6.7). E6.1^{gag+} leukaemic T cells only produce detectable amount of IL-2 and MIP-1β cytokines. They are not known to secrete detectable levels of IL-10.

6.4.1 Minimal differences in IL-2 secretion in Lyp transduced E6.1^{gag+} T cells compared to control E6.1^{gag+} T cells.

An IL-2 ELISA was carried out as described in Materials and Methods using supernatants from four separate experiments. Results from two representive experiments are shown (Figures 6.4-6.5). The result of the first experiment shows a slight reduction in IL-2 secretion by Lyp transduced E6.1^{gag+} T cells compared to the non-transduced control (Figure 6.4). The result from the second experiment (Figure 6.5) shows no difference in IL-2 secretion between any of the four cell lines compared. The conclusion from the data of all four separate experiments is that there appears to be a trend towards a decrease in IL-2 secretion by Lyp transduced E6.1^{gag+} T cells compared to control E6.1^{gag+} T cells.

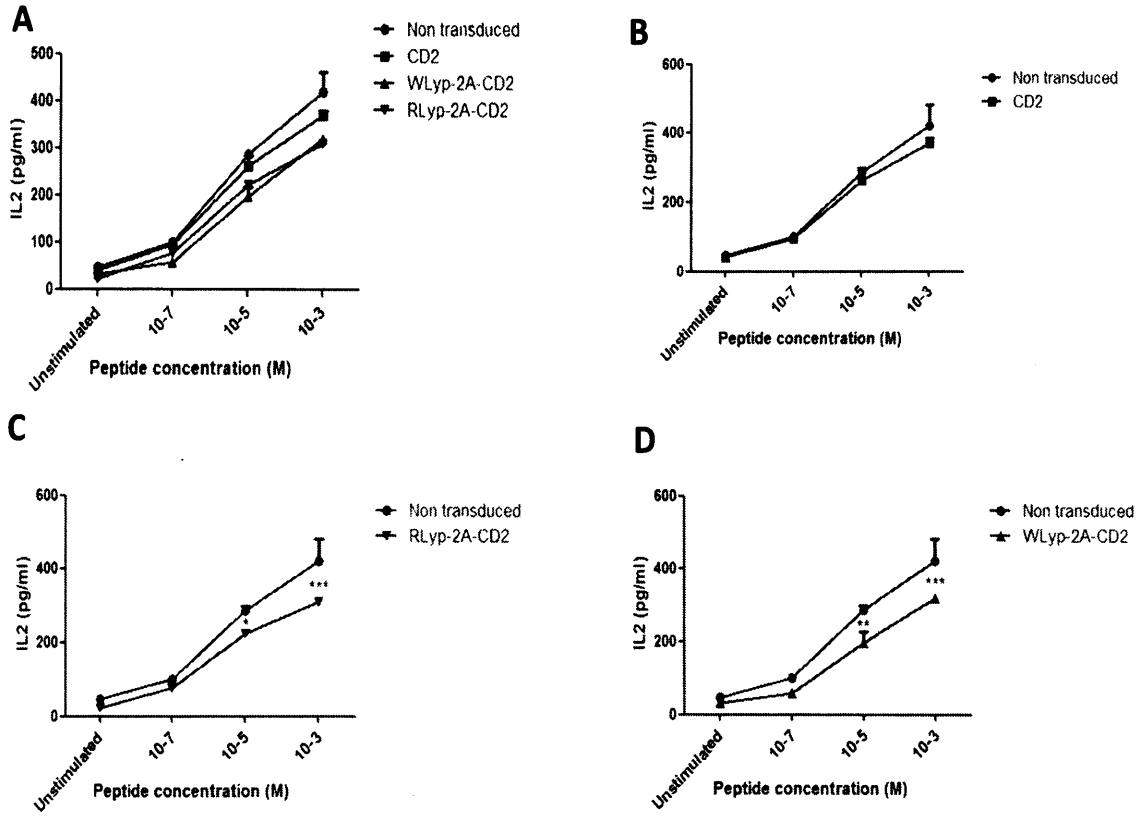


Figure 6.4 Experiment 1: The effect of Lyp isoform expression on peptide pulsed APC mediated IL-2 release in transduced E6.1^{gag+} T cells.

Non transduced E6.1^{gag+} T cells or T cells expressing CD2+/- the indicated isoform of Lyp were activated for 24 hours with different concentrations of SLY peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an IL-2 ELISA kit. A) Effect of varying peptide concentration on IL-2 secretion in all four E6.1^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on IL-2 secretion in E6.1^{gag+} CD2 cells with the non transduced E6.1^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on IL-2 secretion in E6.1$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced E6.1^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on IL-2 secretion in E6.1$^{gag+\ W}$Lyp-2A-CD2 cells with non transduced E6.1^{gag+} T cells. Result from one experiment. The data are presented as mean ± SD. $*P < 0.05$ $** P < 0.01$ $*** P < 0.001$ by Two way ANOVA compared to Non-transduced E6.1^{gag+} T cells.

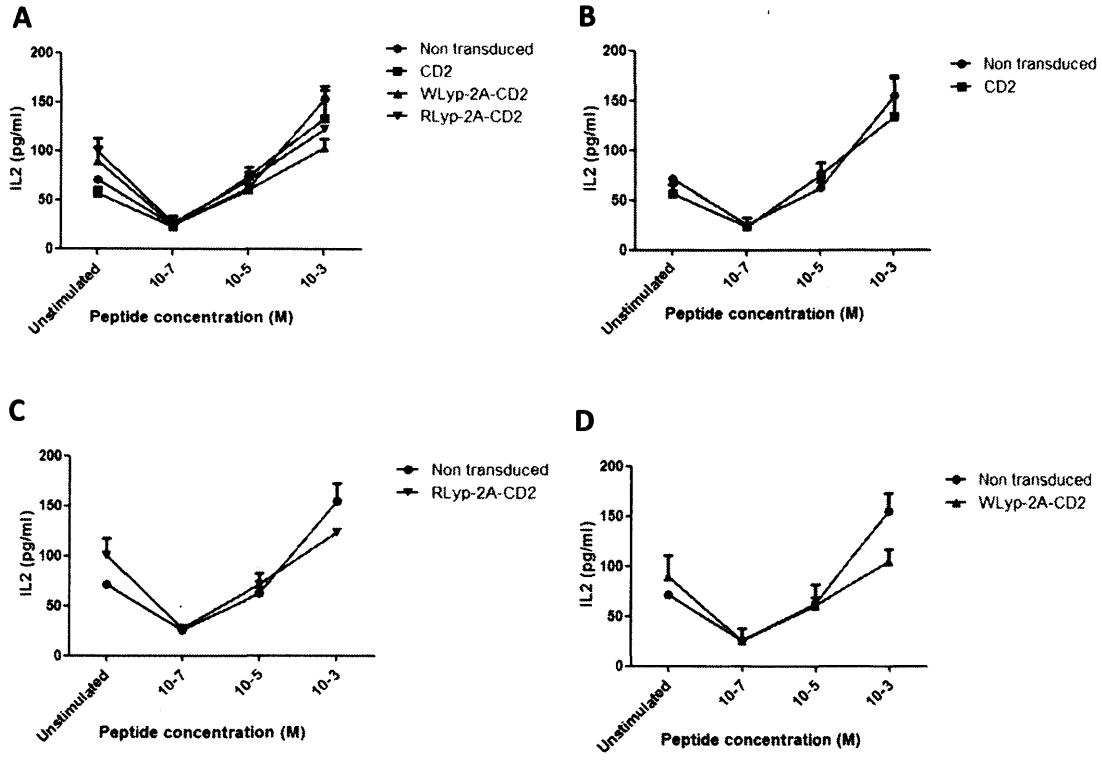


Figure 6.5 Experiment 2: The effect of Lyp isoform expression on peptide pulsed APC mediated IL-2 release in transduced E6.1^{gag+} T cells.

Non transduced E6.1^{gag+} T cells or T cells expressing the indicated isoform of Lyp were activated for 24 hours with different concentrations of SLY peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an IL-2 ELISA kit. A) Effect of varying peptide concentration on IL-2 secretion in all four E6.1^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on IL-2 secretion in E6.1^{gag+} CD2 cells with the non transduced E6.1^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on IL-2 secretion in E6.1$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced E6.1^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on IL-2 secretion in E6.1$^{gag+\ R}$Lyp-2A-CD2 cells with non transduced E6.1^{gag+} T cells. Result from one experiment. The data are presented as mean ± SD.

6.4.2 A lack of difference in MIP-1β secretion in Lyp transduced E6.1^{gag+} T cells compared to non Lyp transduced E6.1^{gag+} T cells

A MIP-1β ELISA was done with supernatants from four separate experiments. Results from all four of the experiments are shown (Figures 6.6-6.7). Experiments shown 6.6 and 6.7 show no difference in MIP-1β secretion by Lyp transduced E6.1^{gag+} T cells compared to the controls (non transduced and rat CD2 transduced E6.1^{gag+} T cells). In addition, there was no significant difference in MIP-1β secretion between the cells transduced with the two isoforms of Lyp.

