The Role of CD5 in T Lymphocyte Activation

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A thesis submitted for the degree of Doctor of Philosophy, Trinity Term 2011

Table of Contents

Abstract

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In order to prevent autoimmune diseases the immune system must be tightly regulated. One mechanism that the immune system uses to prevent autoimmunity is through the use of regulatory receptors on the surface of T lymphocytes. In this thesis I have concentrated on the role of one such receptor, CD5, in T lymphocyte activation. CD5 is a member of the scavenger receptor cysteine rich superfamily and is expressed, along with the closely related CD6, on T cells and a subset of B cells. CD5 is known for its inhibition of thymocyte selection but its function in peripheral T cell activation remains unclear.

In this thesis I demonstrate that OX19, a rat CD5 monoclonal antibody (mAb), will block extracellular engagement of CD5 *in vitro* resulting in an increase in proliferation following T cell activation. In demonstrating the mode of action of OX19 I show that extracellular engagement of CD5 will inhibit T cell activation *in vitro*. I also demonstrate that OX19 does not modulate CD5 from the surface *in vitro* whereas it does modulate CD5 *in vivo.*

Another key finding in this thesis is that differential phosphorylation of the three tyrosines in the CD5 cytoplasmic tail can account for positive and negative signalling by CD5 during T cell activation. Evidence here demonstrates that CD5 Y1 is required for positive signalling which becomes more apparent in the absence of costimulation by the coreceptor, CD2. CD5 Y2 and Y3 are required for the CD5 mediated inhibition of T cell activation. Phosphorylation of these tyrosines is also necessary for CD5 regulation of CD3 down modulation following TCR triggering. These data point to a potential molecular mechanism involving CD5 regulation of CD3 down modulation creating control of T cell activation as well as a potential for cross talk between another coreceptor CD2.

Direct and indirect associations of the phosphorylated tyrosines in CD5 was analysed here using a combination of a screen of binding affinities of SH2 domains with CD5 phospho-peptides and mass spectrometry of CD5 associated proteins. SH2 domains measured in this thesis did not show a high binding capacity to tyrosine phosphorylated CD5 peptides indicating that CD5 mediates transient interactions with signalling proteins. The region in the cytoplasmic tail in CD6, CD6Y3Y4, which lies at the same distance from the membrane as the C-terminus of CD5, will bind to Itk and Lck. Given CD5 and CD6 are often coexpressed this would allow phosphorylation of CD5 by these two kinases providing a molecular mechanism underlying cooperation between CD5 and CD6.

Acknowledgements

I would first of all like to acknowledge my supervisor Marion H Brown for her guidance during my time in Oxford, and Neil Barclay for his support in this project. I would also like to thank my advisor Kevin Maloy for his useful advice and Anton Van Der Merve for all his invaluable support. I could not have carried this project out without the useful hints, fun times, practical jokes and genuine friendship from lab members past and present, namely, Nicholas Clarkson, Clive Metcalfe, Tim Wilson, Lai Shan and Chris Bird. I would also like to acknowledge the never ending support that I have had from members of the Dunn School for letting me borrow reagents and protocols, for the great parties, for listening to my presentations and giving wonderful suggestions, and for making my time here enjoyable. In particular I would like to thank Shaun-Paul Cordoba, Marcus Bridge, all my fellow DPhil students, Kenny Moore and the Powrie/Maloy and Acuto labs. I would like to thank Linacre College for being my home and family for the last four years giving particular mention to Olivier, Helena, Rob, Mick, Dom, Dave, Cat, Maeo, Lizzy, Sian, Phil, Jeanne, the bar, the library crew and of course Thomas Linacre. I would like to give a special thank you to the people, without which, I would most definitely not have survived this process. Thank you, Mum, Dad, Martin, Gift, Anita and my wonderful Justin. Finally I would like to acknowledge the Medical Research Council for funding my DPhil and the Sir William Dunn School of Pathology for allowing another Glaswegian into Oxford.

Abbreviations

APC	Antigen Presenting Cell
ALCAM	Activated Leukocyte Cell Adhesion Molecule
BCR	B Cell Receptor
BCL-L	B Cell Lymphocytic Leukaemia
BMDC	Bone Marrow Derived Dendritic Cell
CHO	Chinese Hamster Ovarian
CD	Cluster of Differentiation
CKII	Caesin Kinase II
Con A	Concanavalin A
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
EAE	Experimental Allergic Encephalomyelitis
EAN	Experimental Allergic Neuritis
ELISA	Enzyme Linked Immunosorbant Assay
FACS	Fluorescent Associated Cell Sorter
Fab	Fragment Antigen Binding
Fc	Fragment Crystallisable
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin G
IgSF	Immunoglobulin Superfamily
IL-2	Interleukin-2
ITAM	Immunoreceptor Tyrosine based Activation Motif
ITIM	Immunoreceptor Tyrosine based Inhibitory Motif
ITSM	Immunoreceptor Tyrosine based Switch Motif
KO	Knock-Out
LAT	Linker for Activation of T cells
Lck	Lymphocyte specific protein tyrosine kinase
mAb	monoclonal Antibody
MCC	Moth Cytochrome C
MHC	Major Histocompatibility Complex
PBL	Peripheral Blood Leukocytes
PD-1	Programmed Death 1
PD-L1	PD-Ligand 1
PI-3K	Phosphoinositide- 3 Kinase
PHA	Phytohaemagglutinin
PH	Pleckstrin Homology
PKCθ	Protein Kinase C theta
PLCγ1	Phospholipase C gamma 1
pMHC	Peptide bound to MHC
PP2A	Protein Phosphatase type 2A
Rac1	Ras-related C3 botulinum toxin substrate 1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2
SH3	Src Homology 3
SHIP	SH2-containing Inositol Phosphatase
SHP-1 and 2	Src Homology region 2 domain containing Phosphatase-1 and 2
SLP-76	SH2 domain containing Leukocyte Protein of 76kDa
SMAC	Supramolecular Activation Complex
SRCR	Scavenger Receptor Cysteine Rich
TCR	T Cell Receptor
TNF	Tumour Necrosis Factor
ZAP-70	Zeta chain Associated Protein kinase 70

1 Chapter : General Introduction

1.1 Regulation of Immune Responses

The immune system is a powerful tool our body uses to fight and kill invading pathogens. In order to do this it has developed multiple methods of releasing toxins, targeting pathogens and killing them. The immune system consists broadly of an innate response which targets pathogens non-specifically and an adaptive response which will remember specific pathogens and prevent subsequent infections. The adaptive immune response, in the main, consists of T and B lymphocytes which are activated and directed by the innate immune cells namely antigen presenting cells (APC) such as macrophages and dendritic cells.

For the purposes of this thesis I will concentrate on T lymphocytes. T lymphocytes or T cells are important for survival. Without T cells we are not able to fight against infection. For example, in the case of human immunodeficiency virus (HIV) infection which kills CD4+ T cells, the body becomes highly susceptible to opportunistic pathogenic infections which would normally not be able to cause disease (1). However, if T cells become over reactive or target self they can be destructive and cause autoimmune conditions. Many of these diseases are T cell driven, such as Multiple Sclerosis (2, 3). In this disease T cells specific for self antigens in the myelin sheath of nerves start attacking the areas around the central nervous system leading to serious disease.

In order to prevent these diseases the immune system must be tightly regulated. There are a number of mechanisms which will stop immune cells reacting against self (Figure 1.1). This is first of all achieved by a careful selection of T cells with T cell receptors (TCR)

which will specifically recognise non self peptides presented on self major histocompatibility complex (MHC) molecules expressed on antigen presenting cells (APC). This selection, or central tolerance, takes place mainly in the thymus where self reactive T cells are eliminated (outlined in Figure 1.1). Complete removal of self reactive cells however is not always achieved and in fact self reactive T cells remain circulating in blood of healthy individuals (4). Some of these have been shown to be part of a subset of T cells which are regulatory in their role and actively prevent reactions against self (5). Added to this, some areas of the body are not normally exposed to the immune system such as the eye, testis and placenta by way of protection from these autoreactive T cells. In addition, when T cells react to peptide bound to MHC (pMHC) without a second 'danger' signal i.e. upregulation of certain receptors when an active infection occurs, they will become unresponsive to repeat stimulations or undergo activation induced cell death (AICD). At the most basic level, peripheral tolerance and regulation are controlled by the receptors expressed on the surface of T cells and on APCs. If a T cell comes into contact with a cognate pMHC on an APC they will only react to this and become activated if there are a number of other accessory receptors present. These are mediated through cell-cell interactions and signals which transmit a positive signal into the T cell. In response to this signal T cells then undergo proliferation and differentiation into mature effector T cells which are then armed and ready to fight infection. These costimulatory signals are counter regulated by inhibitory receptors in order to maintain a tight control over the stimulation. If more inhibitory receptors are stimulated than costimulatory receptors, the T cell will not become activated. Altogether these make up the mechanisms of peripheral immune tolerance to protect against autoimmunity (outlined Figure 1.1).

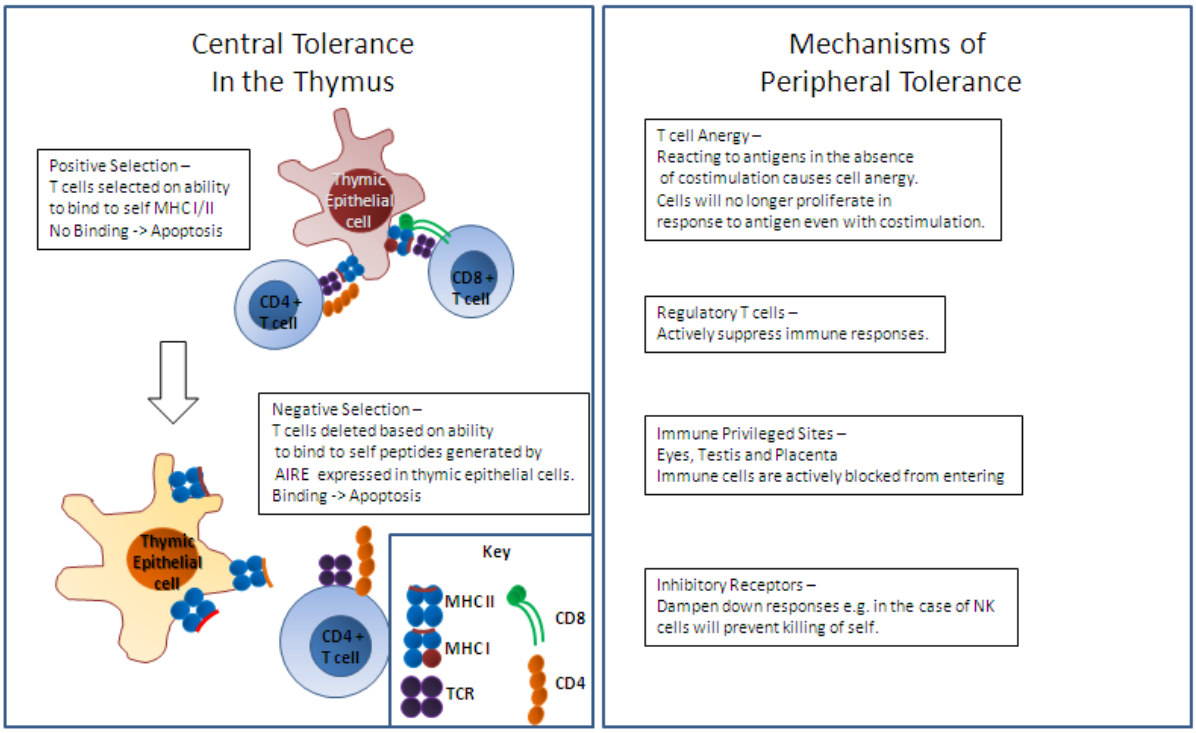


Figure 1.1: Mechanisms of T cell regulation and tolerance. *Summary of the mechanisms of self tolerance divided into central tolerance in the thymus and mechanisms of peripheral tolerance.*

1.1.1 Activating co-receptors regulate T cell activation

T cells are regulated by a vast array of surface receptors (Table 1.1). More and more is being understood about the function of these receptors and how they signal, however there is still a lot which remains unclear. In order to become activated, T cells must receive a signal through its TCR and also via multiple accessory molecules such as CD4, CD8 and CD28. These receptors, if bound to their ligands, MHCII, MHCI and CD80/86 respectively, will transduce a positive signal into the T cell thereby aiding its activation. Following binding of these receptors there is subsequent upregulation of late activation receptors such as CD69 and CD40L along with CD25, the IL-2R α chain. These receptors will then enhance T cell proliferation and the further activation of the APC. If these signals are not received the T cell will become anergic which will leave the cell unresponsive to subsequence stimulation or the cell will undergo clonal deletion.

T Lymphocyte Receptor	Superfamily member	Extracellular Ligand (expressed on APC)	Contribution to T cell activation
CD2	IgSF	CD58 (+CD48 in mice)	Stimulatory/Inhibitory(6, 7)
CD4	IgSF	MHC II	Stimulatory (8)
CD5	SRCR	Disputed	Stimulatory/Inhibitory (9, 10)
CD6	SRCR	CD166 (ALCAM)	Stimulatory (11)
CD8	IgSF	MHC I	Stimulatory (12)
CD28	IgSF	CD80/CD86	Stimulatory (13)
CD45	Receptor type PTP	-	Stimulatory (12)
CD69	Lectin	-	Stimulatory (14)
CD152 (CTLA-4)	IgSF	CD80/CD86	Inhibitory (15)
CD154 (CD40L)	TNF	CD40	Stimulatory – T cell help (16)
PD-1	IgSF	PD-L1	Inhibitory (17)

Table 1.1: T lymphocyte co-receptors. *Details of lymphocyte co-receptors. In particular their structure, ligand and contribution to T cell activation.*

1.1.2 Inhibitory co-receptors regulate T cell activation

In addition to these positive signalling receptors the immune system has negative signalling receptors, some of which are listed in Table 1.1. These are thought to balance the signals delivered through the TCR in order to either prevent activation induced cell death, to block aberrant stimulations against self or simply to balance positive signalling in order to achieve the signalling threshold required for T cell activation. Unlike activating receptors however, the inhibitory receptors' function is not as clear cut and they are often claimed to be costimulatory receptors in certain circumstances. For example, the co-receptor CD2 was first described as an adhesion molecule which aids T cell activation, however subsequent data showed ligation of CD2 by monoclonal antibodies can lead to inhibition of T cell activation (6, 7). A similar contradiction resides around the function of CD5 which was shown to be inhibitory in thymocyte development following many years of evidence showing CD5 ligation could augment T cell proliferation (9, 10). As a lot of literature describing inhibitory receptors is apparently contradictory, theories have arisen as to their true functions in the immune system. In one review Crawford *et al.* describes a theory in which inhibitory receptors will specifically turn off certain pathways allowing other ones to be activated which could account for these apparently contradictory results (18). Further evidence to support this theory remains to be determined.

1.2 T cell receptor signalling

In order to understand the behaviour of these receptors with regards to their regulation of T cell activation it is important to consider the signalling cascades which are involved. T cell signalling has been widely studied and yet there is still a great deal which is not well understood.

1.2.1 T cell receptor activating signalling pathways

In order for T cell activation to occur a number of signalling pathways must be activated. These pathways involve initiation of calcium flux, activation of transcription factors, and cytoskeletal rearrangement all of which are required for successful T cell activation (19, 20). Following ligation of the TCR with pMHC, Lck, a Src family kinase, will phosphorylate tyrosines in the cytoplasmic tail of the CD3 chains which then aids the recruitment of the CD3 associated kinase ZAP-70 (21). These initial events then result in subsequent tyrosine phosphorylation of adaptors such as LAT and SLP76 which recruit and aid the activation of downstream signalling proteins such as PLCγ1, Vav and PI-3K (Figure 1.2.1) (22). The phosphorylation of tyrosines, serines and threonines is central to this process with these modifications providing docking sites for specialised domains, such as the SH2 domain, and also leads to activation of enzymatic cascades (23, 24). Other non phosphorylation dependent interactions involving SH3 domains binding to proline rich regions, aids recruitment of large signalling complexes to the T cell receptor. Together these form a signalling scaffolding which creates the machinery required to transduce a signal to the nucleus and start T cell differentiation and production of certain cytokines.