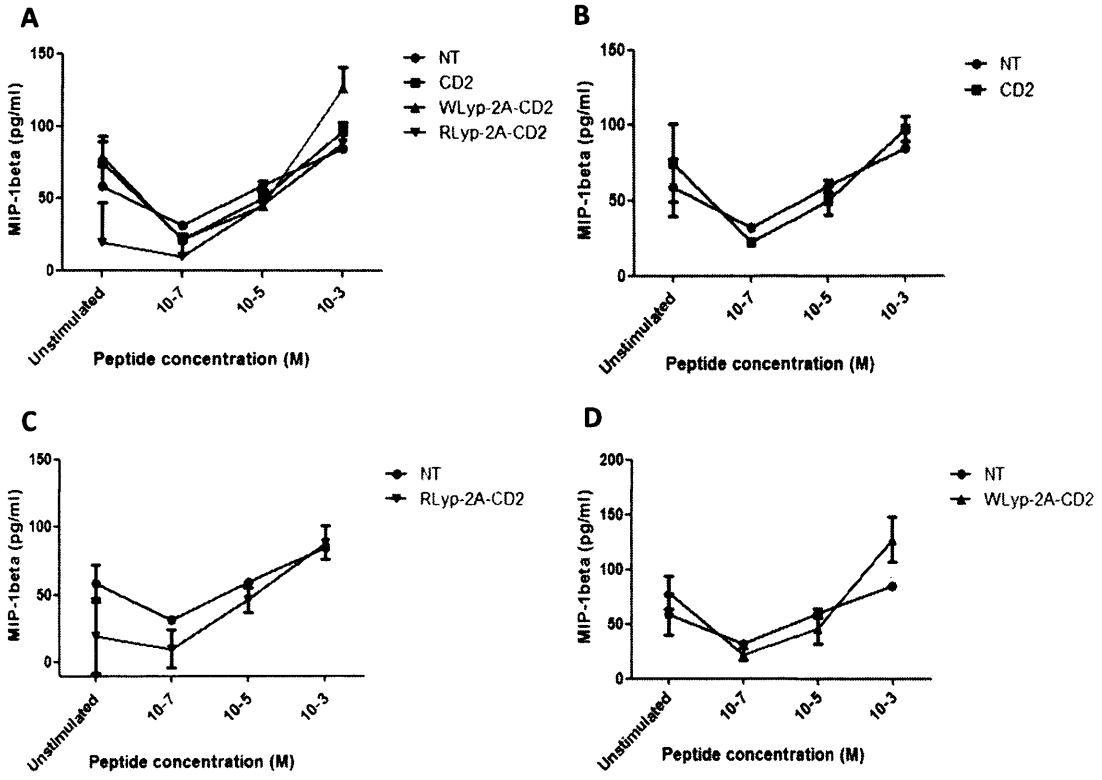


Figure 6.6 Experiment 1: MIP-1β secretions by E6.1^{gag+} cells expressing the two isoforms of Lyp.

Non transduced E6.1^{gag+} T cells or T cells expressing the indicated isoform of Lyp were activated for 24 hours with different concentrations of SLY peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an MIP-1β ELISA kit. A) Effect of varying peptide concentration on MIP-1β secretion in all four E6.1^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on MIP-1β secretion in E6.1^{gag+} CD2 cells with the non transduced E6.1^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on MIP-1β secretion in E6.1$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced E6.1^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on MIP-1β secretion in E6.1$^{gag+\ W}$Lyp-2A-CD2 cells with non transduced E6.1^{gag+} T cells. Result from one experiment. The data are presented as mean ± SD.

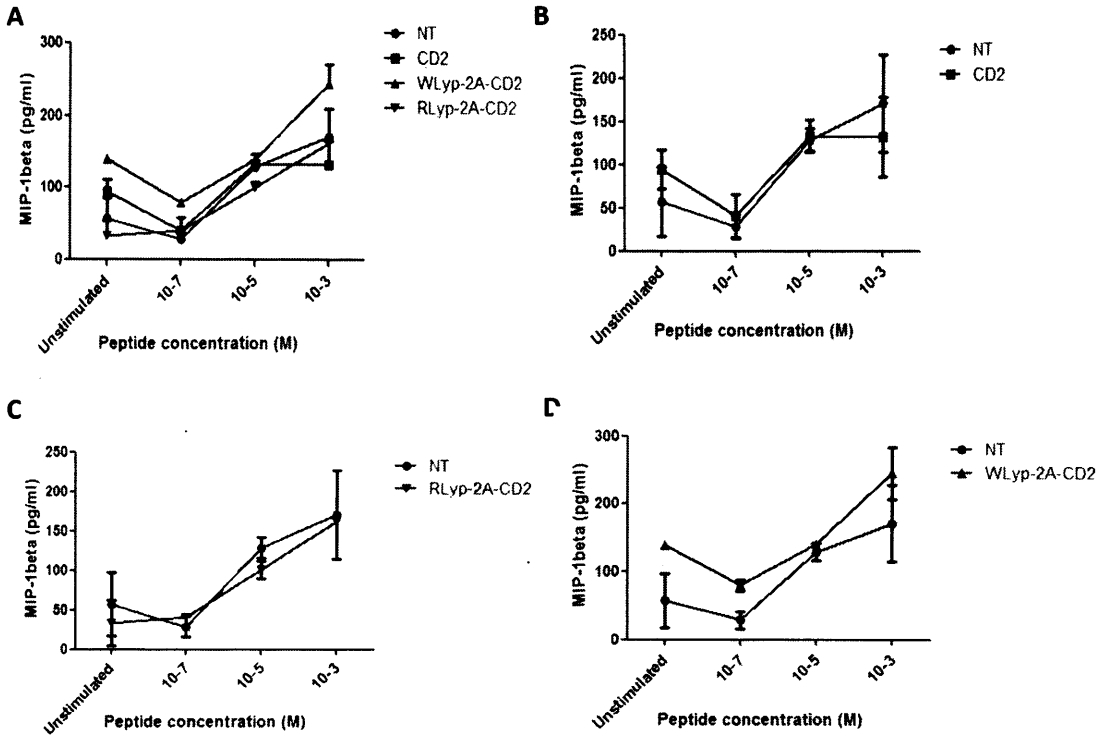


Figure 6.7 Experiment 2: MIP-1β secretions by E6.1^{gag+} cells expressing the two isoforms of Lyp.

Non transduced E6.1^{gag+} T cells or T cells expressing the indicated isoform of Lyp were activated for 24 hours with different concentrations of SLY peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an MIP-1β ELISA kit. A) Effect of varying peptide concentration on MIP-1β secretion in all four E6.1^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on MIP-1β secretion in E6.1^{gag+} CD2 cells with the non transduced E6.1^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on MIP-1β secretion in E6.1$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced E6.1^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on MIP-1β secretion in E6.1$^{gag+\ W}$Lyp-2A-CD2 cells with non transduced E6.1^{gag+} T cells. Result from one experiment. The data are presented as mean ± SD.

6.5 Functional analysis of cytokine production by the panel of Lyp modified $H9^{gag+}$ T cells.

In order to determine whether significant experimental differences could be detected in cytokine production by the Lyp transduced (including both isoforms of Lyp) and control cells, $H9^{gag+}$ T cells were left un-transduced, transduced with truncated rat CD2 or transduced with either the R or W isoforms of Lyp. Following *in vitro* expansion, T cells were stimulated for 24 hours with varying concentrations of SLY peptide pulsed APCs and IL-2 and IL-10 release in culture supernatants was measured using ELISA (Figures 6.8-6.11).

6.5.1 Minimal differences in IL-2 secretion in Lyp transduced $H9^{gag+}$ T cells compared to non Lyp transduced $H9^{gag+}$ T cells

The IL-2 ELISA was done using the culture supernatants of four separate experiments. Results from two representative experiments are shown (Figures 6.8-6.9). Results from experiments 1 and 2 (Figures 6.8 and 6.9 respectively) show a reduction in IL-2 secretion in Lyp transduced $H9^{gag+}$ T cells compared to the non-transduced controls (Figures 6.8C &D and 6.9 C& D) whilst there is no significant difference in IL-2 secretion between the rat CD2 transduced $H9^{gag+}$ T cells and the non transduced $H9^{gag+}$ T cells (Figures 6.8B and 6.9B). The data from these experiments suggest that there is a slight reduction in IL-2 secretion by Lyp transduced $H9^{gag+}$ T cells compared to non Lyp transduced controls (truncated rat CD2 transduced cells). However, there was no difference in IL-2 secretion between the cells transduced with the two isoforms of Lyp (Figures 6.8 and 6.9).

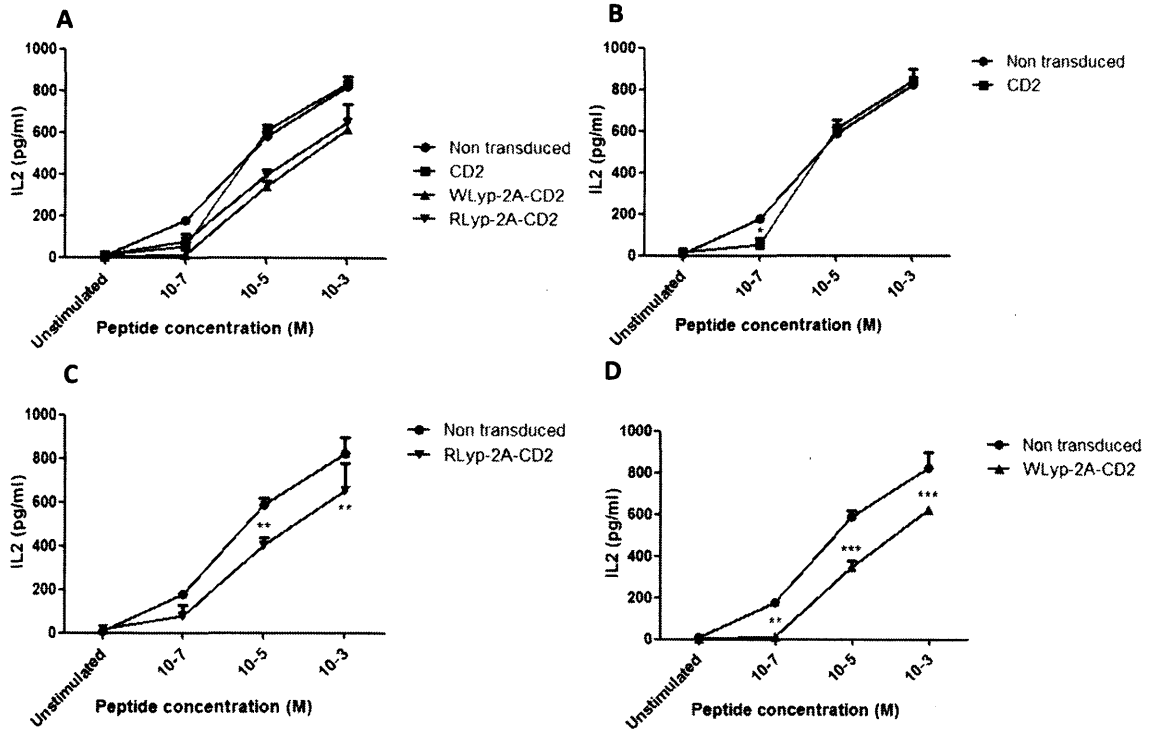


Figure 6.8 Experiment 1: The effect of Lyp isoform expression on peptide pulsed APC mediated IL-2 release in transduced H9^{gag+} T cells.

Non transduced H9^{gag+} T cells or T cells expressing the indicated isoform of Lyp were activated for 24 hours with varying concentrations of SLY peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an IL-2 ELISA kit. A) Effect of varying peptide concentration on IL-2 secretion in all four H9^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on IL-2 secretion in H9^{gag+} CD2 cells with the non transduced H9^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on IL-2 secretion in H9$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced H9^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on IL-2 secretion in H9$^{gag+\ W}$Lyp-2A-CD2 cells with non transduced H9^{gag+} T cells. Result from one experiment. The data are presented as mean ± SD. ** $P < 0.01$ *** $P < 0.001$ by Two-way ANOVA compared to Non-transduced H9^{gag+} T cells.

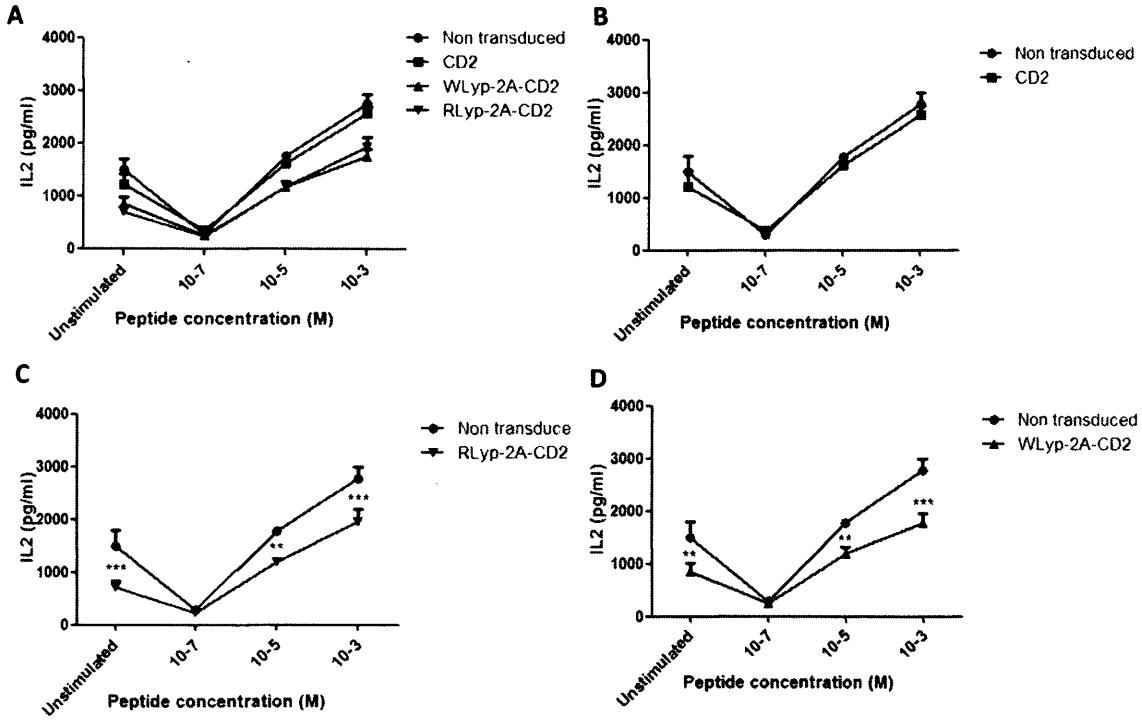


Figure 6.9 Experiment 2: The effect of Lyp isoform expression on peptide pulsed APC mediated IL-2 release in transduced H9^{gag+} T cells.

Non transduced H9^{gag+} T cells or T cells expressing the indicated isoform of Lyp were activated for 24 hours with varying concentrations of SLY peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an IL-2 ELISA kit. A) Effect of varying peptide concentration on IL-2 secretion in all four H9^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on IL-2 secretion in H9^{gag+} CD2 cells with the non transduced H9^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on IL-2 secretion in H9$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced H9^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on IL-2 secretion in H9$^{gag+\ W}$Lyp-2A-CD2 cells with non transduced H9^{gag+} T cells. Result from one experiment. Result from one experiment. The data are presented as mean ± SD. ** $P < 0.01$ *** $P <$ 0.001 by Two-way ANOVA compared to Non-transduced H9^{gag+} T cells.

6.5.2 Reduction in IL-10 secretion in Lyp transduced $H9^{gag+}$ T cells when compared to control transduced $H9^{gag+}$ T cells.

The IL-10 ELISAs were done with supernatants from four separate experiments. Representative results from two of the four separate experiments are shown below (Figures 6.10-6.11). Results from experiments 1 and 2 (Figures 6.10 and 6.11) show a highly significant reduction in IL-10 secretion by Lyp transduced $H9^{gag+}$ T cells compared to the non transduced controls. Although in experiments 1 and 2 (Figures 6.10 and 6.11), there is a reduction in IL-10 secretion observed at unstimulated and 10^{-3}M (Figures 6.10B) or unstimulated and 10^{-5}M (Figures 6.11B) peptide concentration between the non transduced cells and the rat CD2 transduced cells this difference is not as significant as the differences in IL-10 secretion detected between Lyp transduced and control cells. Overall, the significant reduction in IL-10 secretion in the Lyp transduced $H9^{gag+}$ T cells compared to control cells in observed suggests that Lyp reduces IL-10 production in the Lyp transduced $H9^{gag+}$ T cells. However, there was no difference in IL-10 secretion between the cells transduced with the two isoforms of Lyp.