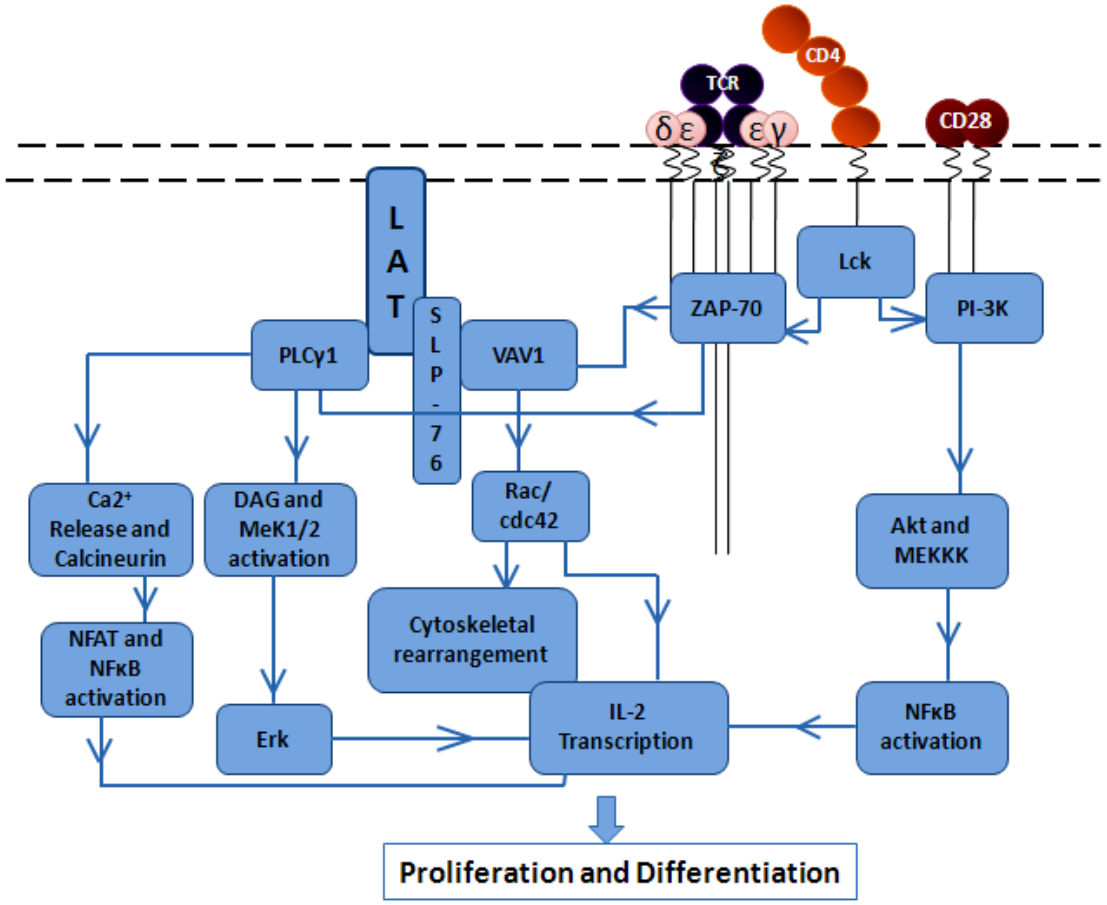


Figure 1.2.1: A Summary of Positive T cell Signalling Pathways. *Arrows indicate phosphorylation/activation of downstream signalling proteins. LAT and SLP76 are adaptors which recruit and bind to PLCγ1 and VAV1.*

1.2.2 T cell inhibitory signalling pathways

The positive signals triggered during T cell activation must be regulated and controlled by inhibitory signals. There are three main mechanisms of inhibition; the balance between phosphatases and kinases, negative feedback loops and degradation of signalling complexes. In the main inhibitory signals are mediated by phosphatases which will dephosphorylate tyrosines, serines and threonines (25). Desphosphorylation will deactivate kinases or prevent docking of signalling proteins via their SH2 domains thereby preventing signal transduction to the nucleus. The main tyrosine phosphatases which regulate T cell signalling in this way are Src homology region domain containing 1 and 2 (SHP-1 and SHP-2). These phosphatases can be recruited to the TCR through direct interactions with inhibitory receptors such as PD-1 (17). The serine protein phosphatase 2A (PP2A) is also recruited to the TCR and where it can inhibit signalling by dephosphorylating serines on downstream kinases. This serine phosphatase is found associated with the inhibitory receptor CTLA-4 (26). However, not all phosphatases are inhibitory. The receptor phosphatase CD45 will dephosphorylate inhibitory tyrosines on kinases such as Lck in order to activate their catalytic activity (27). The tyrosine kinase activity of Csk will inhibit Lck by tyrosine phosphorylation of this specific inhibitory tyrosine (28). In addition to this balance between phosphatase and kinase activity, another inhibitory signalling mechanism in T cell regulation is negative feedback loops. Regulatory proteins, such as Dok-2, are activated and recruited to the TCR by the initial TCR triggering where they can in turn inhibit ongoing signalling (29, 30). TCR signalling is also regulated by ubiquitination and degradation of TCR signalling complexes. This is thought to occur in what is described as the T cell central synapse, where TCR along with the stimulating kinases are tagged for degradation in the proteosome by the E3 ubiquitin ligase, Cbl-C (31).

1.2.3 The T cell synapse and its function

Recently it has become clear that location of TCR mediated signalling events is important in the initiation and regulation of T cell triggering. The T cell synapse was first described by Monks *et al*. in 1998 as a supramolecular activation cluster (SMAC) which forms at the interface between a T cell and APC and was thought to be where TCR triggering occurs (32). The SMAC consists of an accumulation of TCR along with signalling components such as ZAP-70 and a number of other accessory molecules (Figure 1.2.2). Later it was found that the T cell synapse in fact contained signalling proteins which were responsible for the downregulation of TCR, such as Cbl-C, thereby stopping the T cell signal altogether (31). It was determined that T cell triggering in fact occurred prior to formation of the SMAC (33). Using better microscopy techniques it was observed that smaller structures, named microclusters, were formed soon after TCR triggering and contained all of the signalling machinery thought to be required for T cell activation (34). These mircoclusters have been reported to migrate to a central SMAC where it is thought that their signalling is terminated. Since progression of this line of research a number of accessory receptors have been described to be involved in formation and regulation of these structures including CD5 which has been shown to inhibit signalling in the synapse without inhibiting its formation (35).

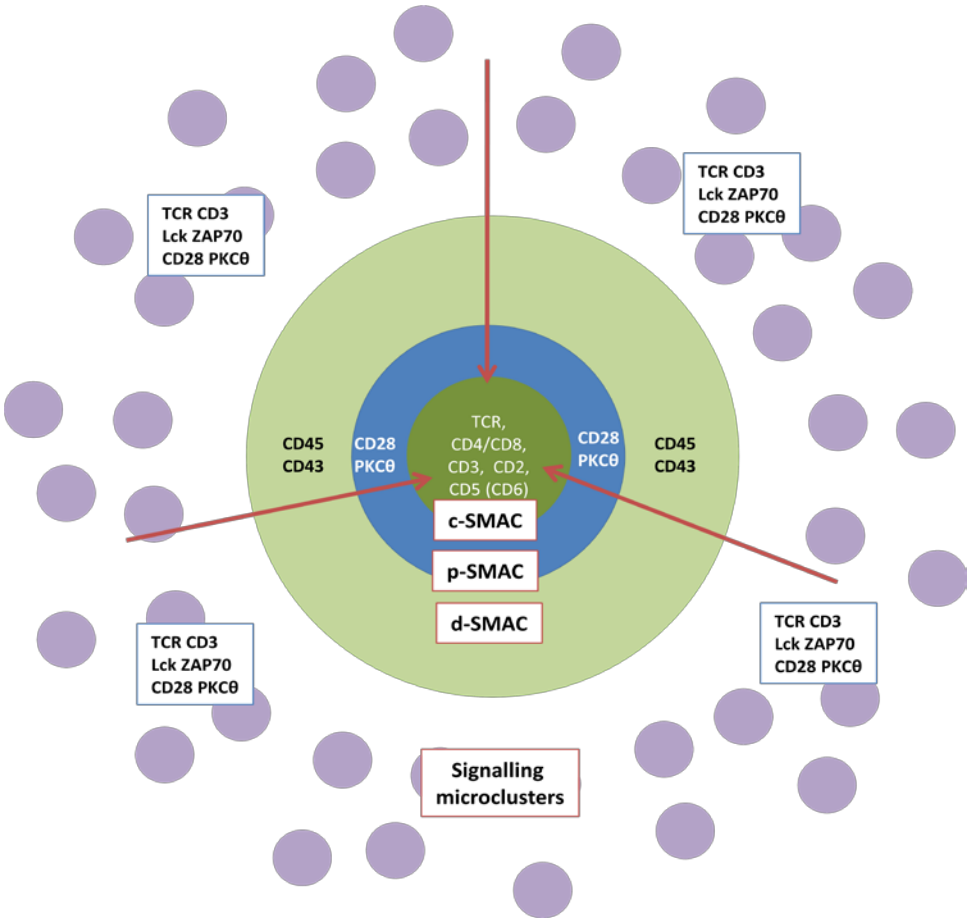


Figure 1.2.2: T cell synapse and receptor organisation. *Components of the c (central) - SMAC, p (peripheral) - SMAC and d (distal) - SMAC and signalling microclusters are illustrated.*

1.3 The T cell co-receptors CD5 and CD6

CD5 and CD6 are two co-receptors expressed on T cells and a small subset of B cells (36). Unlike the vast majority of receptors expressed on lymphocytes, which are members of the IgSF family of receptors, CD5 and CD6 are members of a family of receptors named the scavenger receptor cysteine-rich (SRCR) superfamily. CD6 is co-expressed with CD5 and was cloned around the same time as CD5 in 1986 (37, 38). CD6 is known to costimulate T cells following binding to its ligand, CD166, and signals through the adaptor SLP-76 (11, 39). CD5 was first described as being a marker for identifying T lymphocytes (40). Following this finding CD5 was cloned by Jones *et al*. in 1986 and identified as being important in the augmentation proliferative response of T cells (41, 42). Ten years later CD5 was found to inhibit thymocyte positive selection creating controversy in the field (10). The contradicting literature surrounding the function of CD5 has created a number of questions with regards to the true role of this receptor in peripheral T cell activation.

1.3.1 Extracellular associations and structure

Like the wide spread immunoglobulin superfamily, the SRCR superfamily have roles in the regulation and development of the immune system. This family was defined following the analysis of a domain found in the type I macrophage scavenger receptor (reviewed in (43)). As more members of the family were described, the SRCR superfamily was divided into two subgroups based on the number of cysteines present in the SRCR domains and their genomic organisation. SRCR domains are characterised by containing 6 (Group A) or 8 (Group B) cysteines encoded by 2 to 3 exons or a single exon respectively (43). CD5 and CD6 are members of the group B SRCR family with their 3 extracellular SRCR domains containing 8 cysteines each with the exception of CD5 domain 2 which only

contains 6 cysteines (44). CD5 and CD6 are both encoded on chromosome 11q in humans and chromosome 19 in mice and it is thought that they have arisen due to primordial gene duplication (45). Like CD5 and CD6, other members of the group B SRCR family, including WC1, Spα, CD163 and SCART1, are also expressed in the immune system and have a wide variety of functions (46-49).

Extracellular associations of CD5 have been the subject of much conflict in the literature. Ligands including gp35-37, gp77-80, gp150, the framework of $IgV_{H,}$ CD72, zymosan particles from fungus and CD5 itself have all been suggested in the literature however these have not been verified (50-56). Unlike CD5, the ligand for CD6 was readily identified to be CD166 (57). The well characterised interaction of CD6 with CD166 involves domain 3 of CD6 binding to the domain 1 of the CD166 with a K_D of 0.4 μM (Figure 1.3.1) (11, 58). This interaction has been identified as being required for the costimulation of T cells by CD6 (11). There is some indication of CD6 binding to LPS (59) and another uncharacterised protein named CD6L however these have not been independently confirmed (60).

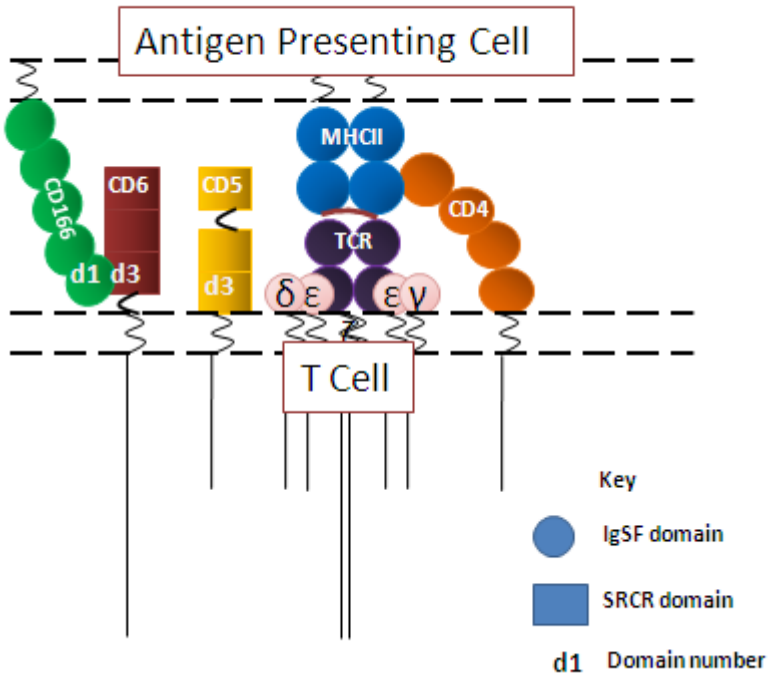


Figure 1.3.1: Representation of domain organisation of CD5 and CD6. *CD5 and CD6 are co-expressed on T cells and found associated with TCR complex. CD6 is known to bind to CD166 via domain 3 on CD6 and domain 1 on CD166. CD166 is expressed on antigen presenting cells.*

1.3.2 CD5 signalling and sequence homology

Amino acid alignments of CD5 from different species shows this receptor is highly conserved from chicken to man (Figure 1.3.2 A). Of note there is high sequence homology in the intracellular region of CD5 across species including the regions surrounding three tyrosines and the serine rich sequence at the C terminus which points to a possible conserved role of these regions. In mature peripheral T cells there is strong evidence showing that CD5 is phosphorylated on tyrosines early (2 mins) following TCR stimulation (61). There is also evidence showing CD5 is capable of becoming phosphorylated on serines (62). The physiological relevance of the phosphorylation events with regards to CD5 regulation of T cell activation however remains controversial.