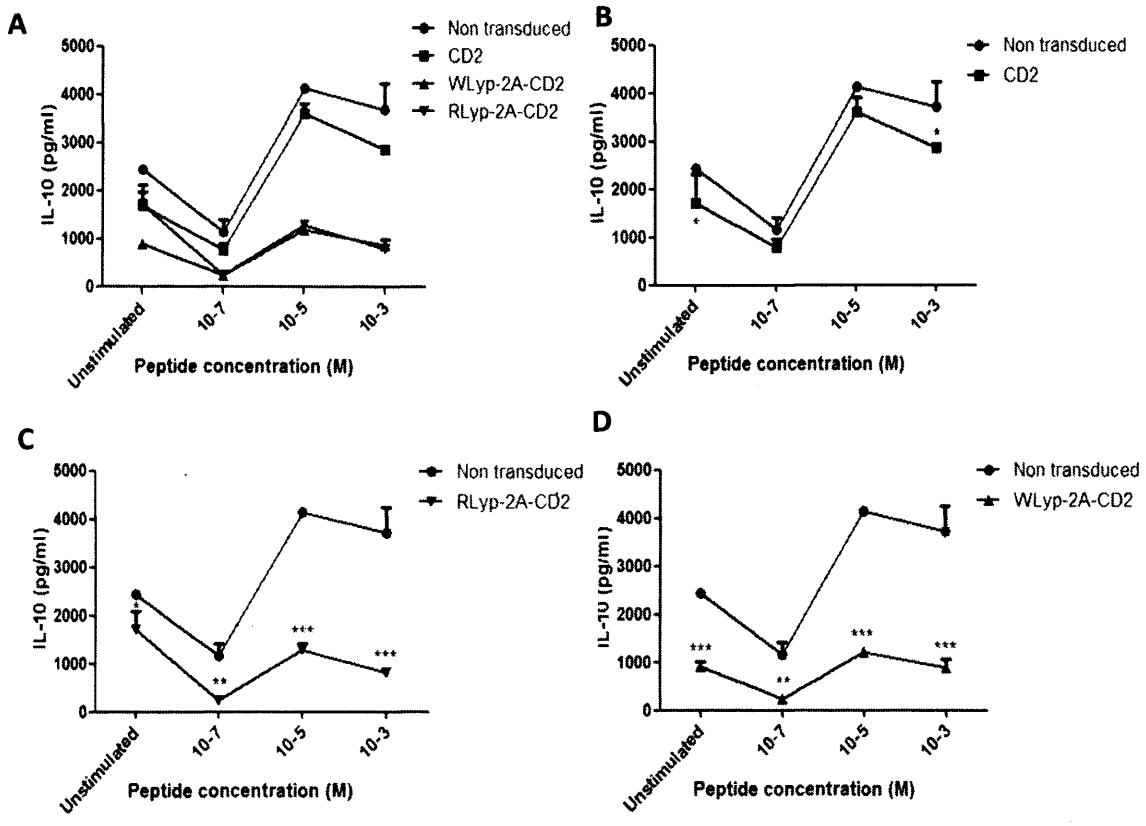


Figure 6.10 Experiment 1: IL-10 secretion by H9^{gag+} cells expressing the two isoforms of Lyp.

Non transduced H9^{gag+} T cells or T cells expressing the indicated isoform of Lyp were activated for 24 hours with varying concentrations of SL9 peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an IL-10 ELISA kit. A) Effect of varying peptide concentration on IL-10 secretion in all four H9^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on IL-10 secretion in H9^{gag+} CD2 cells with the non transduced H9^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on IL-10 secretion in H9$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced H9^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on IL-10 secretion in H9$^{gag+\ W}$Lyp-2A-CD2 cells with non transduced H9^{gag+} T cells. Result from one experiment. The data are presented as mean ± SD. The data are presented as mean ± SD. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ by Two-way ANOVA compared to Non-transduced H9^{gag+} T cells.

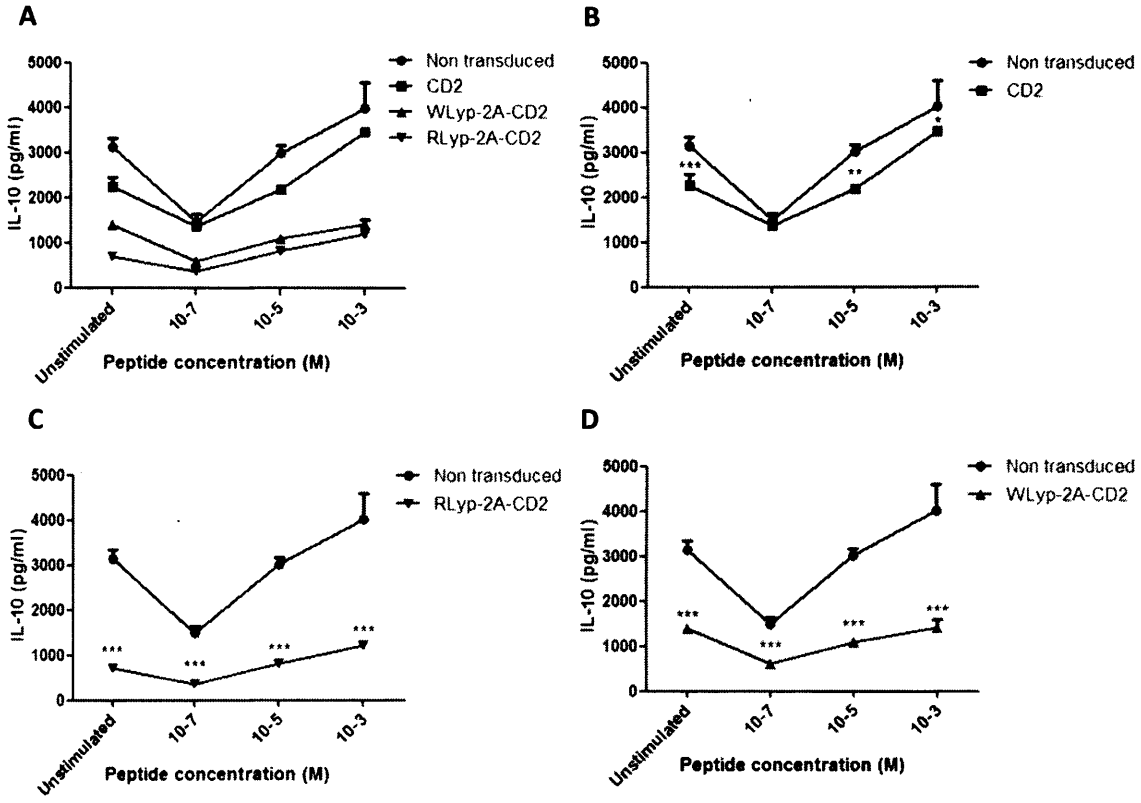


Figure 6.11 Experiment 2: IL-10 secretion by H9^{gag+} cells expressing the two isoforms of Lyp.

Non transduced H9^{gag+} T cells or T cells expressing the indicated isoform of Lyp were activated for 24 hours with varying concentrations of SL9 peptide pulsed APCs. Following incubations, the media was removed and tested for cytokine secretion using an IL-10 ELISA kit. A) Effect of varying peptide concentration on IL-10 secretion in all four H9^{gag+} T cell lines. B) Comparison of the effect of varying peptide concentration on IL-10 secretion in H9^{gag+} CD2 cells with the non transduced H9^{gag+} T cells. C) Comparison of the effect of varying peptide concentration on IL-10 secretion in H9$^{gag+\ R}$Lyp-2A-CD2 cells with the non transduced H9^{gag+} T cells. D) Comparison of the effect of varying peptide concentration on IL-10 secretion in H9$^{gag+\ W}$Lyp-2A-CD2 cells with non transduced H9^{gag+} T cells. Result from one experiment. The data are presented as mean ± SD. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ by Two way ANOVA compared to Non-transduced H9^{gag+} T cells.

6.6 Discussion

Lyp has been previously demonstrated to act as a negative regulator of TCR signalling and to reduce cytokine production by T cells (Aarnisalo et al. 2008; Rieck et al. 2007; Vang et al. 2005). In this chapter, the role of Lyp as a negative regulator of T cell activation and the potential effect of Lyp isoform expression on cytokine production by leukaemic T cell lines was investigated. No difference in CD69 activation marker expression was observed in E6.1^{gag+} and H9^{gag+} cells expressing the two isoforms of Lyp compared to controls. The required signalling thresholds for activation marker upregulation is different to that required for cytokine production (Bucy et al. 1995). Minimal activation of the T cell via their TCR is enough to upregulate activation markers. This minimal signalling requirement may have masked any subtle differences in activation due to Lyp mediated suppression of signalling. Rieck et al (2007) did observe a decrease in the level of CD25 activation marker expression by normal PTPN22$^{1858C/1858T}$ expressing CD4$^+$ T cells relative to PTPN22$^{1858C/1858C}$ expressing CD4$^+$ T cells. However there was no transduction and over-expression of Lyp in the Rieck study and there were also differences in the method of stimulation of the T cells; stimulation by MHC/SLY peptide and stimulation using anti CD3/CD28 beads or APCs/anti CD3 antibody may account for the lack of difference in Lyp mediated CD69 expression in this study compared to Rieck et al (2007). Hence, the role of Lyp in reducing TCR activation marker expression will require further investigation.

Previous studies have examined cytokine expression by T cells expressing different isoforms of Lyp. Vang et al (2005) investigated the effect of Lyp on IL-2 production and demonstrated that primary T cells from T1D patients with PTPN22 $^{1858C/1858W}$ genotype secrete lower levels of IL-2 compared to T1D patients with PTPN22 $^{1858C/1858C}$ genotype. In the same study, it was also shown that primary T cells nucleofected with the two isoforms of Lyp produce significantly lower amounts of IL-2 when compared to the control and the ^{W}Lyp nucleofected primary T cells produce even lower levels of IL-2 when compared to ^{R}Lyp nucleofected primary T cells (Vang et al. 2005). Likewise, Aarnisalo et al (2008) observed a significant difference in IL-2 secretion by PTPN22$^{1858C/1858T}$ CD4$^+$ T cells from T1D patients relative to PTPN22$^{1858C/1858C}$ expressing CD4$^+$ T cells from T1D patients. Interestingly, Rieck et al (2007) also investigated the functional effect of the Lyp R620W polymorphism on CD4$^+$ T cells from healthy individuals by examining the secretion of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α and only detected a significant decrease in IL-10. In the same study, subtle

decreases in IL-2 and IL-4 secretion, which did not reach statistical significance, and no differences in IFN-γ and TNF-α secretion in PTPN22 $^{1858C/1858T}$ versus PTPN22 $^{1858C/1858C}$ expressing $CD4^+$ T cells were also observed (Rieck et al. 2007).