CD5 has also been shown to associate with CD3 and a number of other receptors found in the immune synapse and is therefore in a position where it could regulate TCR signalling either positively or negatively (35, 63, 64). In attempting to define the function of CD5 a lot of work has concentrated on determining how CD5 signals. This research however appears to have created more questions than it has answered. CD5 appears to associate with a wide variety of signalling pathways which are involved in activation of T cell proliferation, cytoskeletal rearrangement, receptor degradation and T cell inhibition (Figure 1.3.2 B). These include direct and indirect associations with kinases ZAP-70, Lck, Fyn, PI-3K and casein kinase II, calcium signalling CamKII and cytoskeletal regulator Vav (64-70). CD5 has been shown to associate with signalling proteins involved in receptor downregulation and degradation including Cbl C and AP2 (71, 72). CD5 is thought to inhibit T cell signalling through an association with SHP-1 (73). Most of this work has been carried out using immunoprecipitation with some mutational analysis which

could be argued as being biased and does not clearly distinguish between direct and indirect associations. In addition many of the associations described have been induced by antibody ligation of CD5 and does not address the effects of CD5 signalling on T cell activation. Clearly more work is required in order to ultimately define the nature of CD5 signalling and how it regulates T cell activation.

In defining the role of CD5 signalling on T cell activation, one can consider the function of the related receptor CD6, which has been defined better than CD5. These two receptors share structural and amino acid homology. Like CD5, CD6 is found associated with TCR/CD3 complex in the centre of the immunological synapse (74). The last tyrosine in the intracellular region of CD6 is shown to interact with and bind with 0.5 μM affinity to the adaptor SLP-76 (39). There is evidence to suggest that CD6 also associates with tyrosine kinases (75). These could include Lck, Fyn, ZAP-70 and Itk protein tyrosine kinases which have all been shown to pull down with CD6 (76). Considering these two receptors are co-expressed, it is not surprising that there is evidence showing crosstalk between the two. CD6 has been shown to regulate CD5 phosphorylation by recruitment of specific kinases (76). This regulation may extend further to a combined role for regulation of T cell activation.

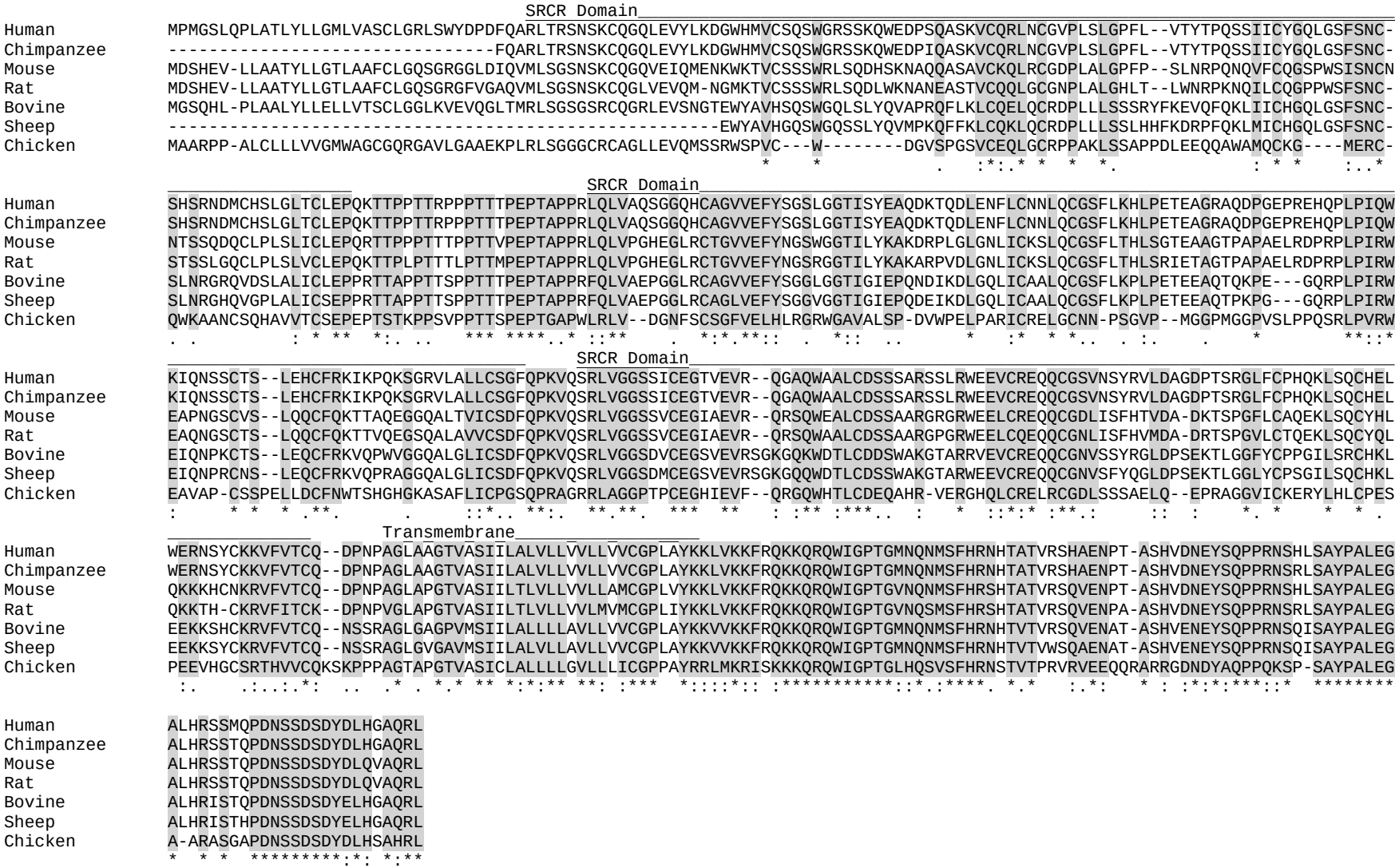


Figure 1.3.2 A: Amino acid alignment of CD5 across different species. *Conserved sequences are shaded and indicated below with identical a.a. (*), conserved substitutions (:) and semi conserved substitutions (.). The 3 SRCR domains are denoted above along with the transmembrane region.*

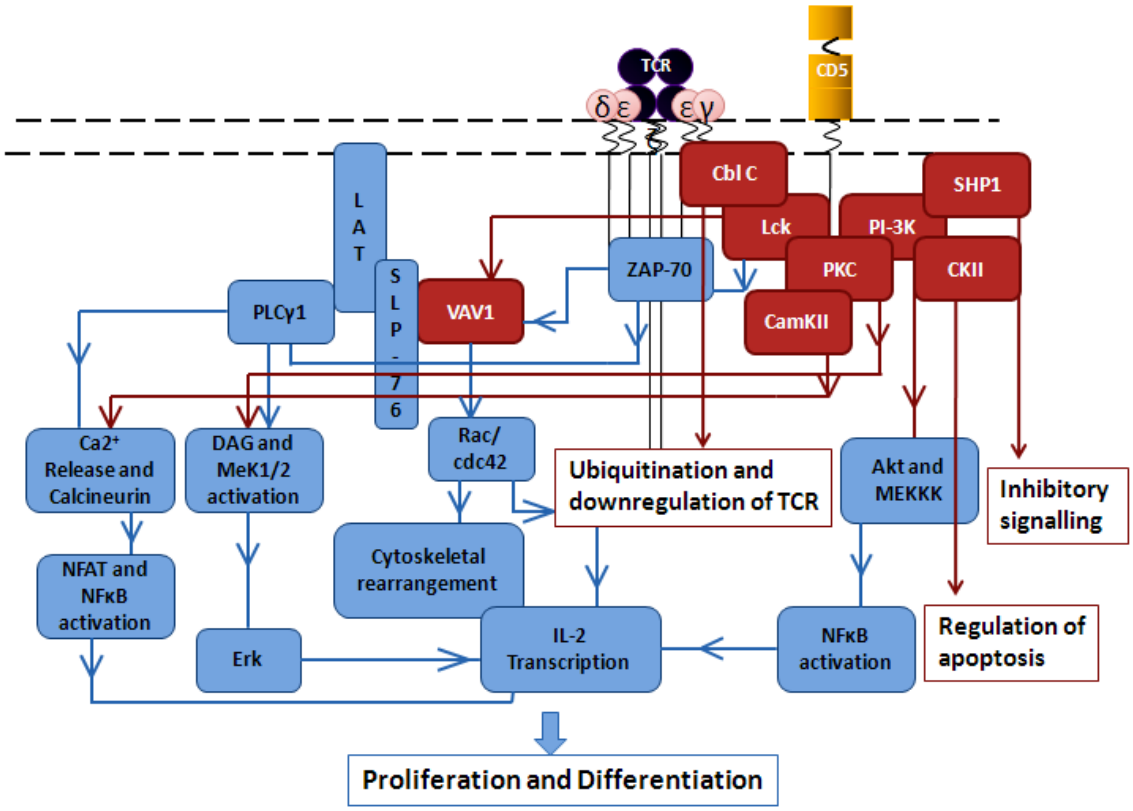


Figure 1.3.2 B: Summary of suggested CD5 signalling pathways. *Signalling pathways affected by CD5 ligation or found to associate with CD5 are highlighted in red. The TCR signalling pathways are indicated in blue. Arrows indicate phosphorylation or activation of downstream signalling proteins.*

1.3.3 The function of CD5 *in vitro* and *in vivo*

There are strong data demonstrating a role for CD5 in thymocyte development (10, 77). CD5 KO mice showed no gross defect but on closer inspection there was a defect in the selection of TCR specificities (78). This pointed to CD5 being inhibitory to thymocyte stimulation thereby aiding the selection of TCRs which bind with high affinity to pMHC. This inhibition by CD5 was also shown to be independent of its' extracellular domain (77). Moreover the expression of CD5 itself appears to be tightly regulated by signal strength delivered through the TCR namely via signals from PKC theta (78-81). Azzam *et al.* demonstrate that strong T cell signals result in an upregulation of CD5 expression in thymocytes (81).

As far as the role of CD5 in the peripheral T cells, however, the evidence remains less clear. Peripheral T cell proliferation is enhanced following CD5 ligation and crosslinking with either CD3 or CD28 demonstrating a positive effect of CD5 on T cell activation (82-86). However, in CD5 knock-out (KO) mice the T cells are hyperresponsive to stimulation *ex vivo* pointing to an inhibitory role of this receptor (78). Contradictory to this, the CD5 KO mice display a delayed disease onset along with decreased disease severity when EAE is induced (10, 87). The authors go on to suggest this is due to increased AICD which is indicated by developing resistance to stimulation by the antigen and increased apoptosis in the CD5 KO T cells (87). This was later described as being due to signalling through CK2 which will inhibit apoptosis (88). However, when this interaction was first characterised, Raman *et al*. show CK2 activation only occurs in thymocytes and does not occur upon CD3 stimulation of peripheral T cells (69). Further work on these CD5 KO mice showed

that CD5 may in fact provide inhibition to regulatory T cells adding further complexity to this story (89).

Due to the lack of a concrete definition of CD5 function in the periphery it has been difficult to produce appropriate therapies using CD5 mAbs. Interestingly CD5 is highly upregulated in B cell chronic lymphocytic leukaemia (BCL-L) (90, 91). However the use of CD5 mAbs to remove these specifically has been unsuccessful (92). Moreover, the use of CD5 mAbs in treatment of autoimmune disease models such as EAE and collagen induced arthritis have provided contradicting results (93-97). One such mAb, OX19, has been used extensively and is thought to recognise domain 1 of rat CD5 (51). This antibody has been shown to exacerbate disease in Experimental Allergic Neuritis (EAN), inhibit disease in Experimental Allergic Encephalomyelitis (EAE) and also have no effect in EAE (93-95). The differential results seen with this and other mAbs may be due to isotype of the antibody, the epitope of CD5 which it recognises or the timing of administration. Further work needs to be carried out in order to define the role these mAbs are playing in order to further define the function CD5 has in relation to T cell activation.

The function of CD6 with regards to its role in T cell activation is relatively well defined. Expression of CD6 alone will limit the responsiveness of T cells. However, upon binding to CD166, CD6 will stimulate T cells to proliferate (11). CD6 can also replace the requirement for CD28 costimulation of CD3 stimulated T cells (98). CD6 mAbs have been used as therapeutic agents to prevent bone marrow graft rejection by depleting CD6 positive T cells (99-101). By considering the role of both CD5 and CD6 together may help shed more light on the role of CD5 in regulation of T cell activation.

1.4 Aims of this thesis

It is clear that there is a great deal of unanswered questions regarding the nature of the role of CD5 in peripheral T cell activation. It is not clear whether CD5 in fact inhibits or aids activation of T cells following their stimulation. It is also not clear how CD5 signals in order to do this. In this thesis I aim to readdress the contradictions in the literature surrounding the role of CD5 during peripheral T cell activation.

In order to do this I will:

1. Define the mode of action of OX19, the rat CD5 mAb, in manipulation of CD5 regulation *in vitro* and *in vivo.*
2. I will also analyse the role of tyrosine phosphorylation of CD5 with specific regard to its effect on CD5 regulation of T cell activation.
3. I will characterise potential direct and indirect interactions of the CD5 cytoplasmic tail.

The purpose of this research is to investigate the role of CD5 in the regulation of T cell activation in the periphery.

Chapter 2: Materials and Methods

2.1 Reagents and Solutions

2.1.1 Buffers

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.3 was made by dissolving tablets (Oxoid Ltd.) in ddH_2O or bought in Dulbecco's PBS (PAA Laboratories Ltd.)

PBS/BSA: PBS containing 0.5 % (v/w) bovine serum albumin (BSA) (Sigma-Aldrich Co.).

FACS buffer: 10 mM NaN_3 was added to PBS/BSA.

Mammalian cell lysis buffer 2 fold concentrated (2X): 20 mM Tris:HCl pH 7.4 supplemented with: 280 mM NaCl, 2 mM EDTA, 20 % (v/v) Glycerol, 0.4 % (v/w) NaN_3, 2 % NP40, 2 mM NaF (Sigma-Aldrich Co.) and 20 µg/ml DNAse (Sigma-Aldrich Co.), 100 µl/10^8 cells of mammalian protease inhibitor cocktail (Sigma-Aldrich Co.).

Bacterial Lysis Buffer: 1 % (v/v) Triton X-100 in Hepes buffered saline (500mM) 10mM Hepes, 500mM NaCl, 0.02 % (v/w) NaN_3.

Hepes buffered saline (HBS): 10 mM Hepes pH 7.5 supplemented with: 140 mM NaCl. 0.02% NaN_3 was added for gel filtration.

SDS PAGE running buffer: 50 mM MOPS pH 7.3, 50 mM Tris, 3.5 mM SDS, 1 mM EDTA

Reducing and Non Reducing Protein Sample Buffer 2X: Reducing 2X - 4 % (v/w) SDS, 10 % (v/w) 2-mercaptoethanol, 20 % (v/v) glycerol, 0.004 % (v/w) bromophenol blue in 125 mM Tris:HCl. For non reducing buffer do not include 2-mercaptoethanol.

Western blotting transfer buffer: 25 mM Tris pH 7.7, 200 mM Glycine, 0.1 % (v/w) SDS and 20 % (v/v) methanol.

Wash buffer: PBS, 0.05% (v/v) Tween 20.

Blocking buffer: For Western Blotting: Wash Buffer 1% BSA, For ELISA: PBS supplemented with 10% FCS

Digestion buffer for preparation of Fab fragments from IgG: 0.04M EDTA 0.04M Cysteine in PBS.

ACK red cell lysis buffer: 150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA, adjusted to pH 7.2-7.4 with 1N HCl

2.1.2 Tissue Culture Media

RPMI 1640 and DMEM (PAA Laboratories Ltd.) were supplemented with 2 mM L-Glutamine (GibcoBRL), 50 µM 2-β mercaptoethanol, penicillin 50 U/ml(PAA Laboratories), streptomycin 50 µg/ml (PAA Laboratories) and heat inactivated fetal calf serum 5-10% (v/v) as indicated (PAA Laboratories). All supplements were passed through 0.22 µm filters (Millipore Ltd). Supplemented media are referred to as RPMI-C and DMEM-C.