The result from this study suggests that there may be a trend towards reduction in IL-2 secretion by the Lyp transduced E6.1^{gag+} T cells when compared to controls. This data is consistent with that expected from literature review. However, there were no differences in MIP-1β cytokine secretion observed between the Lyp transduced and control cells. In addition, there were no differences in cytokine secretion observed between the E6.1^{gag+} T cells expressing the two isoforms of Lyp.

A functional study of the Lyp modified H9^{gag+} leukaemic T cells demonstrated a slight reduction in IL-2 secretion by H9^{gag+} T cells expressing Lyp compared to the non-transduced controls. This result is consistent with the previous studies discussed above which also demonstrated an Lyp-mediated decrease in IL-2 secretion. However, in this study there was no difference observed in the IL-2 secretion between the two isoforms of Lyp contrary to that observed in the Vang et al (2005) study. Vang et al (2005) demonstrated a significant reduction in IL-2 secretion by T1D T cells from individuals with PTPN22 $^{1858C/1858W}$ genotype compared to those with PTPN22 $^{1858C/1858C}$ genotype. This discrepancy may be due to difference in the methods used for activating T cells in this study compared to Vang et al (2005) study. SLY peptide pulsed APC, a more physiologic means of activation was used to activate T cells in this study whereas anti-CD3/anti-CD28 coated beads were used to activate T cells in the Vang et al (2005) study. In this same study Vang et al (2005) also demonstrated that ^{W}Lyp nucleofected primary T cells showed a significant reduction in IL-2 secretion compared to the ^{R}Lyp nucleofected cells. Rieck et al (2007) also demonstrated lower levels of IL-2 secretion by $CD4^+$ T cells with the PTPN22 $^{1858C/1858W}$ genotype compared to $CD4^+$ T cells with the PTPN22 $^{1858C/1858C}$ genotype that did not reach statistical significance. However, Rieck et al (2007) also used a less physiologic method of activating T cells using anti-CD3/anti-CD28 coated beads which may account for the lack of difference in IL-2 secretion between the ^{R}Lyp and ^{W}Lyp expressing cells in this study.

IL-10 secretion was significantly reduced in the Lyp expressing H9^{gag+} T cells compared to controls. However, there was no difference observed in IL-10 secretion between cells expressing the R and the W isoforms of Lyp. This may be because the overexpression of R

and W isoforms of Lyp using lentiviruses in this study is so exaggerated that it masks a subtle difference in IL-10 secretion that may exist under physiological conditions between the cells expressing the R and the W Lyp isoforms. The extent of IL-10 reduction is much higher than that detected for IL-2. Intriguingly, this data is consistent with the Rieck et al (2007) study where it was demonstrated that $CD4^+$ T cells with the PTPN22 $^{1858C/1858W}$ genotype showed a significant reduction in IL-10 secretion compared to $CD4^+$ T cells with a PTPN22 $^{1858C/1858C}$ genotype. However, no difference was observed in IL-10 secretion between the two isoforms of Lyp in my study. The significantly reduced IL-10 level due to Lyp expression is very interesting and may have implications for therapeutics. Several studies have found altered IL-10 expression levels to strongly correlate with many ADs (Cohen et al. 1995; Cush et al. 1995; Houssiau et al. 1995; Llorente et al. 1993; Lopatin et al. 2001; Mongan et al. 1997). IL-10 is produced by multiple cell types that include $CD4^+$, $CD8^+$ T cells, $CD4^+CD25^+$ cells, activated B cells, monocytes, macrophages and keratinocytes but the four major producers of IL-10 are thought to be T_H2 cells, T_R1 cells, T_H1 cells and T_H17 cells (Akdis et al. 2011). IL-10 exhibits a multiple modulatory effect on the immune system. As an anti-inflammatory and immunosuppressive cytokine, IL-10 reduces production of pro-inflammatory mediators and results in diminished T cell stimulation (de Waal Malefyt et al. 1991a; Peguet-Navarro et al. 1994). Studies using genetically IL-10 deficient mice have illustrated the importance of IL-10 in limiting autoimmune pathologies. Mice lacking IL-10 or treated with blocking anti-receptor antibodies succumb to what would normally be sublethal doses of lipopolysaccharides (Berg et al. 1995). Furthermore, normally self-contained bacterial and parasitic infections can result in lethal autoimmune mortality in IL-10-deficient mice (Gazzinelli et al. 1996; Hunter et al. 1997) and in almost every mouse model of autoimmunity, including experimental autoimmune encephalitis, RA, and inflammatory bowel disease, disease is dramatically exacerbated in mice lacking IL-10. These studies demonstrate the crucial role of IL-10 in limiting an over-exuberant immune response and preventing autoimmunity. Therefore, Lyp mediated reduction in IL-10 levels could be a major contributing factor leading to predisposition to autoimmunity. The molecular mechanism of how Lyp results in a reduction in IL-10 levels potentially holds great promise in the development of treatments for AD.

Interstingly, a reduction in IL-2, MIP-1β and IL-10 cytokine secretion by H9 and E6.1 cells was observed at the lowest concentration of the peptide stimulation compared to unstimulated. This dip in cytokine secretion when transitioning from unstimulated to

stimulation with low peptide concentration could an effect of B cells which were used as APCs. It may be that because unstimulated T cells still contain APCs but no SLY peptide, the B cells are also producing the respective cytokines, however addition of the once the B cells are pulsed with SLY peptide they are targeted by T cells resulting in B cell death leading to a cessation in cytokine production by B cells therefore an overall decrease in cytokine levels from unstimulated to stimulation with lowest peptide concentration. In addition, there may also be an added tonic inhibition effect. There may be an allogenic stimulation taking place between the B cells and T cells which maybe dominant when unstimulated leading to the production of cytokines, this effect would cease once the B cells are peptide pulsed leading to B cell death and reduction in cytokine production.

In summary, in this chapter a functional analysis of Lyp-modified leukaemic T cells was performed. This analysis revealed several interesting findings. Firstly, no difference in CD69 activation marker expression was observed between the exogenous Lyp expressing and control E6.1^{gag+} and H9^{gag+} T cells. In addition, no difference was observed in MIP-1β secretion between the exogenous Lyp expressing and control E6.1^{gag+} T cells however a trend towards reduction in IL-2 was observed in Lyp trasduced E6.1^{gag+} T cells compared to controls. A small reduction in IL-2 expression was observed in Lyp transduced H9^{gag+} cells compared to control cells. In addition, a highly significant reduction in IL-10 secretion was observed in Lyp expressing H9^{gag+} cells compared to controls. The data presented in this chapter highlight a potential preferential effect of Lyp activity on cytokine secretion, particularly IL-10 which is known to play an important anti-inflammatory role in autoimmunity and reduction or impairment of IL-10 could pre dispose to autoimmunity.

Chapter 7

Final Discussion

The notion of autoimmunity has been around for a very long time, although the pathogenic mechanisms leading to ADs are not clearly understood. The focus of the immune response in ADs, namely self antigens, makes them difficult to cure at present. ADs affect otherwise relatively healthy individuals, often young adults, making it a huge physical, physiological and economic burden (Rioux and Abbas 2005). The current available treatments target the resulting organ damage rather than the underlying cause of the disease (Rioux and Abbas 2005). Despite some of these treatments having some remarkable success, they tend to come with the huge risk of side effects due to the general nature of the treatment. Additionally, as the underlying causes of the ADs are usually unknown the available treatments can only slow down disease progression and provide relief from the symptoms. Genetics is known to strongly influence the development of autoimmunity and understanding the mechanism of this influence will increase our understanding about the causal derangements and may lead to development of better treatment strategies (Rioux and Abbas 2005).

Whilst the genetic contribution to ADs is now a widely accepted phenomenon, the influence of any individual predisposing allele is modest and therefore the relationship between the predisposing variant and the disease state is influenced by other factors such as other genes and the environment. Despite this modest effect, understanding the effect of these genes in predisposing to ADs would help piece together the mechanism of ADs. MHC polymorphisms are the major contributor to ADs (Simmonds and Gough 2005). However as MHC genes exert their effect on multiple components of the immune system, how they lead to ADs is likely to be complex. In addition, it has been difficult to confidently associate many of the causal MHC genes to ADs due to the high degree of linkage disequilibrium between the MHC alleles. Genes outside of the MHC region predisposing to ADs are alternatively much easier targets for studying their contribution to autoimmunity and for developing treatments that can target the disease mechanism resulting from the genetic polymorphisms. Single gene defects leading to ADs have provided understanding of the pathways of disease and normal physiology. However, most ADs result from the interactions of several predisposing genes, which affect multiple immune parameters, and deciphering the link between genetics and molecular mechanisms leading to ADs poses a significant challenge.

A recently identified SNP in the gene, PTPN22, implicated in several ADs has aroused considerable interest. Outside of the MHC region, the PTPN22 C1858T polymorphism represents the most robust association of any genes linked to ADs. Much work has been directed at finding the association between this SNP and various ADs. However, studies involving the normal physiological role of the Lyp protein encoded by the PTPN22 gene and the functional effect of this R620W polymorphism on Lyp are less well developed. Experiments described in this thesis were devised to investigate the functional effect of the PTPN22 C1858T polymorphism in T cells. Specifically, does over-expression of the $^{R/W}$Lyp proteins result in altered physiologic activation of T cells? Is there a difference in cytokine production by T cells over-expressing the R and W isoforms of Lyp?

In addition, it was of great interest to determine whether a monoclonal antibody generated specifically against the ^{W}Lyp isoform would have a selective blocking effect on Lyp activity. If so, this would mean that there are sufficient differences in the conformational epitopes formed by the intact R and W Lyp proteins to be distinguished by the antibody. This in turn would provide confidence that other reagents, such as a small molecule inhibitor could be designed to specifically target either the R or W Lyp protein. Potentially such a reagent could be very useful in assisting in understanding the molecular basis of R620W polymorphism in predisposing to ADs. In addition, the reagent may also assist in development of therapies. Therefore, an attempt was made to generate polymorphism specific monoclonal antibodies against the R and W isoforms of Lyp. Unfortunately, this proved difficult and only on the third attempt was it possible to generate monoclonal hybridomas against the Lyp peptide. Three different hybridoma clones were generated but antibody secreted by each of these clones was unable to distinguish between the R and W Lyp peptides. Furthermore, none of the three secreted antibodies was able to detect denatured full length Lyp protein by immunoblotting. Therefore, attempts to generate polymorphism specific monoclonal antibodies were unsuccessful in this instance.

In order to examine the effect of the PTPN22 C1858T polymorphism in T cells, it was important to design a suitable system to over-express the two Lyp isoforms. The rationale for over-expression of Lyp was based on the fact that the W620 Lyp isoform is known to behave in a dominant manner (increased risk of AD in individuals who are heterozygous for ^{W}Lyp isoforms). Therefore, it is conceivable that introducing exogenous Lyp W620 into ^{R}Lyp/^{R}Lyp

homozygote T-cell populations would partially mimic the cellular environment in T cells isolated from heterozygote individuals and allow examination of the effect of the R620W polymorphism. To achieve this goal, recombinant lentivirus plasmids for use in over-expression of Lyp in T cells were designed and generated. Recombinant lentivirus plasmids encoding either the R or the W isoform of Lyp together with a "self-processing" TaV derived 2A peptide and either GFP or truncated rat CD2 reporter genes were generated. The recombinant R/WLyp-2A-GFP and R/WLyp-2A-CD2 plasmids generated in this thesis are ideal tools for over-expressing Lyp isoforms in T cells and the rat CD2 reporter gene allows direct sorting of transduced cells from the non transduced population.

To carry out critical evaluation of the functional effect of over-expressing the two isoforms of Lyp in T cells it was important to generate T cell lines over-expressing the Lyp isoforms. Human leukaemic T cell lines, E6.1 (Jurkat) and H9 (Hut78) previously transduced with an SLY specific class I restricted TCR, were further transduced with lentiviruses particles encoding either ^{R}Lyp-2A-CD2, ^{W}Lyp-2A-CD2 or CD2 alone hence generating E6.1 and H9 T cell lines expressing ^{R}Lyp-2A-CD2, ^{W}Lyp-2A-CD2 and CD2 respectively. The cell lines were successfully generated and shown to have comparable SLY TCR expression. In addition, the two cell lines expressing the R and the W isoform of Lyp were shown to have comparable reporter gene expression. The E6.1 and H9 cell lines were used as initial model systems due to their advantage over primary human T cells in terms of ease of maintenance in culture and their relative ease of transduction (especially Jurkat T cells). Maintaining primary T cells in culture requires periodic restimulation, which may affect surface expression of activation markers such as CD69 and CD25. Furthermore, the cells often need to be rested for long periods before the expression of activation markers returns to basal levels. In addition, there was also an advantage of the speed with which functional assays could be conducted using T cell lines once the exogenous Lyp expressing cell lines had been generated. Indeed, any interesting observations made with the T cell lines could be taken forward for further investigation into primary human T cells and other specialised subsets of T cells such as T reg cells and T follicular helper cells for examination of proliferation, suppression and cytokine secretion.

There were a trend towards reduction observed in this study in IL-2 production between Lyp transduced and control E6.1 cells. This result was consistent with other studies, which have observed a decrease in IL-2 secretion upon Lyp over expression by transduction (Vang et al.

2005) and a reduction in IL-2 production in heterozygous ^{W}Lyp expressing T cells when compared to homozygous ^{R}Lyp expressing T cells (Aarnisalo et al. 2008; Rieck et al. 2007; Vang et al. 2005). However, there was no difference observed in MIP-1β production between the Lyp transduced and control E6.1 T cells.

The key findings of particular significance obtained during this study were the effects of Lyp over expression on H9 cells. Results described in this report suggest that there may be a slight reduction in IL-2 secretion by H9 T cells over-expressing the R and W isoforms of Lyp when compared to H9 T cells not expressing Lyp. There was however no difference observed in IL-2 secretion between the H9 T cells expressing the two isoforms of Lyp. The over-expression of the two isoforms of Lyp may be so exaggerated in this model that it may have obscured a subtle difference in cytokine secretion between the two isoforms of Lyp. This overexpression may account for the lack of differences observed in IL-2 and IL-10 cytokine secretion between the ^{R}Lyp and the ^{W}Lyp expressing cells in this study. Despite the expression of Lyp in this model being more exaggerated than the normal physiologic levels of Lyp expression in the cell, it nevertheless has potentially provided potential important indications as to how Lyp may be functioning. The potential use of zinc finger nucleases to promote homologous recombination at nucleotide position 1858 in the PTPN22 gene of a T cell homozygous for the PTPN22 C1858 allele in order to create a cell with a PTPN22 $^{C1858/T1858}$ genotype or further conversion to a PTPN22 $^{T1858/T1858}$ genotype would provide a much more physiologically relevant model (Rahman et al. 2011). This could be achieved by co-introduction of zinc finger nucleases and a PTPN22 cDNA encompassing the desired nucleotide change at position 1858 into a T cell with a PTPN22 $^{C1858/C1858}$ genotype. The zinc fingers in the zinc finger nuclease would recognise and bind to the target sequence in the PTPN22 gene enabling the attached non specific cleavage domain from the *FokI* endonuclease to cleave the spacer region and create a double stranded break in the DNA (Rahman et al. 2011). This double stranded break would induce a homology directed repair between the defective DNA and the co-transfected PTPN22 T1858 encoding DNA, resulting in a C to T change in the PTPN22 gene (Rahman et al. 2011). Following limiting dilution cloning, the monoclonal cells could be genotyped and potentially cell lines established for all three genotypes (PTPN22 $^{C1858/C1858}$, PTPN22 $^{C1858/T1858}$ and PTPN22 $^{T1858/T1858}$). This method would avoid the need for overexpression of the PTPN22 T1858 allele in order to mimic a heterozygous cell and would eliminate the limitations associated with overexpression in the current model.

This study also revealed that H9 cells expressing Lyp showed a significant reduction in IL-10 secretion when compared to H9 cells not expressing Lyp. This report is not the first highlighting a role of Lyp in reduction of IL-10 production. However, the previous study was conducted in $CD4^+$ T cells isolated from healthy controls who were either ^{R}Lyp/^{R}Lyp homozygous or ^{R}Lyp/^{W}Lyp heterozygous and therefore the effect observed could not necessarily be attributed to a intrinsic effect of Lyp in $CD4^+$ T cells. In addition, a different method of activating T cells was used (Rieck et al. 2007). Results from the current study suggest a direct cell intrinsic effect of Lyp on the production of the cytokine IL-10. Although no differences in IL-10 secretion were observed between the H9 cells expressing the R and W isoforms of Lyp in this study, if ^{W}Lyp is indeed a "gain of function" variant as indicated by several studies then it is conceivable that the ^{W}Lyp isoform would result in further decreases in IL-10 production when compared to ^{R}Lyp expressing cells. Hence the enhanced reduction in IL-10 production that would be predicted for the ^{W}Lyp isoform may implicate reduced IL-10 levels as the key pathogenic mechanism by which the ^{W}Lyp variant predisposes to ADs.

Although over-expression of R and W isoforms of Lyp in H9 cells in this report has highlighted an important intrinsic effect of Lyp expression on IL-10 production, further work is required to provide supporting evidence that this effect is consistently observed in other T cell lines and primary T cells as well as to identify any other effects of Lyp expression in T cells. Generation of other T cell lines and primary T cells over-expressing ^{R}Lyp and ^{W}Lyp by using lentivirus particles encoding R and W isoforms of Lyp produced using the method optimised in this study will allow analysis of IL-10 and IL-2 production by these cells and may confirm if the effect of Lyp seen with H9 cells is indeed a major effect of Lyp on T cells in general. It would also be interesting to investigate the effect of Lyp expression on cellular production of other cytokines in addition to IL-2 and IL-10. Rieck et al (2007) in their study observed a small non-significant reduction in IL-4 but no difference in interferon-γ and TNF-α secretion by $CD4^+$ T cells from heterozygous (^{R}Lyp/^{W}Lyp) individuals when compared to $CD4^+$ T cells from homozygous (^{R}Lyp/^{R}Lyp) individuals (Rieck et al. 2007). The authors in the Rieck et al (2007) study also reported that the Lyp R620W polymorphism resulted in a decrease in anti-inflammatory cytokine IL-10 reduction, whereas the production of pro-inflammatory cytokines was not affected as much. It would be interesting to investigate if indeed the effect of Lyp is more profound in the production of anti-inflammatory cytokines by T cells than the production of pro-inflammatory cytokines and if so how Lyp may mediate

this selective effect on cytokine production. Additionally, the role of Lyp in cytokine production and T and B cell biology could be further explored in a more physiological context. Funcational analysis of cellular proliferation, activation and cytokine producion of immune cells from C57Bl/6 R620W Lyp knock-in mouse or $Pep^{-/-}$ mouse would provide a more physiological model for exploring the role of Lyp in these cells. Additionally, generation of R620W conditional knock out or inducible knock-in mouse with inducible expression of Lyp in specific cell types would provide attractive models for exploring the role of Lyp in T and B cells biology.