2.1.3 Bacterial Culture Media

Luria-Bertani (LB) broth: 171 mM NaCl, 1.0 % (v/w) bactotryptone and 0.5 % (v/w) yeast extract pH 7.0. 100 µg/ml sodium ampicillin was used as a selective agent. Supplemented with 100 µg/ml sodium ampicillin and 30 µg/ml chloramphenicol (Sigma-Aldrich Co.) for

protein expression and induced with 100 µg/ml Isopropyl β-D-1-thiogalactopyranoside IPTG (Sigma-Aldrich Co.).

2.1.4 Bacterial Cells

Ecoli DH10B™ derivative NEB 10-beta competent ecoli were used for plasmid amplification and cloning (New England Biolab Inc.). Ecoli BL21 competent bacteria were used for protein expression.

2.2 DNA Methods

2.2.1 PCR and Expression Cloning

Primers were synthesised, desalted and lyophilised (Sigma-Aldrich Co.). PCR reactions were carried out using Platinum® Pfx DNA Polymerase (Invitrogen Ltd.) supplemented as per the manufacturer's instructions. 30-40 cycles of PCR amplification were carried out consisting of: denaturation step 95 °C for 15 sec, annealing step 52 °C for 30 sec and elongation step 68-76 °C for 1min/1000 bases. Reactions were carried out in an automated thermo cycler with peltier-heated block and heated lid (Biometra).

2.2.2 Generation of CD5 constructs for transfection

The retroviral mammalian expression system vector pBABE-puro was used (Figure 2.2) (102). hCD5 WT pBABE, hCD5Y1F pBABE and hCD5 Y3F pBABE clones were made previously in the lab (MH Brown). The 5' BamH1 site in the multiple cloning site (MCS) in pBABE was previously destroyed by cloning hCD5 WT into pBABE using a 5' BglII site. hCD5 Y1Y3 double mutant was cloned using hCD5 Y1F pBABE as a template.

PCR fragments using a convenient sense domain 1 hCD5 M124Q primer 5' GCAGAAATGACCAATGTCACTCTCT 3' and the antisense hCD5Y3F 5'CTTAAGGTCGACTTACAGCCTCTGAGCCCCATGCAGATCAAAGTCACTGTCG GAGGAGTTG 3' with SalI digestion site underlined. An internal BamH1 site exists in human CD5 at the N terminus of the transmembrane region. PCR fragments were therefore digested using BamH1 and Sal1 for insertion into BamH1/Sal1 digested hCD5WT pBABE vector thereby swapping the intracellular regions. Vectors were then sequenced using pBABE primers, sense 5' ATCCAGCCCTCACTCCT 3' and antisense 5' AACTGGGCGGAGTTA 3'. For hCD5 Y2F pBABE mutant double PCR was carried out. PCR fragments were generated using the sense M124Q primer and antisense Y2F primer 5' GCCTGTCAGCTTTTCCAGCTCTGG 3' and hCD5WT pBABE as a template. A second set of PCR fragments were generated using sense Y2F primer 5' CCAGACTGGAAAAGCTGACAGGC 3' antisense hCD5 WT primer 5' CTTAAGGTCGACTTACAGCCTCTGAGCCCCATGC 3' with Sal1 site underlined using hCD5WT as a template. PCR fragments were then generated using the first 2 PCR fragments annealed together as a template with the sense M124Q primer and hCD5WT antisense primer. PCR fragments were then digested BamH1 and Sal1 for cloning into hCD5WT cut with BamH1 and Sal1 to swap the intracellular regions as before. hCD5 serine mutants were generated using hCD5 WT pBABE as a template and the antisense primers as follows hCD5AA 5' GTCGACTTACAGCCTCTGAGCCCCATGC AGATCATAGTCAGCGTCGGCGGAGTTGTC 3' and hCD5 AAF 5' GTCGAC TTACAGCCTCTGAGCCCCATGCAGATCAAAGTCAGCGTCGGCGGAGTTGTC 3'.

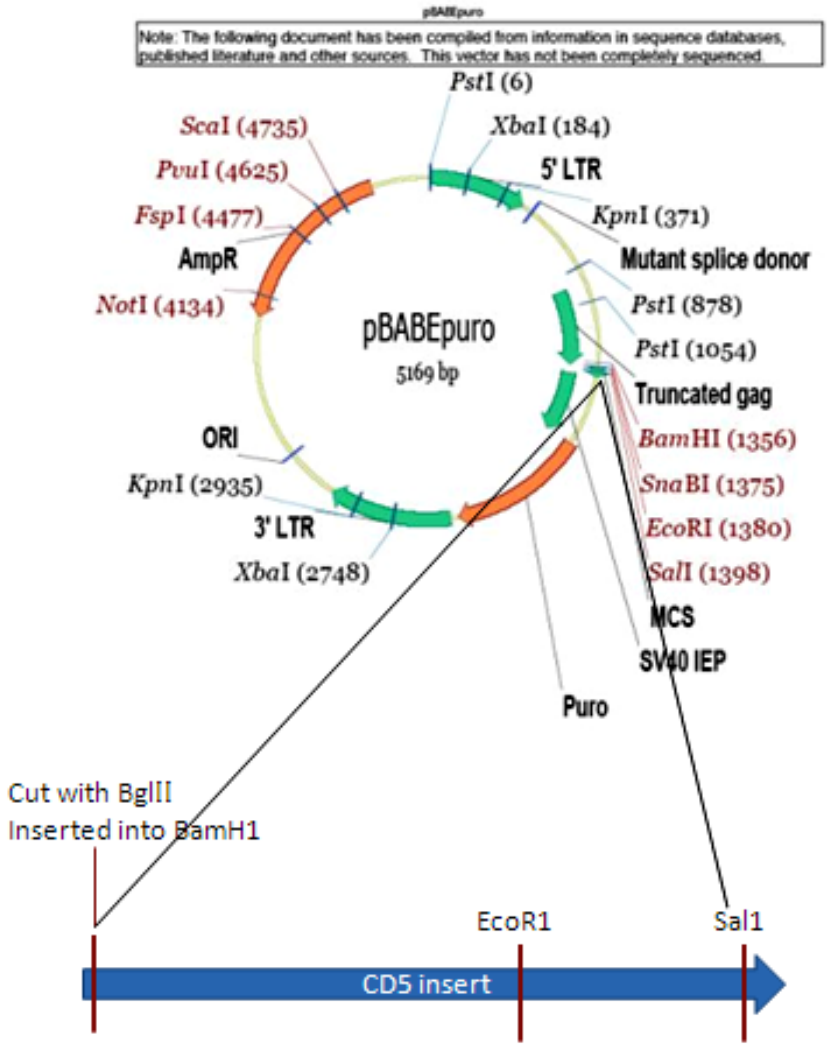


Figure 2.2: Vector map of pBABE-puro. *Adapted from www.addgene.com. Includes the CD5 WT insert indicating the restriction sites used originally.*

2.2.3 Ligations

Molar vector:insert ratios of 1:4 or 1:1 of appropriately digested vector and insert were incubated with 1 unit of T4 DNA ligase (Boehringer, Mannheim) in the supplied buffer at a final volume of 10 µl. Ligations were incubated overnight at room temperature and then used to transform competent bacteria.

2.2.4 Restriction Digestion

Restriction enzymes were purchased from New England Biolab Inc. and stored at -20 °C. 1-2 units of enzyme was used to cut 0.2-1 µg DNA in manufacturers' recommended buffer and incubated in a water bath at 37 °C for 1 hour. Digested fragments were run on a DNA gel and gel purified using Qiagen Gel Purification Kit.

2.2.5 Transformation of competent bacteria, DNA Amplification and Purification

15µl NEB Ecoli DH10B™ derivative competent cells were mixed with 10µl ligation mix or 1µl purified plasmid DNA and incubated for 30 mins on ice. Bacteria were then heat-shocked for 90 sec at 42°C and were placed on ice for a further 5 mins. Transformed bacteria were then incubated in 100µl LB at 37°C for 10 mins before cells were plated out on agar plates supplemented with appropriate selection antibiotic (Ampicillin 100µg/ml) and incubated for 16 hours at 37°C. Colonies were then picked and grown up in the appropriate volume of LB with selection antibiotics for 16 hours at 37°C shaking. Volume depended on the size of Qiagen kit used. Plasmid DNA was purified using Qiagen columns as recommended by the manufacturer (QIAGEN). Purity and quantity of DNA

was measured using Nanodrop ND100 spectrophotometer measured by absorbance at 260 and 280 nm.

2.2.6 DNA sequencing

Samples for sequence reactions were sent as separated vials of 100-500ng DNA template and 3.2 pmol primers. DNA sequencing was carried out externally using Geneservices (Oxford). This was carried out using the BigDye™ Sanger dideoxynucleotide method. Chromatograms were analysed and assembled using Chromas Lite v2.01 and ApE-A plasmid Editor v1.17.

2.3 Protein Methods

2.3.1 Expression and Purification of Recombinant Proteins

All constructs and most recombinant SH2 domain proteins were produced by the Oxford Module Consortium in the Oxford Protein Purification Facility (Louise Bird) (Table 2.3.1). Cbl C was made by expression of pMAL construct from the OMC consisting of the Cbl C PTB domain in BL21 bacteria. BL21 cells transformed with Cbl C construct were grown in 5ml of LB supplemented as mentioned before for 16 hours at 37°C in a shaking incubator. This culture was then added to a further 200ml of LB for protein expression and incubated until an O.D. 600 nm had reached 0.4. Bacteria cells were then induced with 1 mM of IPTG and left to express for 4 hours at 37°C in a shaking incubator. Cells were pelleted and could be stored at -20°C until further use. Pelleted cells were defrosted and 10 ml of bacterial lysis buffer was added and incubated with the cells for 30 min. Lysates were sonicated at amplitude 10 for 30 secs x3 with incubations on ice in between each

cycle. Lysates were then centrifuged in an ultracentrifuge 30,000 rpm for 40 mins at 4°C. Supernatants were diluted 1:3 with HBS 500 mM before being loaded on a 2 ml pre-equilibrated nickel agarose column and allowed to flow through under gravity. The nickel column was then washed with 10 column volumes of HBS 500 mM. The protein was then eluted using 250mM immidazole in PBS. 200 µl samples were collected and protein concentration was determined by absorbance at 280 nm on Nanodrop ND 100 using theoretical extinction coefficients determined on Protein Calculator v3.2 (http://www.scripps.edu/~cdputnam/protcalc.html).

Protein	Domains	Species	Amino Acid number
Lck	SH2	Human	123-230
Lck	SH2 SH3	Human	58-230
Fyn	SH2 SH3	Human	82-248
Itk	SH2	Human	228-345
Itk	SH2 SH3	Human	162-345
ZAP 70	SH2 SH2	Mouse	2-261
Cbl C	PTB	Human	5-351
Vav1	SH2	Human	665-770
PI-3K p85	SH2 (N)	Human	327-435
PLCγ1	SH2 (N)	Human	545-660
SHP-2	SH2 SH2	Human	2-219
CSK	SH3 SH2	Human	2-182

Table 2.3.1: Details of proteins and their domains used in this thesis. *All supplied by Louise Bird (Oxford Module Consortium (OMC)) except ZAP-70 which was made by Marcus Bridge and Cbl C which was made by myself from construct provided from the OMC.*

2.3.2 Immunoprecipitation

2B4.CD2WT hCD5 transduced cells or activated human PBLs were either pervanadate stimulated for 5 mins or not and then lysed with mammalian lysis buffer on ice for 30 mins. Lysates were spun at 13,000 rpm for 10 mins at 4°C. 5µl of isotype control antibody Cyanogen Bromide (CNBR) coupled Sepharose beads with CD5 mAb (Leu1) were added to lysates for 1 hour at 4°C on a rotor in order to pre-clear non specific binding

proteins. Pre-clear lysates were then incubated with human CD5 mAb (Y2-178) CNBR coupled Sepharose beads and incubated for 2 hours at 4°C on a rotor. All beads were washed 3X with cold PBS and samples were boiled in reducing sample buffer and loaded on gels for analysis by western blotting or mass spectrometry.

2.3.3 Peptide Pull Down

Streptavidin coupled Dynabeads® were coated with 16µg biotinylated peptides for 30 mins at 4°C. Peptides used for this and for surface plasmon resonance are listed in Table 2.3.3. 10^8 stimulated human peripheral blood lymphocytes (PBLs) per peptide pull down sample were washed in PBS and lysed in total volume of 500µl with mammalian lysis buffer for 1 hour at 4°C. Lysates were spun at 15000 G for 15 mins at 4°C and then precleared with streptavidin coupled Dynabeads® for 30 mins at 4°C on a rotor. Using a magnet, supernatants were removed and peptide coated beads were added and incubated on a rotor for 2 hours at 4°C. Beads were then washed 3X in cold PBS and resuspended in 20µl of reducing protein sample buffer and boiled for 10 mins. Beads were spun and supernatant removed and run 1cm on a gel. The gel band was then cut out and processed as described section 2.3.8 for analysis by mass spectrometry.

Peptide name	Amino acid number of tyrosine	Peptide sequence	Species
CD5 Y0	402	Bio- CGPLApYKKLVK	Human
CD5 Y1Y2	429, 441	Bio-YDNEpYSQPPRNSHLSApYALEG	Human
CD5 Y1	429	Bio-HVDNEpYSQPPRN	Human
CD5 Y1 non phos	429	Bio-HVDNEYSQPPRN	Human
CD5 Y3	463	Bio-PDNSSDSDpYDLHGAQRL	Human
CD5 Y3 non phos	463	Bio-PDNSSDSDYDLHGAQRL	Human
CD244 ITSM 1	297	Bio-GCSTIpYSMIQS	Human
CD6 Y3	486	Bio-GSDSDpYEHYDFSAQ	Human
CD6 Y4	489	Bio-GSDSDTEHpYDFSAQ	Human
CD6 Y3 Y4	486,489	Bio-GSDSDpYEHpYDFSAQ	Human
CD6 Y9	662	Bio-PDSTDNDDpYDDISAA	Human
CD3 zeta ITAM 1	72,83	Bio-DPNQLpYNELNLGRREEpYDVLE	Mouse
ZAP-70	290	Bio-TLNSDGpYTPEPA	Human
CSK positive control peptide	-	Bio-EISAMpYSSVNK	Human

Table 2.3.3: Peptides and their sequences used in this thesis. *pY depicts phosphorylated tyrosine. Peptides were supplied by Peptide Protein Research Ltd.*