It is conceivable that a reduction in IL-10 production due to Lyp expression may aid development of ADs as IL-10 seems to have a protective role in several ADs. Patients with deficient IL-10R expression develop severe form of IBD (Glocker et al. 2009). Furthermore, several polymorphisms in the IL-10 promoter region have been identified that are associated with altered IL-10 expression and altered IL-10 expression as a result of such genetic variation has been linked to a number of ADs (Akdis et al. 2011). IL-10 is a potent anti-immune and anti-inflammatory cytokine and is a member of a family of cytokines that include IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29.

IL-10 plays a crucial role in the development of an immune response. It is therefore of great importance that production of IL-10 is carefully regulated. Several layers of regulation of IL-10 expression exist and some of these mechanisms are conserved among all IL-10 producing cells, while others appear to be cell specific (Mosser and Zhang 2008). IL-10 gene expression is controlled by constitutively and ubiquitously expressed transcription factors Sp1 and Sp3 (Tone et al. 2000). Chromatin modification at the IL-10 locus is another proposed mechanism of IL-10 regulation. Histone deacetylase 11 protein has been shown to bind to the IL-10 promoter region resulting in a formation of a more compact chromatin upon deactylation further leading to impaired accessibility of this region for IL-10 inducing transcription factors such as STAT3 (Villagra et al. 2009). Post-trancriptional mechanisms of regulating IL-10 production also exist. This was first suggested by the finding that T cell clones actively transcribing IL-10 were unable to yield detectable levels of IL-10 mRNA (Naora et al. 1994). In addition, IL-10 mRNA levels were found to vary between cell lines showing comparable promoter activity (Tone et al. 2000). Multiple copies of mRNA destabilizing motifs are found to be present in the 3' untranslated region (UTR) of the IL-10 mRNA (Powell et al. 2000). Powell et al (2000) cloned three potential mRNA destabilising motifs AUUA located in the

3' UTR region of IL-10 mRNA individually and together into luciferase reporter plasmids with SV40 promoter/luciferase expression unit. In this system, if the cloned AUUA sequence contained an RNA-destabilising activity then it would generate a reduced luciferase activity when compared to the luciferase activity of the control plasmid. Following luciferase reporter assays, they observed a 50% reduction in luciferase activity when plasmids encoding each of the three AUUAs were individually transfected in non stimulated T cells. The reduction in luciferase activity increased to 80% when the T cells were transfected with a plasmid encoding all three of the AUUA sequences (Powell et al. 2000). This suggests that constitutively expressed IL-10 mRNA is unstable unless stabilised by post-trancriptional modification. Although IL-10 is ubiquitously transcribed, the actual production and secretion of IL-10 protein depends on the post-transcriptional signal.

The result showing the reduction in IL-10 production by H9 cells transduced with Lyp leads to the proposal of a hypothesis presented in Figure 7.1 and 7.2. These figures suggest how Lyp expression may result in a reduction in IL-10 production. It is hypothesised that the reduction in IL-10 production may be the effect of Lyp at the proximal TCR signalling cascade or alternatively Lyp may affect other signalling cascades that play a role in regulating IL-10 production. Lyp may directly dephosphorylate and therefore inactivate transcription regulators of IL-10 leading to reduced production of IL-10 (Figure 7.2). This hypothesis could be tested by carrying out Immunoprecipitation studies on the R and W Lyp overexpressing T cells to investigate if Lyp interacts with regulators of IL-10 transcription such as Sp-1 or Sp-3. Alternatively, Lyp may have as yet unknown binding partners that may interact with IL-10 regulators influencing production of IL-10. Once again Immunoprecipitation of Lyp in exogenous Lyp expressing T cells may help identify as yet unknown binding partners. In addition, Lyp may interact with proteins that affect the post transcriptional IL-10 mRNA. Lyp may affect the phosphorylation status of these proteins thereby inactivating or activating them which may in turn affect their function in stabilising IL-10 mRNA resulting in degradation of IL-10 mRNA leading to reduction in IL-10 production. Lyp may directly bind to microRNAs that play a role in post-transcriptional regulation of IL-10 or interact with proteins that regulate such microRNAs, resulting in a decrease in IL-10 production. Yet again, Immunoprecipitation, Co-immunoprecipitation of Lyp in Lyp over-expressing cells may identify such substrates of Lyp.

Maximal expression of IL-10 following re-stimulation of T_H1 cells has been shown to require a strong TCR signalling (Saraiva et al. 2009). It may be that a similarly strong TCR signal is required for IL-10 production in H9 cells and therefore Lyp mediated suppression of TCR signal strength may have resulted in the impaired IL-10 production observed in this study (Figure 7.1). As there was a comparatively much larger reduction in IL-10 secretion compared to IL-2 secretion in Lyp transduced cells, this may indicate a minimal effect of Lyp in the transcription of cytokine genes. Further, if the reduction in cytokine secretion is not due to an effect of Lyp on the transcription of cytokines an alternative suggestion is that Lyp may be playing a role in post-transcriptional modification. This is supported by the observation that IL-2 has not been observed to be post-transcriptionally modified whereas IL-10 has been reported to be post-transcriptionally modified. This is consistant with the observation in this study that whereas IL-2 is only minimally reduced, the reduction in IL-10 secretion is more profound. Currently there is no evidence to directly support either of these hypotheses.

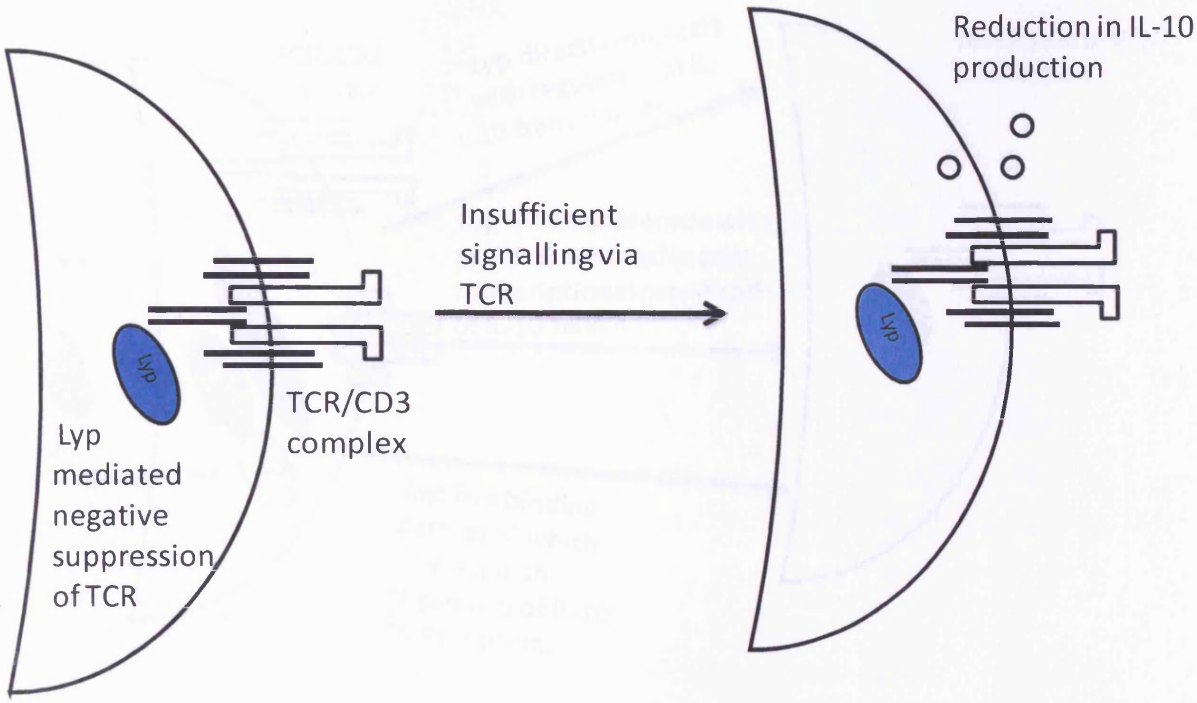


Figure 7.1 Hypothesis for Lyp mediated suppression of IL-10 production

The reduction of IL-10 production by H9 cells expressing Lyp may be due to the effect of Lyp in TCR signalling. Lyp mediated dephosphorylation of Src family kinases, Lck and FynT, and Zap70 which is thought to result in a suppression of TCR signalling may lead to a reduction in IL-10 production by the suppressed T cells.

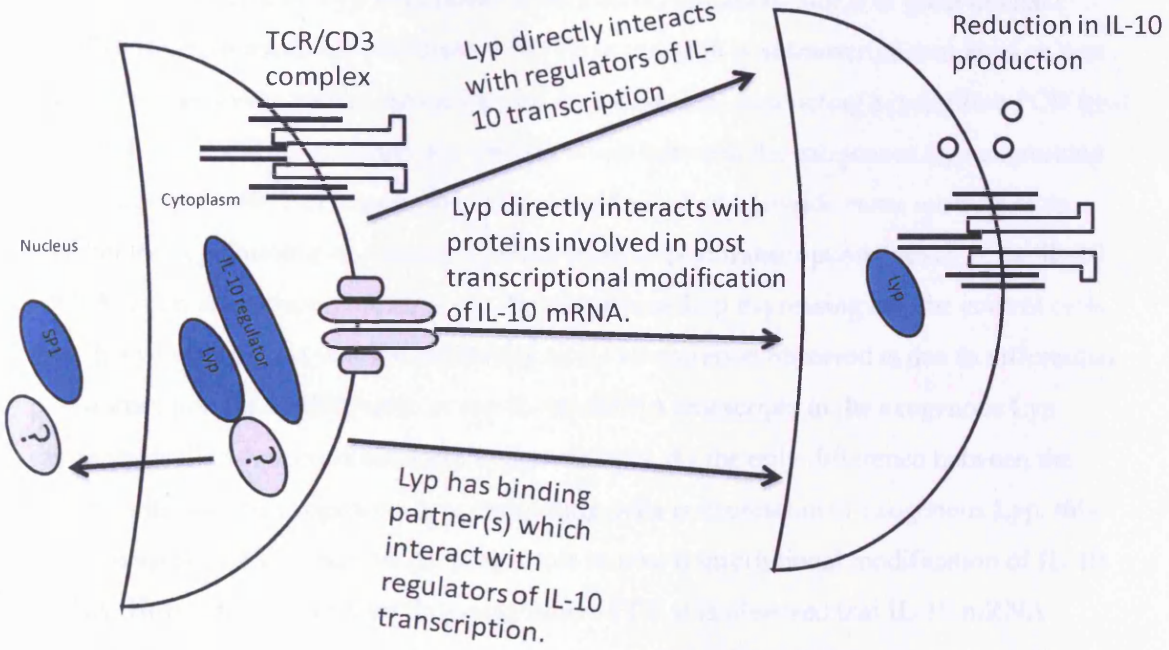


Figure 7.2 Hypothesis for the effect of Lyp on IL-10 transcription and translation

The reduction in IL-10 production by exogenous Lyp expressing cells suggests a potential role for Lyp in directly affecting IL-10 transcription and translation. Lyp may interact with IL-10 transcriptional regulators and dephosphorylate and inactivate them directly or indirectly via its as yet unknown binding partner (represented by a circle with a question mark). In addition, the effect of Lyp may be due to its known effects on post transcriptional modification mediators of IL-10. Lyp may dephosphorylate these post transcriptional modification mediators thereby affecting their function in stabilising the IL-10 mRNA. In addition, Lyp may affect IL-10 production by acting as a competitor or binding partner of these transcription and translation mediators and thereby affect their availability to cells to regulate IL-10 transcription or stabilise IL-10 mRNA.

The mechanism of how Lyp may have influenced IL-10 production is of great interest. Whether this Lyp mediated reduction in IL-10 production is at transcriptional level or post transcriptional level requires investigation. As a first step, conducting a quantitive PCR (real time PCR, RT-PCR) to compare the mRNA levels between the exogenous Lyp expressing and the control cells (not expressing exogenous Lyp) could provide some answers as to whether the regulation is at a transcriptional level or post transcriptional level. If the IL-10 mRNA levels are comparable between the exogenous Lyp expressing and the control cells than this would suggest that the difference in IL-10 secretion observed is due to differential post transcriptional modification of the IL-10 mRNA transcripts in the exogenous Lyp expressing cells when compared to the control cells. As the only difference between the control cells and the exogenous Lyp expressing cells is expression of exogenous Lyp, this would suggest that Lyp may be playing a role in post transcriptional modification of IL-10 mRNA. However, if by conducting quantitative PCR it is observed that IL-10 mRNA transcription in the cells expressing Lyp and the control cells is different and consistent with the differences seen for IL-10 protein, then this would suggest a role for Lyp at the level of transcription of IL-10 mRNA. Lyp may play role in directly or indirectly regulating IL-10 transcriptional regulators.

It is feasible that the effect of this polymorphism may be different in different T cell subsets. This hypothesis has not been explored previously, therefore would be of great interest to explore if the R620W polymorphism has different effects in different T cell subsets in an in-vitro setting. Identifying the cell populations that are altered by the ^{W}Lyp is a critical step in understanding how this polymorphism eventually results in autoimmunity. T reg cells have been shown to be a key player in suppression of autoreactive T cells in the periphery and have been implicated in autoimmunity (Costantino et al. 2008; Laurent et al. 2009). If indeed ^{W}Lyp is a "gain of function" polymorphism as suggested by most studies then it is plausible that the impact of ^{W}Lyp on TCR signalling may have an effect on the development or/and functioning of T reg cells. Fewer natural T reg cells may be generated in W620 Lyp expressing individuals or they may not be as effective in suppressing effector T cells as T reg cells from homozygous R620 Lyp expressing individuals because their TCR signalling is more negatively suppressed. The question of whether T reg cells in ^{W}Lyp expressing individuals are less able to exert suppressive effect on effector T cells than T reg cells from ^{R}Lyp homozygous individuals can be addressed by introducing R and W isoforms of Lyp into the T reg cells using infectious lentivirus particles generated using the recombinant R and W

Lyp encoding lentivirus plasmids generated in this study. Subsequently, these R and W Lyp expressing T reg cells may be analysed by suppression assays to observe if indeed a difference in their ability to suppress exists.

Furthermore, it may be the case that the R620W polymorphism does not significantly affect T reg cell function, but rather has a profound effect on T follicular helper (Tfh) cells which are known to play an important role in providing help to B cells for homing into germinal centre and immunoglobulin class switching (Laurent et al. 2009). Results from Pep knockout study carried out by Hasegawa et al (2004) observed spontaneous generation of germinal centres in $Pep^{-/-}$ mice which was seen to depend on cooperation between T and B cell as administration of an anti CD40L monoclonal antibody disrupted the enhanced germinal centre formation (Hasegawa et al. 2004). This suggested that the spontaneous germinal centre formation is unlikely to be due to a B cell intrinsic defect but may be due to defects in T cell mediated help to B cell. Therefore, as Tfh cells play a role in B cell maturation, they are good candidates for further investigation. The impact of Lyp expression on Tfh cells could be assessed by isolating Tfh cells from tonsils and lymph nodes of patients, transducing with lentivirus particles encoding R and W isoforms of Lyp and assessing for altered cell function. Expression of cell surface markers such as CXCR5, ICOS and CXCR7 by Tfh cells over-expressing either the R or the W isoforms of Lyp could be assessed by flow cytometry. The ability of Tfh cells over-expressing either the R or the W isoforms of Lyp in producing cytokines such as IL-21 and IL-6 cytokine production could also be assessed. Tfh cells are known to induce IgG production by B cells, therefore it would be interesting to assess the ability of transduced Tfh cells to drive B cell proliferation and IgG production by co culturing with B cells.

Alternatively, the B cell population rather than T cell population might be more significantly affected by the R620W polymorphism. Rieck et al (2007) observed an association of the ^{W}Lyp variant with a diminished response to B cell receptor stimulation suggesting that B cell-intrinsic processes may be directly altered by the ^{W}Lyp variant. Therefore, further assessment of the effect of R620W polymorphism on other lymphocyte population such as the B cells to identify the cell populations where cell function may be critically altered, would help to better understand the effect of Lyp in cellular immunology.

In summary, the generation of ^{R}Lyp-2A-CD2 and ^{W}Lyp-2A-CD2 encoding recombinant lentivirus plasmids which were used to produce infectious lentivirus particles and the

subsequent over expression of Lyp in $E6^{gag+}$ and $H9^{gag+}$ T cells together with the sorting of the transduced cells based on CD2 reporter gene expression has proven to be a useful model system to assess Lyp mediated effect on cytokine production. A limitation of this model system has also been identified in this current study. The exaggerated expression of exogenous Lyp in this system may be the reason for the lack of differences observed between the R and the W Lyp expressing cells. The use of zinc finger nucelases for conversion of the homozygous ^{R}Lyp/^{R}Lyp expressing cell into a heterozygote cell (^{R}Lyp/^{W}Lyp) or a homozygous cell (^{W}Lyp/^{W}Lyp) would address this issue and provide a more physiologic model for further study.

The use of this model system has however, allowed identification of a critical role of Lyp in cytokine production by H9 cells. There was a trend towards reduction in IL-2 production observed in Lyp expressing cells compared to control cells, but more importantly there was a significant reduction in IL-10 production by Lyp expressing cells when compared to control cells despite there being no difference in CD69 expression between these cells. It would be interesting to further analyse the effect of Lyp on IL-10 production in other T cell lines and in primary cells and if so analyse whether the differences in IL-10 expression are at a transcriptional or post transcriptional level as it may provide further insights into the role of Lyp in T cells. Notwithstanding, this result suggests an important role for the Lyp in the immune regulation of IL-10 and provides a potential mechanism for association of the PTPN22 gene to ADs.

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