2.3.4 Antibodies

Isotype	Reactivity	Clone	Format	Application	Company
Ms IgG1	Hu CD5	Leu1	TCS	FACS	In house
Ms IgG1	Hu CD5	UCHT2	Purified	Biacore	In house
Rat IgG2a	Ms CD3	KT3	TCS	FACS	In house
Rat IgG2a	Hu CD52	YTH 34	TCS	FACS negative control	In house
Goat polyclonal IgG	Ms IgG	N/A	Purified: FITC	FACS secondary	AbD Serotec
Goat polyclonal ($F(ab)^2$)	Rat IgG	N/A	Purified: FITC	FACS secondary	AbD Serotec
Goat polyclonal IgG	Ms IgG	N/A	Purified: DyLight®649	FACS secondary	AbD Serotec
Hamster IgG1	Ms CD3e	145-2C11	Purified and biotinylated	Stimulation assays	BD biosciences UK
Ms IgG1	Rat CD5	OX19	Purified, TCS	Animal and FACS	In house
Ms IgG1	Rat CD5	OX19	Purified:RPE	FACS	AbD Serotec
Ms IgG1	Hu Factor I	OX21	Purified, TCS	Animal and FACS negative control	In house
Rat IgG2a	Ms CD2	RM2.1	TCS	FACS	In house
Rat IgG2a	Ms CD5	53.7.313	TCS	FACS	In house
Ms IgG1	Rat CD8	OX8	Purified: FITC	FACS	AbD Serotec
Ms IgG1	Hu CD45RA	F8-11-13	Purified: FITC and RPE	IgG1:FITC/RPE negative control FACS	AbD Serotec
Ms IgG1	Rat CD4	W3/25	Purified: FITC	FACS	AbD Serotec
Ms IgG1	Rat TCR	R73	FITC	FACS	AbD Serotec
Ms IgG1	Rat TCR	R73	Purified	Stimulation	In house
MsIgG1	Rat CD45RA	OX33	Purified: RPE	FACS	AbD Serotec
Ms IgG2a	Rat CD6	OX52	Purified: Biotin	FACS	AbD Serotec
Ms IgG2a	Hu CD45	F10-89-4	Purified: Biotin	IgG2a:Biotin negative control FACS	AbD Serotec
Ms IgG1	Hu CD6	OX126	TCS	FACS	In house
Ms IgG2a	Ms CD6	OX128	TCS	FACS	In house
Rat IgG2a	Ms IL-2	JES6-1A12	Purified	ELISA capture	BD biosciences UK
Rat IgG2b	Ms IL-2	JES6-5H4	Biotin	ELISA detection	BD biosciences UK
Ms IgG1	Hu IL-2	5344111	Purified	ELISA capture	BD biosciences UK
Ms IgG1	Hu IL-2	B33-2	Biotin	ELISA detection	BD biosciences UK
Ms IgG1	Phospho-tyrosine	PT66	Purified	BIACore	Sigma
Ms IgG2b	Phospho-tyrosine	4G10	TCS	Western blots (Thanks to Dr Acuto, SWDSOP)	In house
Rabbit IgG	ZAP-70	99F2	Purified	Western blots	Cell Signalling Technology
Rabbit IgG	Phospho ZAP70 Tyr 319	N/A	Purified	Western blots and FACS	Cell Signalling Technology
IgG	Ms IgG	N/A	HRP linked	Western blots	Cell Signalling Technology
Ms IgG1	Hu CD5	Y2-178	CNBR coupled sepharose	immunoprecipitation	In house (courtesy of Dr MH Brown)
Ms IgG2a	Ms NK1.1	PK136	Purified: Biotin	FACS	BD biosciences UK
Ms IgG2a	Ms IE^k	17-3-3	Purified: Biotin	FACS	BD biosciences UK
Rat IgG2a	Ms CD48	OX78	TCS	FACS	In house

Table 2.3.4: List of antibodies and applications for they were used. *Mouse (Ms) and Human (Hu).*

2.3.5 Fab production

The required amount of papain was dissolved in a volume of digestion buffer equal to the volume of OX21 antibody. This mixture was incubated for 12 hours at 37degrees. The reaction was stopped with Crystalline Iodoacetamide 0.03 M. Protein mixture was then dialysed over night in 2 litres of PBS with dialysis tubing at 4 degrees. The protein mixture was then concentration using 4k-10kDa cut membrane spin filter.

Fab fragments were resolved by FPLC (Pharmacia Biotech Ltd) using a Superdex S-200 column to exclude higher order aggregates. 42-43kDa fragment represent the correct size for Fab fragments and were isolated and pooled. Fc fragments were not removed in this case with protein A purification. OX19 Fab fragments had been generated previously in the lab and were resolved by FPLC to remove higher order aggregates.

2.3.6 SDS PAGE and Western Blotting

Protein samples were boiled for 10 mins in 2X non reducing or reducing sample buffer. Proteins were then loaded and separated according to their molecular weight using NuPAGE® Novex Bis-Tris pre-cast gradient (4-12%), 12 well, 1mm thick (Invitrogen Life Technologies). Gels were run in an X-Cell II gel tank (Novex) in MOPS running buffer. For western blotting SDS-PAGE gels were blotted onto Hybond™ P-PVDF transfer membrane (Amersham Pharmacia Biotech) using the Novex Excell blotting module according to the manufacturer's instructions. Blotted membranes were blocked overnight in blocking buffer at 4°C. Antibody detection was carried out using SnapI.D. (Millipore) system according to the manufacturer's instructions. Antibodies were used as described in Table 2.3.4.

2.3.7 BIACore™ and ProteON™ Analysis

Surface Plasmon resonance uses laser reflection from a surface to determine the amount of a given substance bound to a gold plated chip surface. Surface Plasmon resonance experiments were carried out using BIACore™ 3000 and for high throughput analysis, the ProteON™ using HBS supplied by the manufacturer. Streptavidin 0.2 mg/ml (Pierce Chemical Co) in 10 mM sodium acetate pH 5 was directly coupled to CM5 chip or ProteON equivalent (BIACore AB) following activation according to manufacturer's instructions resulting in 3000 response units (RU). Following deactivation of the chip, around 25 RU of biotinylated phosphorylated peptides diluted in HBS were immobilised onto the chip (Table 2.3.3). In all cases one flow cell was used as a negative control with no peptides immobilised. Equilibrium measurements were then determined as described (103). For the BIACore™, increasing and then decreasing concentrations of SH2 domains were run over each of the four flow cells at a flowrate of 20 µl/min. At each 5 µl injection the RU were recorded at the point of equilibrium and these were subtracted from the negative control flow cell. Binding affinities were then determined by plotting these RUs against concentration of protein injected. Curve fitting was done using one site specific binding equation in Prism 5 software (GraphPad). For the ProteON™, increasing concentrations of SH2 domains were run over the 6X6 flow cell matrix in a similar fashion as the Biacore. The ProteON™ Manager software (Bio-Rad) was used to subtract background response units using interspots (regions in between flow lanes as described by the manufacturer) and binding affinities were determined again using the equilibrium binding measurements and fitting them against protein concentration using this same software.

2.3.8 Mass Spectrometry

All mass spectrometry samples were processed by Benjamin Thomas in the Central Proteomics Facility. Proteins were extracted from gel pieces and digested with trypsin, desalted on a C18-packed pipette tip, before injection into Ultimate 3000 nano HPLC (Dionex) system coupled to a Thermo LTQ Orbitrap mass spectrometer. Samples were resolved on a 12 cm by 75 micron inner diameter picotip column (New Objective) which was packed in-house with ProntoSIL 120-3 C18 ace-EPS (3 micron) phase (Bischoff chromatography). Samples were resolved using a 30 min to 2 hour gradient, at a flow rate of 300nLmin^{-1}. The mass spectrometer was operated in a data dependent acquisition mode, in which 2+, 3+ and 4+ ions were selected for fragmentation. Precursor scans were performed in the Orbitrap at a resolving power of 60,000 (FWHM), from which five precursor ions were selected and fragmented in the linear ion trap ("top-5 method"). Charge state +1 ions were rejected. For data analysis RAW data files were converted to the mzXML format using ReAdW (version 4.2.1), and submitted to the in-house Central Proteomics Facilities Pipeline (CPFP, version 1.3.0) (104). Searches were performed against version 3.64 of the IPI mouse or human protein sequence database utilising Mascot (Matrix Science), X!Tandem with the k-score plugin, and OMSSA search engines. The resulting peptide identifications from each search engine were validated with PeptideProphet. ProteinProphet inferred protein identifications from the resulting peptide list. All searches were performed against a concatenated target/decoy database, providing an empirical false discovery rate (FDR) that can be compared with the estimated FDRs from the prophet tools to confirm the validity of results. Spectral index normalised quantitation (SINQ) at the protein level were performed on grouped datasets to provide quantitative estimates of the relative protein abundance between samples. Localisation of chemical modifications was determined by running a ModLS localisation algorithm within

the CPFP (Trudgian et al unpublished). Protein identifications were exported from the CPFP and uploaded to ProteinCenter (ver 3.7.10003, Proxeon) for filtering, comparison and interpretation.

2.4 Cellular Methods

2.4.1 Rat Tissue

Red cells from rat splenocytes were first lysed using ACK lysis buffer and filtered to remove red blood cells and dead cells before staining.

2.4.2 Human PBL Isolation and Activation

Human PBLs were isolated by centrifugation on Ficoll-Hypaque for 30 mins at room temperature. Mononuclear cells were collected from the interface and washed twice with RPMI-C. Cells were maintained in RPMI-C at 5 % CO_2 and 37 °C. For activation and expansion, PBLs were treated with 1 µg/ml of PHA (Sigma) in media supplemented with 100 U/ml of recombinant human IL-2 and incubated for up to 10 days at 5 % CO_2 and 37 °C. Cells were fed with new media every 5 days.

2.4.3 Cell Lines

2B4 T cell hybridoma cells expressing transfected mouse CD2 or a tailless CD2 (CD2Δ) under 0.5 mg/ml G418 selection (105) were transduced with hCD5 wt or mutants constructs indicated. Cells were selected 1-2 µg/ml puromycin and grown in DMEM-C 10

% FCS and maintained at 10 % CO_2 and 37 °C. A chinese hamster ovarian cell line (CHO) expressing major histocompatibility complex (MHC) class II IE_K previously described by Wild *et al*. (105) were maintained at 10 % CO_2 and 37 °C in DMEM-C 5% FCS supplemented with 0.5 mg/ml G418. CHO IE_K cells expressing mouse CD48 used by Wild *et al*. (105) were maintained in DMEM-C + 5% FCS supplemented with 20 µM methylsulfoximine and 0.5 mg/ml G418 at 10 % CO_2 and 37 °C.

2.4.4 Retroviral Transduction

The various hCD5 pBABE constructs were transfected into the Phoenix ecotrophic- helper free retroviral producer cell line (Phoenix-eco) which stably expresses gag/pol genes required for retrovirus production (106). Transfection of Phoenix-Eco cells grown to 70% confluency with 1µg of DNA was carried out using FuGene® 6 standard protocol. Cells were incubated at 37 degrees and supernatant containing virus was harvested at 24 and 36 hours after transfection. Virus supernatant was added to $5x10^5$ 2B4.mCD2WT or 2B4.mCD2Δ cells with 8 mg/ml of Polybrene which aids sticking of viral particles to cells. These were incubated for 4-6 hours at 37 degrees, the media changed and cells incubated for a further 24 hours before 1-2µg/ml of puromycin selection was added. Cells were then maintained in puromycin selection.

2.4.5 Flow Cytometry and Cell Sorting

For flow cytometry, cells were stained with primary and secondary antibodies for 30-60 mins on ice and washed in 200-500 µl FACS buffer between each staining. Cells were fixed in 0.5% paraformaldehyde before analysis as required. FACS analysis was carried

out on FACS Calibur and FACS Sort machines using Cell Quest software for acquisition and Flowjo™ for further analysis.

For cell sorting, cells were washed in sterile PBS/BSA and stained with primary and secondary antibodies diluted in sterile PBS/BSA and washed in between each stain. Cells were sorted by Nigel Rust for matched expression levels using a Dako Cytomation MoFlo Legacy machine**.**

2.4.6 Dynal Bead Cell Sorting

Transduced cells were washed once in media and then resuspended in 500µl of sorting Buffer (5µM EDTA, 10% FCS in PBS). Approximately 9µg of selecting biotinylated antibody (BD biotin anti-CD5) was added and incubated on ice for 1 hour. Cells were then washed once with sorting buffer and incubated for 30 minutes on ice with prewashed streptavidin coated Dynabeads® (Sigma) were added Dynal beads were washed using dynal magnet to remove any unbound cells. Dynal beads were resuspended in selection media and left to grow off the beads.

2.4.7 Confocal Microscopy

CHO IE^K mCD48 cells were plated out in poly-d-lysine coated, 35mm glass bottomed petri dishes with No. 1.5 coverglass (MatTek Corporation) at 10^5 cells/well. 1µM of Moth Cytochrome C (MCC) peptide (amino acid no. 88–103 ANERADLIAYLKQATK – made by Peptide Protein Research Ltd) was incubated in 500 µl with the CHO cells for 5 mins at 37°C 10% CO_2. 2B4.CD2WT cells with hCD5WT transduced were added to these wells at $4x10^5$ cells/well and incubated for 30 mins at 37°C 10% CO_2. After incubation media

was gently removed and cells were fixed with 3.7% paraformaldehyde and blocked with 0.5%BSA/PBS. Cells were washed 2X with cold PBS and permeabilised with 0.1% Triton X100 in 5 mM TrisHCl. Cells were washed 2X and stained with primary antibodies specific for mouse CD3 (KT3) and human CD5 (Leu1) diluted in blocking buffer for 1 hour at room temperature in a humidified chamber. Cells were washed 3X 5 mins with cold PBS and secondary antibodies (anti-rat IgG FITC and anti-mouse IgG DyLight 649) were incubated for 1 hour at room temperature in dark humidified chamber. Cells were washed 3X 5 mins in PBS and mounted with DAPI stain using Vectasheild® with DAPI (Vector Laboratories Inc.). Cells were imaged using Olympus flowview FV1000 and FV10-ASW software in the Bioimaging facility.

2.5 Cellular Assays

2.5.1 CD3 Stimulation of Cell Lines

For CD3 stimulation assays 96 well plates were coated with mouse CD3 mAb (BD 143-2C11) in PBS at stated concentrations for 1 hour at room temperature. The antibody solution was flicked out of the plate by quick inversion prior to addition of cell lines. 2B4 transduced cell lines were counted and resuspended in DMEM 10%FCS without selection and plated out at $1x10^5$ cells/well in a volume of 200µl of media and incubated for 24 hours or otherwise stated at 10% CO_2, 37 °C. Following incubation plates were spun at 300 G and 150µl of supernatant were removed for analysis by ELISA. The remaining cells were fixed in 0.5% Paraformaldehyde for FACS analysis.

2.5.2 Antigen Specific Stimulation of Cell Lines

For antigen specific stimulation assays, CHO cells were added to round bottomed 96 well plates at $5x10^4$cells/well in a volume of 50µl of DMEM 10% FCS. MCC peptide was added at stated concentrations and 2B4 transduced cells were added at $1x10^5$cells per well. Total volumes in the wells were made up to 200µl total and incubated for 24 hours at 10% CO_2, 37 °C. Following incubated cells were harvested as for the CD3 stimulation.

2.5.3 Ex Vivo Antigen Specific Stimulation

Matured bone marrow derive dendritic cells (BMDCs) were plated at $1x10^5$cells/well in a 96 well plate with and without myelin oligodendrocyte glycoprotein peptide (33-55) (MOG) at 5µg/ml. Draining lymph nodes were acquired from diseased rats and single cell suspensions and plated out at $1x10^6$cell/well with and without mAb or Fab fragments at 5µg/ml or Con A at 5µg/ml. Cells were incubated for 3 days at 37°C 5% CO^2 and pulsed for the last 18 hours with ^{3}H-Thymidine and harvested.

2.5.4 IL-2 ELISA

ELISA plates (Nunc) were coated with 50µl of capture antibody (see Table 2.3.4) diluted 1:500 in PBS and incubated overnight at 4°C. Plates were washed 2X in washing buffer and blocked in ELISA blocking buffer for 1 hour at room temperature. 50µl of samples to be assayed were added neat or diluted in blocking buffer. 50µl of recombinant IL-2 standard was added starting at 2000pg/ml with doubling dilutions and measured in triplicate wells. Plates were incubated for 2 hours at room temperature and washed 3X in wash buffer. 50µl of detection antibody diluted 1:1000 mixed with ExtraAvidin®-

peroxidase (Sigma-Aldrich Co.) at 1:500 in blocking buffer was added to the plate and incubated for 1 hour at room temperature. Plates were washed 4-5X in wash buffer. 50µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich Co.) was added to each well and left until colour developed. Reaction was stopped with 50µl of 2 M sulphuric acid and read at 450 nm using an automated ELISA plate reader.

2.5.5 Proliferation Assays

Cervical, inguinal and mesenteric lymph nodes were removed from 10 week old female Lewis rats and pooled. Single cell suspensions were made and red cells lysed using ACK lysis. Cells were incubated with antibody or Fab fragments for 30 mins on ice. Concanavalin A at 5µg/ml and R73 TCR mAb at 5µg/ml were incubated along with the cells, which were plated at a concentration of $2.5x10^6$cells/ml for 3 days at 37°C 5% CO_2 in a 96 well plate. For the last 18 hours cells were pulsed with ^{3}H Thymdine and its incorporation was measured using microbeta plate reader following harvesting of the cells onto a membrane. The radiation is proportional to the amount of thymidine that is incorporated into the cells which reflects DNA replication in the cells.

2.6 Animal Experiments

2.6.1 Animals

Inbred 8 week old female Lewis rats were obtained from Jackson Laboratories. Male rats were not used because disease is too severe in these animals.

2.6.2 Rat EAE Induction

Rats were injected at the base of the tail bone with a homogenised mixture of complete Freunds Adjuvant and Guinea pig MOG peptide at 10µg/rat. Following first signs of disease rats were injected intraperitoneally with 250 µg/rat of antibodies. Animals treated in this manner were examined daily for clinical disease. Results were scored as follows: 0, no EAE; 1, loss of tail tonicity; 2, Loss of hind leg tonicity; 3, Unilateral hind limb paralysis; 4, Bilateral hind limb paralysis; and 5, Lower body paralysis. In later experiments animals were weighed daily also. Animals were euthanized if remained on score of 4 for more than three days or progressed to score of 5. Animals were euthanized following recovery from paralysis and spleens and draining lymph nodes were removed and cell suspensions were made before freezing samples at -80 degrees in 10% DMSO in FCS.

2.6.3 Bone marrow removal and BMDC generation

Rats were euthanized by CO_2 and hind legs were removed. Bone marrow was extracted from the legs using a needle and cells were then washed and resuspended in ACK solution for 3 mins at room temperature. Cells were then plated at $1x10^7$ cells/well in 6 well plates in R5 media supplemented with flt-3 (donated from Gordon MacPherson) at 100ng/ml and incubated 9 days at 37°C, 5% CO_2 to allow dendritic cell formation. Media was changed every 3 days (107).

Chapter 3: Manipulation of CD5 regulation *in vitro* and *in vivo*

3.1 Introduction

One way of understanding the role of a receptor and to elucidate the molecular mechanisms underlying this is through the use of monoclonal antibodies (mAbs). mAbs can affect receptor function in four different ways: triggering and crosslinking the receptor to induce signalling, blocking interactions with a ligand, inducing cell death via antibody mediated cytotoxicity, and finally triggering modulation of the receptor from the surface of cells (Figure 3.1). It is important to determine which of these mechanisms the mAb is operating through in order to accurately interpret any results. The mouse IgG1 anti rat CD5 antibody named OX19 has been used in the literature in order to help gain insight into the role of CD5 and yet this still remains a mystery. This mAb has had opposing effects *in vivo* and *in vitro* leading to contradictory conclusions about CD5 function (93, 94). Conclusions of CD5 being inhibitory or stimulatory have been drawn from other experiments also using CD5 mAbs (108, 109). Demonstrating the mode of action of OX19 may therefore help elucidate the role of CD5 in the immune response. This area of research is important when considering the use of mAbs as a therapeutic agent.

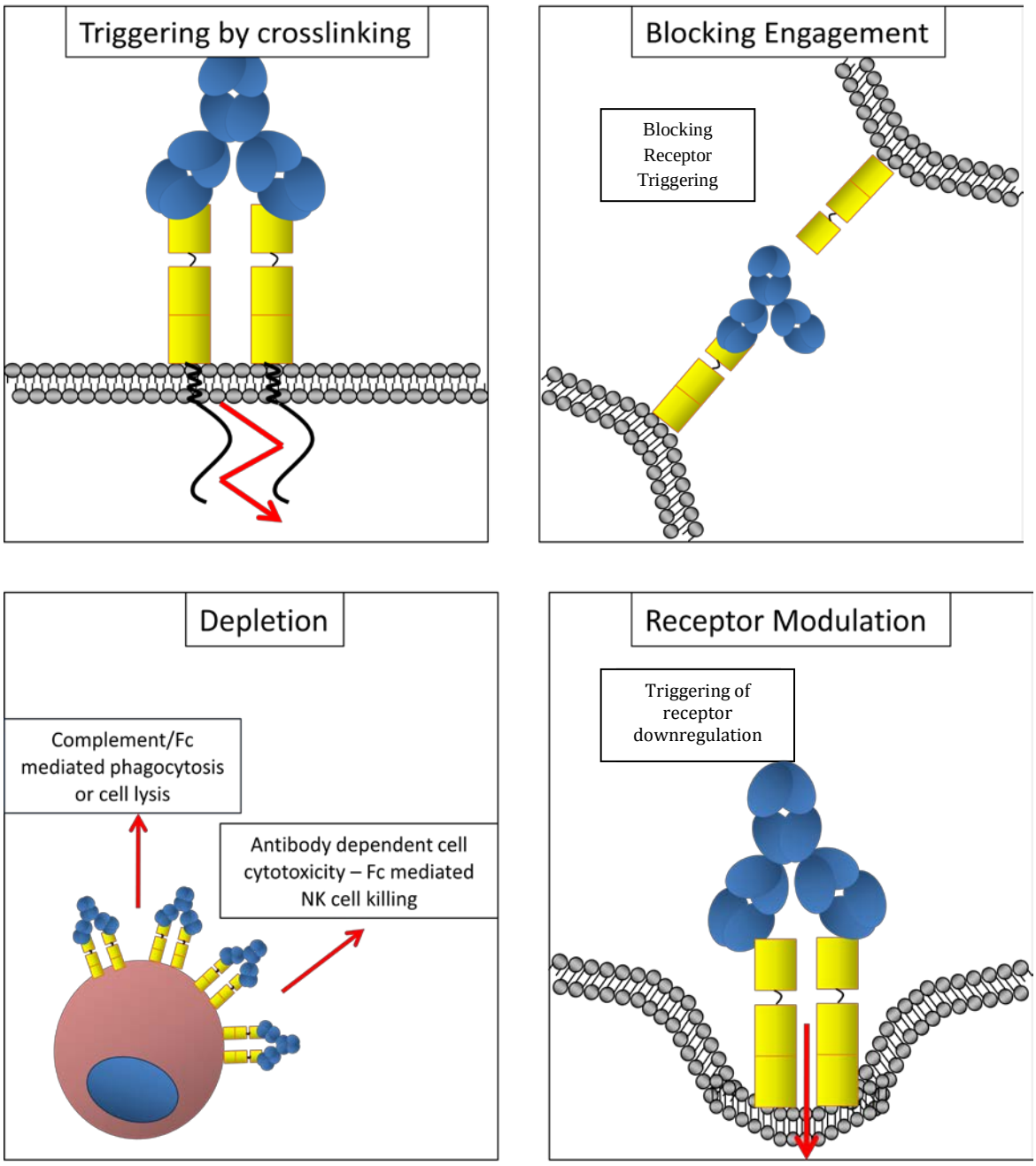


Figure 3.1: Four modes of actions of mAbs. *Antibodies can crosslink receptors and induce signalling, block receptor engagement with its ligand, stimulate depletion of the cells through various mechanisms and can induce receptor downmodulation.*

3.1.1 Evidence of CD5 mAbs triggering CD5

It is often assumed that mAbs will crosslink receptors and induce signalling. OX19, the rat CD5 mAb, has been used in the rat model of multiple sclerosis, EAE and has been shown to block development of disease by administration before the disease induction (94). Sun *et al.* have proposed that OX19 induces an active suppressive activity in T cells which is mediated by CD5. These suppressive cells were shown to prevent transferred pathogenic T cells causing disease. Contrary to these results, OX19 was shown to induce proliferation of T cells *in vitro* (86). OX19 itself is not mitogenic, but when added alongside mitogenic stimulation will increase the response produced further. In addition to OX19, there are examples in the literature of other CD5 mAbs (anti human CD5 mAbs Cris-1, F145GF3 and OKT1) triggering signalling through CD5 and augmenting T cell proliferation and activation (110, 111). These antibodies are described as mitogenic but also often require the presence of monocytes or immobilisation in order to exert these effects. If these antibodies are triggering CD5 by crosslinking and inducing CD5 signalling one could conclude that CD5 induces T cell activation, but at the same time inducing an active suppression.

3.1.2 Evidence of CD5 mAbs blocking CD5

The evidence proposing OX19 is crosslinking could at the same time be interpreted as blocking extracellular engagement. There have been many ligands proposed for CD5 and therefore it is possible that a mAb could block the engagement of CD5 and therefore prevent it from triggering. The mouse CD5 mAb (TIB104) is thought to prevent pathogenicity in rheumatoid arthritis by blocking accumulation of CD5 expressing pathogenic T cells (97). This was based on the assumption that CD5 binds to CD72, an

interaction which has not been repeated elsewhere (unpublished data Brown) (51, 54). Stronger evidence that demonstrates the effect of blocking CD5 uses recombinant soluble CD5Fc fusion which could sequester all available binding sites in a similar fashion as an antibody (87). Human or mouse CD5 Fc was expressed in mice using adenovirus. The mouse, but not human CD5 Fc, managed to block the development of disease, which the authors predicted was due to blockade of ligand engagement. Given the assumption that CD5 mAbs are blocking, these data suggest that CD5 promotes accumulation of pathogenic T cells at the site of inflammation and can also enhance disease.

3.1.3 Evidence of CD5 mAbs modulating or deleting CD5

Another explanation for the effects of mAb is the induction of receptor modulation or specific deletion of the cells expressing the receptor. OX19 has been thought to have the ability to modulate CD5 and therefore eliminate its function. In EAN, T cells target the peripheral nervous system. Administration of OX19 at the height of the clinical response increases disease severity and coincides with a loss of CD5 expression (93). Strigard *et al*. discuss the possibility that CD5 is therefore required for downregulation of the immune response because removal of CD5 results in an increased inflammation. Contrary to this Sun *et al.*, provided evidence showing CD5 removal before disease induction resulted in decreased disease severity seen in EAE (94). Evidence for CD5 modulation in this model came from OX19 treatment of healthy animals which resulted in a loss of CD5 expression on T cells from the CD4 T cell compartment. Sun *et al*., however, do not manage to distinguish whether this is due to induced proliferation of the CD8 T cell compartment, altering the CD4:CD8 ratio or specific antibody induced cell death. This loss of CD5 could be due to deletion or receptor modulation, however no modulation of the CD5 from

the surface of the cells was found *in vitro*. Contrary to these results Brostoff *et al.* show no effect of OX19 introduction in the disease outcome of EAE when OX19 was given following disease induction (112). Altogether these two authors highlighted the importance of timing of CD5 mAbs introduction. Whether OX19 is given during, before or after disease induction can result in different clinical outcomes. Other CD5 mAbs (Cris-1) can act to modulate CD5 from the surface of cells (113). Alberola-lla *et al.*, however, had previously demonstrated that stimulation with Cris-1 triggered CD5 to increase T cell proliferation highlighting the importance of understanding the mechanisms used by mAbs (110). Considering this later result with the evidence showing Cris-1 in fact removes CD5 one may then conclude CD5 may in fact inhibit T cell proliferation.

3.1.4 Aims

There has been no clear demonstration of the mode of action of any of these antibodies and in particular the OX19 CD5 mAb. In light of this, the conclusions drawn about the role of CD5 in the immune response may have been wrongly interpreted (Table 3.1). In order to understand this better I aim to show whether OX19 can work by triggering, blocking, modulating or deleting the receptor and the functional outcomes of this *in vitro* and *in vivo*.

Activation by CD5 versus inhibition by CD5	
CD5 Stimulates	CD5 Inhibits
Deletion of CD5 by modulation or deletion with OX19 blocks EAE induction	Deletion of CD5 with OX19 during EAN enhances disease
Blocking CD5 engagement (CD5 Fc) blocks EAE induction	OX19 triggers active suppression of EAE reducing disease
OX19 enhances proliferation	

Table 3.1: Weighing up the evidence for CD5 activation versus CD5 inhibition of the immune response.

3.2 CD5 mAb increases proliferation of splenocytes by blocking CD5 engagement *in vitro*

mAbs can only be used to help elucidate the function of a receptor once the mode of action of the antibody has been determined. OX19, the rat CD5 mAb, has apparently opposing effects depending on how it is used and how the data is interpreted (Table 3.1). Therefore it is important to determine the mechanism through which this antibody is operating. Previous work has shown that OX19 can increase proliferation of lymph node cells (86). Whether OX19 was crosslinking or blocking CD5 could not be confirmed in this case however because only whole antibody or $F(ab)^2$ fragments were used.

In order to determine if OX19 is working by crosslinking or by blocking engagement of CD5 the effect of whole antibody must be compared to the effect of Fab fragments which are unable to crosslink. To this end, lymph node cells were isolated from a healthy rat and stimulated with Con A and the rat CD3 mAb, R73, with and without rat OX19 mAb or Fab fragments. Proliferation in response to this stimulation of lymph node cells was then determined by measuring the level of tritiated thymidine incorporation into the cells after three days. When intact OX19 was introduced there was an increase in the proliferative response to ConA and CD3 mAb when compared to a control mAb, OX21 (Figure 3.2 A). Fab fragments of OX19 were generated by enzymatic digestion using papain and purified by FPLC in order to prevent aggregation. These fragments are unable to crosslink CD5. Addition of OX19 Fab fragments to the stimulated lymph node cells also enhanced proliferation when compared to the isotype control Fab (Figure 3.2 B). These data indicate that, in this system, OX19 works by blocking CD5 engagement. One can then assume CD5 is delivering an inhibitory signal to the cell.

In addition, OX19 mAb and Fab fragments were also titrated into unstimulated lymph node cells. OX19 mAb stimulation resulted in an increase in proliferation of the lymph node cells when compared to control mAb (Figure 3.2 C). This concentration dependant increase in the proliferative response is not seen when OX19 Fab is introduced into the system. These data indicate that OX19 can trigger by crosslinking when the cells are not being stimulated.

Altogether these data show that when lymph node cells are stimulated with ConA and CD3 mAb, OX19 can block CD5 inhibition of proliferation. By using the Fab fragments this shows the OX19 does not trigger the receptor thereby helping us draw the conclusion that CD5 is inhibitory in this setting. However when the cells are not activated by a strong stimulus OX19 appears to be weakly mitogenic and can trigger an increase in proliferation.

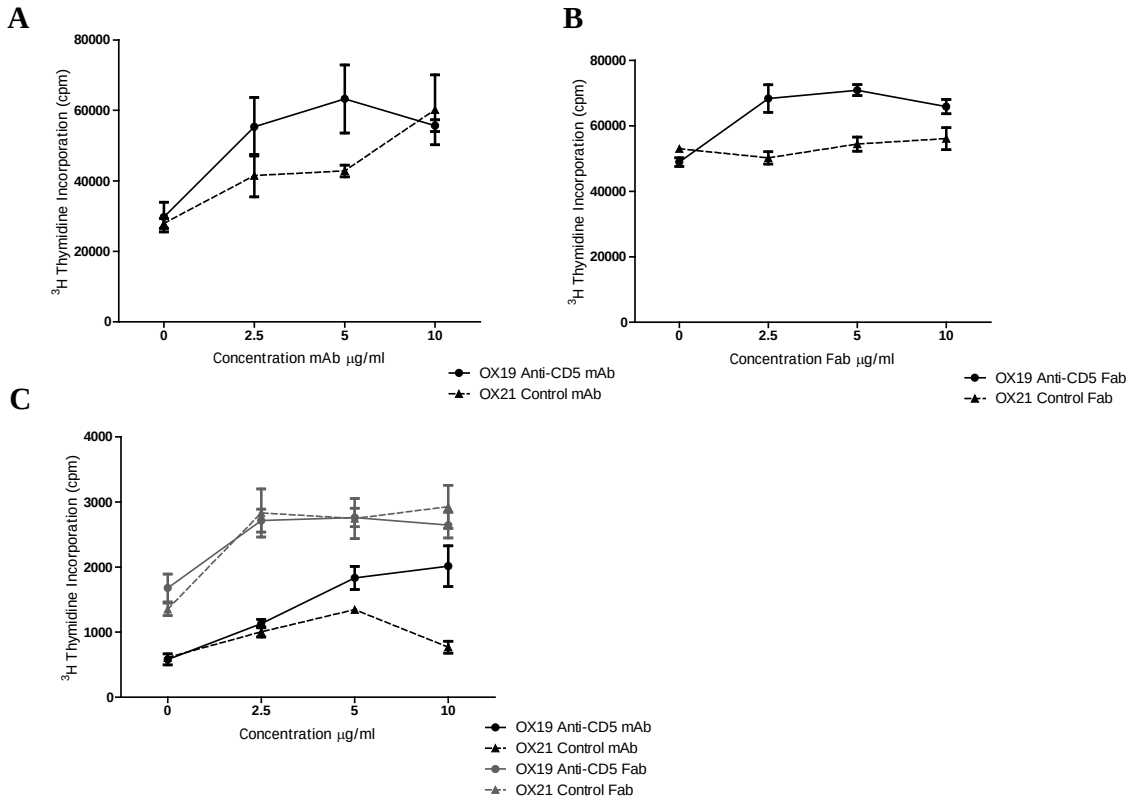


***Figure 3.2*: Rat Anti-CD5 mAb, OX19 acts by blocking not crosslinking CD5.** *^{3}H Thymidine incorporation in counts per minute of cervical, inguinal and mesenteric lymph node cells (2.5 x 10^6 cells/ml) from 10 week old female Lewis rats stimulated in vitro with Con A (5μg/ml) and R73 TCR mAb (5μg/ml) for 3 days following preincubation with increasing amounts of A) IgG whole antibody fragments and B) with Fab fragments. C) Unstimulated lymph node cells with OX19 or control mAb and Fab fragments. Results are represented as the mean of triplicate wells with standard error of the mean. These are representative of 3 separate experiments.*

3.3 CD5 mAb does not modulate CD5 from the surface of T cells *in vitro*

Here it has been shown that CD5 inhibition of T cell stimulation can be blocked by OX19. This evidence does not however rule out modulation of the receptor from the cell surface by the antibody. OX19 has been shown to modulate CD5 *in vivo* so it is important to determine whether CD5 modulation is occurring *in vitro* (94). To determine this flow cytometry was used to measure CD5 expression levels before and after Con A plus CD3 mAb stimulation in the presence of OX19. The FACS plot in figure 3.3 A shows that 70% of the rat lymph node cells are TCR positive and of these 89 % co-express CD5 before the experiment which is to be expected. Following stimulation with ConA and CD3 mAb in the presence of OX19 or a control mAb, lymph node cells were stained with OX19 to determine the levels of CD5 expression. It is important to consider that any residual bound OX19 from the stimulation of the lymph node cells could be masking CD5 from OX19 staining. In order to measure the presence of any residual OX19 bound to the cells, staining with OX19 directly conjugated to RPE was compared to staining with a polyclonal anti-mouse IgG. Considering that the anti-mouse IgG picked up staining on the OX19 mAb treated cells indicated that there was indeed residual bound OX19 remaining after the stimulation. To account for this, levels of CD5 expression were hereafter determined using the secondary anti-mouse IgG alone. The histograms in figure 3.3 B show CD5 expression following ConA and CD3 mAb stimulation in the presence of increasing concentrations of the mAb or Fab fragments as indicated. The top left panel shows a uniform level of CD5 expression which remains unchanged following incubation with increasing amounts of OX19. The top right panel shows the CD5 expression using OX19:RPE to stain following Fab fragment stimulation. There is a slight decrease in

apparent CD5 expression in this case however this is most likely due to increased masking of the CD5. Considering that the secondary anti-mouse IgG is unable to bind to Fab fragments it was not possible to distinguish between reduced CD5 expression and increased bound OX19 Fab. The two bottom panels show the levels of CD5 present on the control mAb stimulated cells which remains unchanged. These data are represented with the mean fluorescence levels of CD5 expression following ConA and CD3 mAb stimulation in the presence of OX19 or Control mAb (Figure 3.3 C). This shows no significant modulation of CD5 from the surface following OX19 treatment and an increase in the level of CD5 on the surface of these cells when compared to Control mAb treated cells. Altogether these data show that OX19 does not work by modulating CD5 *in vitro* and therefore will most likely block CD5 engagement.

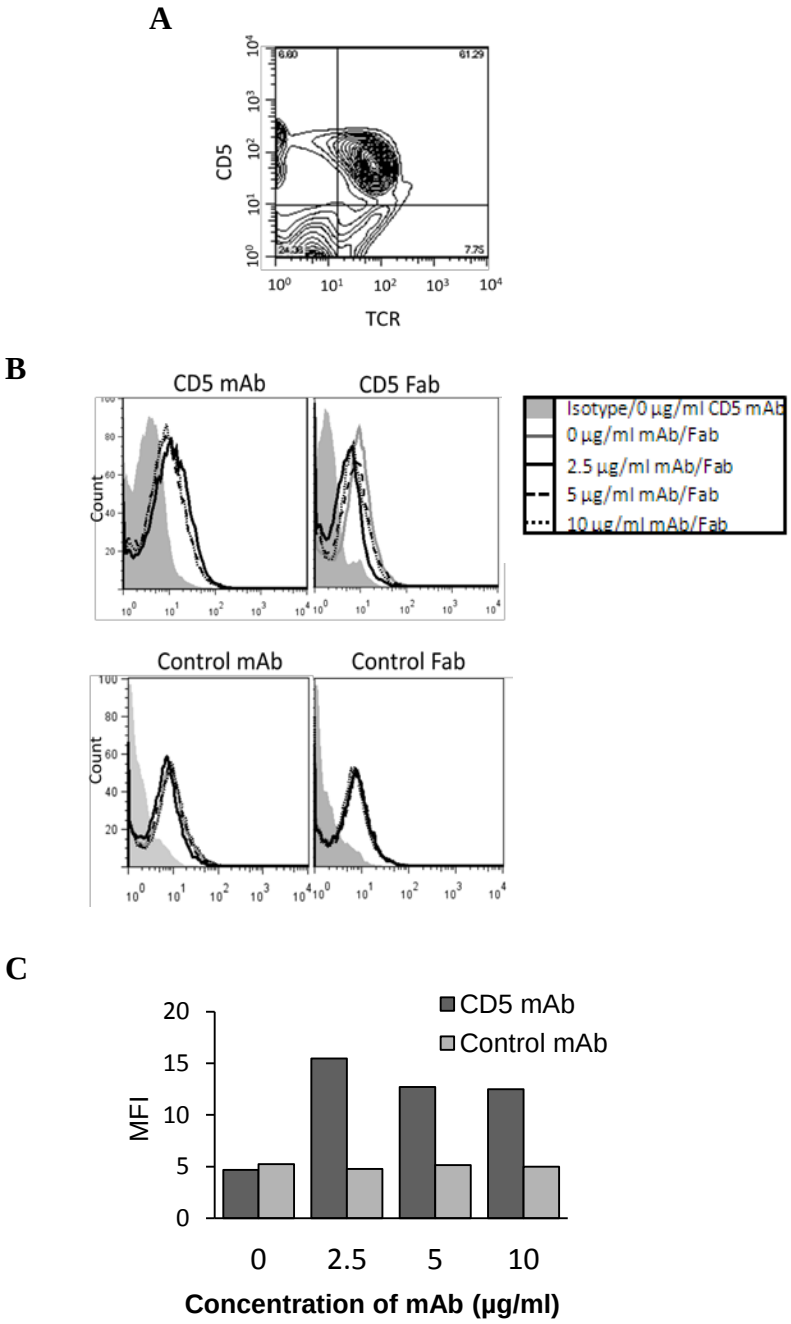


Figure 3.3: OX19 does not modulate CD5 from the surface of T cells. *A) FACS plot showing CD5 and TCR expression on lymph node cells freshly isolated from 10 week old female Lewis rat before the experiment. B) Histograms showing CD5 expression levels on these same lymph node cells stimulated with Con A (5µg/ml) and R73 (5µg/ml) for 3 days following preincubation with increasing amounts of CD5 and Control mAb and Fab fragments. C) Graph showing the mean fluorescence intensity (MFI) of CD5 staining of the left hand panels in B).*

3.4 CD5 mAb *in vivo* treatment can partially reduce disease in EAE

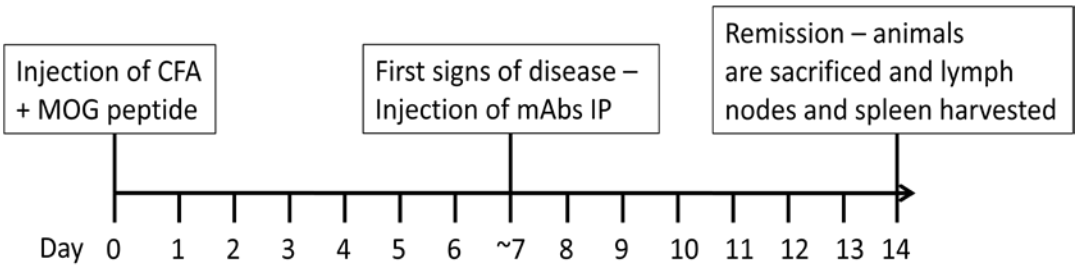
I have shown that OX19 does not crosslink and trigger CD5 nor does it modulate CD5 from the surface *in vitro*. Given the variable results from the literature involving the use of OX19 *in vivo* it is important to readdress whether this mAb is working in a similar fashion *in vivo* (93, 94). In the case of EAE, previous work has shown both inhibition and no effect on the outcome of disease following treatment by OX19 (94, 112). This may have been due to the time of antibody injection. If OX19 was given before disease induction it showed inhibition of disease (94) whereas if it was given after disease induction it either had no effect (112) or enhanced disease (93). Therefore it was important to first of all confirm the outcome of treatment of EAE with OX19. To determine this, female Lewis rats were injected with MOG peptide plus Complete Freunds Adjuvant (CFA) on either side of the tail bone to induce EAE. After 7 days the rats developed varying degrees of paralysis starting from the tail and working upwards. Figure 3.4 A shows a timeline of the experimental procedure. At the first signs of disease mAbs were injected intraperitoneally and the disease severity was scored as described in the materials and methods. The rats go into remission at around day 14 regardless of mAb treatment. There is a trend towards earlier remission when rats are treated with OX19 when compared to control mAb however this is not statistically significant when tested using a Mann Whitney statistical test (Figure 3.4 B).

Considering that using a CD5 mAb to treat ongoing EAE had no dramatic effect perhaps the addition of an antibody against the related CD6 would have a greater effect. Determining whether there is interplay between these two highly similar receptors may also help in further defining the role of CD5. In order to evaluate the combined role of

CD5 along with the closely related CD6, OX19 was used in combination with two CD6 mAbs, OX52 (a mouse IgG2a anti-rat CD6) and OX126 (a mouse IgG1 anti-human CD6 which cross reacts with rat CD6). OX126 will block CD6 interacting with its' ligand, CD166 but OX52 will not (11). When OX19 is used with a control mAb or one of the CD6 mAbs there is again a trend for earlier remission however to a lesser extent than the OX19 treatment alone. This again is not statistically significant (Figure 3.4 B).

It is clear from these data that the effect of the OX19 *in vivo* is not as dramatic as it is *in vitro*. The tendency to reduce disease severity is also contradictory to the *in vitro* results from which you would predict an increase in disease severity due to the increase in proliferation of the T cells. In addition the combination of using CD6 mAbs of two different isotypes made no difference to disease outcome indicating that CD6 does not play an important role in aiding CD5 regulation of this particular disease. Altogether these data highlight that CD5 is unlikely to be a useful therapeutic target in the treatment of multifactorial autoimmune conditions.

A



B

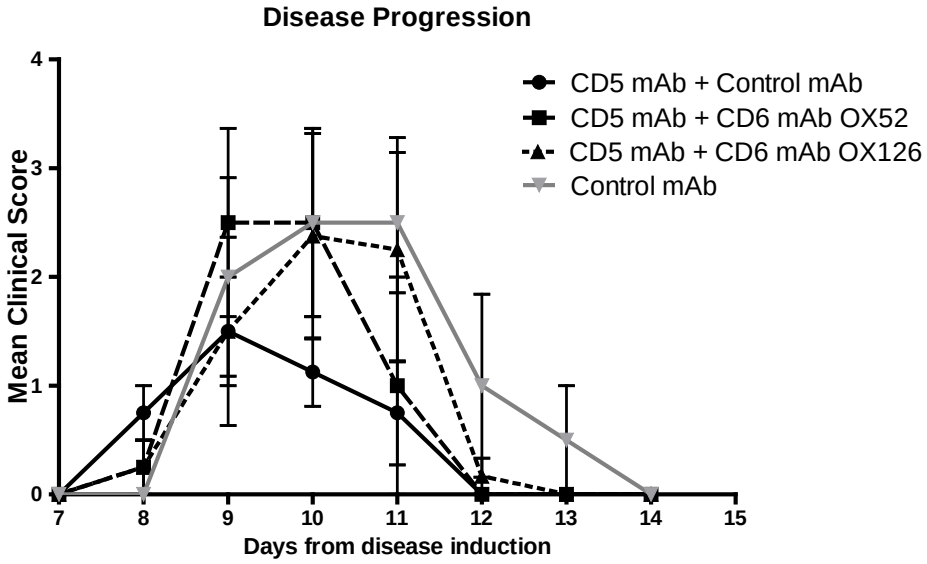


Figure 3.4: CD5 mAb partially inhibits disease severity in rats with EAE. *A) Timeline of the experiment. Female Lewis rats were immunised with 1:1 homogenised mixture of complete Freunds adjuvant with 10 µg/rat MOG peptide then injected with 250 µg/rat (total) mAb following first signs of disease. B) Mean clinical score of 4 rats in each group indicated above, representative of 3 separate experiments. Means are plotted from the day of antibody injection. Error bars represent the standard error of the means and a Mann Whitney was carried out and no significant difference between the clinical outcomes following different treatments.*

3.5 CD5 mAb treatment results in a modulation of CD5 expression *in vivo*

OX19 can block CD5 engagement and it does not modulate CD5 from the surface of cells *in vitro*. Also the functional effect of OX19 is more dramatic *in vitro* than in an *in vivo*. Previous work has shown OX19 was both modulating CD5 expression and depleting CD5 positive T cells *in vivo* when injected into a healthy rat (94). This however was never examined following disease induction. To determine whether OX19 modulates CD5 expression or depletes CD5 positive T cells following disease, flow cytometric analysis of CD5 expression on splenocytes from rats which had been given EAE was carried out. CD5 expression was determined on T cells following treatment of EAE with OX19, OX52 or control mAb. Splenocytes were stained for TCR, CD4 and CD8 to determine whether any dramatic deletion of the T cells was taking place. Staining of CD5 and CD6 was also carried out to determine whether there was any modulation of these from the surface. There was no dramatic difference in the percentage of T cells present which remained at 54 – 65% regardless of disease state or mAb treatment (Table 3.5). The CD4:CD8 ratio also remained around 1:1 with a slight increase in CD8 cells when animals were treated with OX19 or OX126 which could be due to cell death or proliferation of CD8 T cells (Table 3.5). Further work would be required to confirm this. These results are consistent between two independent experiments. There was dramatic down regulation of CD5 expression on both CD4 and CD8 T cells when rats were treated with OX19 and not control mAb (Table 3.5 and top FACS plots in Figure 3.5). It should be noted that this lack of CD5 staining could be due to residual bound OX19 blocking staining by OX19 however this effect has been shown reproducibly by other groups indicating this is unlikely to be the case. Interestingly there is no alteration of CD6 expression following treatment

with OX126 (bottom FACS plots in Figure 3.5). There was also no alteration in CD6 levels following treatment with OX52 (results not shown). These results tell us that the addition of OX19 to rats following EAE induction results in a down modulation of CD5 expression. This is contrary to the results found *in vitro* with this same mAb.

	Healthy Rat	Rats with EAE in Remission		
Treatment	No Treatment	Control mAb	OX19	OX19 + OX126
% T cells	54	65	57	59
CD4:CD8 Ratio	1:1	1:1	1:2	1:1.7
% CD5 + T cells	44	31	10	16
% CD6 + T cells	51	25	41	40
% CD5 + CD4 + cells	33	27	11	17
%CD6 + CD4 + cells	42	44	41	35
%CD5 + CD8 + cells	N/A	21	11	17
%CD6 + CD8 + cells	N/A	11	40	41

Table 3.5: Expression profiles of splenocytes from stimulated rats. *Percentages and ratios of cells expressing T cell receptor, CD4, CD8, CD5 and CD6 in healthy rat spleens and following EAE and mAb treatments. Representative results from 2 experiments.*

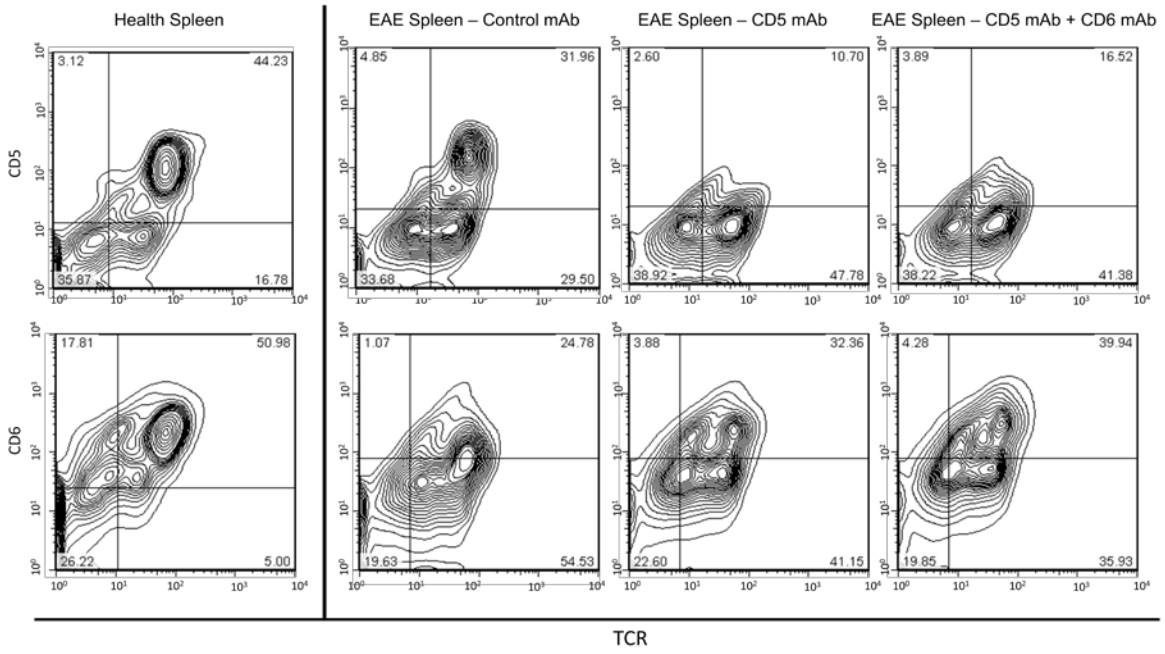


Figure 3.5: CD5 mAb treatment results in modulation of CD5 expression on T cells in spleen of diseases rats without depleting T cells. *Contour of FACS plots showing CD5 or CD6 expression as indicated on T cells in healthy rat spleens or EAE rat spleens treated with the mAbs indicated. Percentage of cells in each quadrant is indicated.*

3.6 CD5 mAb enhances proliferation of restimulated lymph node cells *ex vivo*

OX19 can dramatically enhance proliferation of ConA and CD3 mAb stimulated cells *in vitro* when preincubated before the T cells had been activated. This is representative of a primary immune response when naïve T cells are stimulated. It is important to investigate the effect of OX19 on a secondary immune response. Secondary immune responses are stronger and produce a faster response in order to prevent reinfection. Elucidating the effect of OX19 during these two very different responses may help to gain a clearer picture of the temporal role of CD5 during T cell activation. To this end, lymph node cells were taken from two rats which had gone into remission following EAE. Draining lymph node cells were then removed and preincubated with OX19 or control mAb or Fab fragments and then stimulated with MOG peptide loaded BMDCs. This antigen specific stimulation should only reactivate those previously matured antigen specific cells.

The disease severity was different in the two rats. Rat 1 had less severe disease scores accompanied with slower weight loss but did not go into complete remission (Figure 3.6 A and B). Rat 2 had much greater disease severity with a higher percentage weight loss and did go into complete remission by day 14 (Figure 3.6 A and B). Interestingly, flow cytometric analysis of the lymph nodes from these rats show a higher level of CD5 expression on the T cells from Rat 1 than from Rat 2 (Figure 3.6 C). This may be a marker of how activated the cells were at the time of analysis.

Proliferation of lymph node cells was determined following stimulation with peptide loaded BMDCs in the presence or absence of OX19 mAb and Fab fragments (Figure 3.6 D). Cells from rat 2 were not tested in this way due to the very low CD5 expression. There is a small increase in proliferation of the lymph node cells in response to antigen alone. This proliferation is increased 3 fold when cells were stimulated in the presence of OX19 mAb and not the control mAb. The same increase in proliferation was observed when cells were stimulated in the presence of OX19 Fab fragments. Overall these results show OX19 can block CD5 inhibition of a secondary stimulation of antigen specific lymph node cells. Further experiments comparing the effect of antibody without antigen stimulation versus stimulation with antigen, would have to be carried out to determine whether this was a direct affect on antigen specific T cell activation in this instance.

Flow cytometric analysis of CD5 expression levels was carried out following restimulation with antigen in the presence or absence of OX19 mAb or Fab fragments. Interestingly restimulation of lymph node cells with antigen alone results in a loss of CD5 expression (Figure 3.6 E). CD5 expression levels reduce even more when cells were restimulated with antigen in the presence of OX19 mAb or Fab when compared to the control mAb (Figure 3.6 F). This staining however could be due to masking of the receptor by residual bound OX19. Altogether these results indicate that during a secondary immune response OX19 can enhance proliferation in a similar fashion to a primary immune stimulation. In this instance it appears that OX19 is blocking receptor engagement in addition to modulation of CD5 from the surface unlike the results seen previously *in vitro* where CD5 expression is in fact increased. In both cases however one can conclude that these results show an inhibitory role for CD5 in this system.

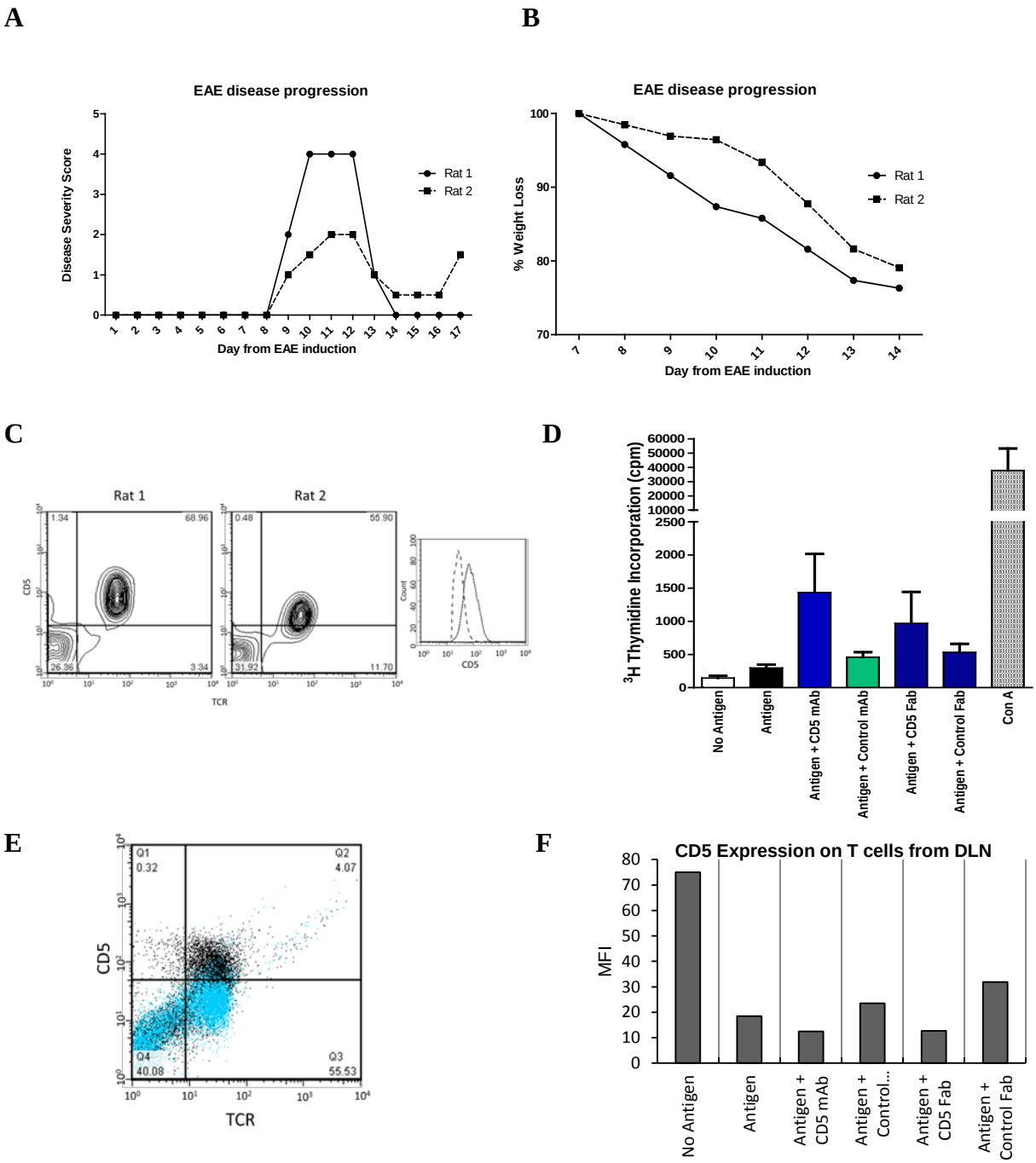


Figure 3.6: CD5 mAb enhances proliferation of diseased lymph node cells restimulated ex vivo. *A) Disease severity score of two rats given EAE. B) Percentage weight loss of two rats given EAE. C) FACS plot showing the CD5 expression on T cells of two rats following remission and histograms comparing the MFIs of CD5 expression on the T cells. D) ^{3}H Thymidine incorporation in counts per minute of cells preincubated with the mAb or Fab fragments indicated and stimulated by antigen loaded dendritic cells or Con A. E) Dot plot showing CD5 expression on Rat 1 lymph node cells before (black) and after (blue) antigen stimulation. F) MFI of CD5 expression on T cells treated as indicated from rat 1.*

3.7 Discussion

OX19 will block CD5 inhibition of primary T cell proliferation *in vitro*. Given that both whole antibody and Fab fragments of OX19 had the same functional outcome to increase proliferation of lymph node cells it can be assumed that this antibody is blocking engagement of CD5. When OX19 was first characterised, Dallman *et al.* are careful not to conclude that OX19 induced augmentation of proliferation and increased IL-2 production was due to a triggering of CD5 by the antibody (86). Dallman *et al*. only tested whole antibody and $F(ab)^2$ fragments which are still capable of crosslinking and so it could not have been assumed that CD5 engagement was being blocked. Results shown here also demonstrate that blocking only occurs in cells that are being stimulated by Con A and CD3 mAb. When cells are unstimulated, OX19 appears to trigger, and not block, proliferation in these cells. This proliferation is not dramatic and therefore it is possible that these mitogenic properties could have been previously overlooked by Dallman *et al*.

The act of blocking assumes that there is a ligand at play and as discussed previously, the ligand for CD5 remains disputed. However, recently Brown *et al.* have shown that one ligand for CD5 can be itself and results here demonstrate that interrupting this self interaction may in fact disrupt its regulatory function (51). This is contrary to results demonstrating CD5 inhibition of thymocyte activation does not require the extracellular region of CD5 (77). These differences in dependence on the extracellular domain of CD5 between thymocytes and peripheral T cells may account for the differential behaviour of CD5 in these cells.

I have shown that CD5 is not modulated from the surface by OX19 *in vitro*, however a dramatic loss of CD5 expression is observed *in vivo* following injection of OX19. These differential effects of OX19 have been shown previously (94). This is a mechanism specific to CD5 because the highly similar CD6 is not modulated from the surface in the same way with either OX52 or OX126. The removal of CD5 expressing cells is unlikely to be due to any Fc mediated effects either because the mouse IgG1 is unlikely to bind rat Fc receptors. It is interesting that CD6 is not downregulated along with CD5 and points to a different regulation of expression of these two receptors despite the theory that they are a result of gene duplication (45). The differential evidence where CD5 is present or absent however can help towards a better understanding of its function.

Having demonstrated clearly that OX19 can block CD5 inhibition, it would be predicted that this antibody will enhance a disease such as EAE. However I have shown that there is a trend for inhibition of disease. This is more dramatic if the antibody is administered before the disease and prevents disease progression (94). One hypothesis of why this is the case is that the cells are proliferating so much that they undergo AICD. In CD5 KO mice with EAE, there were a reduced number of peripheral CD4 T cells with an increased susceptibility to apoptosis (87). It was proposed by these authors that CD5 regulates the cells resistance to AICD (114). Axtel *et al.* later showed an involvement with a serine threonine kinase, CK2, in this process which is a known to provide pro survival signalling factor (88). Evidence I have shown here however demonstrates no apparent loss of T cells despite the loss of CD5 expression. Moreover this does not explain the enhancement of disease seen when OX19 is given during EAN (93). In addition, this effect of OX19 antibody was not repeated by other authors who did not find a dramatic effect when

compared to other T cell antibodies (112). It is possible that due to the multifactorial nature of autoimmune disease, multiple domains of CD5 would have to be targeted in order to prevent disease and that CD5 expression on cells other than T cells are affecting disease outcome.

Given the results shown here and by closer analysis of the literature it is clear there are differences in the role of CD5 on the different stages of the immune response. It appears that in the primary immune response, OX19 can enhance proliferation showing CD5 inhibits T cell stimulation. This coincides with an increased expression of CD5 on the surface of the T cells which may reflect the increased proliferation of these cells. During the immune response the absence of CD5 appears to result in less disease in the case of EAE which is reminiscent of the response seen with CD5 KO mice and may indicate a requirement for CD5 expression in order to mount an immune response (87). During a secondary stimulation, CD5 is downregulated which appears to enhance proliferation once again contrary to the results seen *in vitro* during a primary immune response. It is possible that the level of CD5 expression, the differentiation state of the cell and the strength of stimulation given can account for differences seen in the literature from treatment with CD5 mAbs. This could be addressed by using T cell markers for different stages of differentiation and then look at CD5 expression.

To summarise, the results here clearly demonstrate that OX19 can block receptor engagement and therefore block regulation of T cell activation by CD5 *in vitro*. *In vivo* the effect of OX19 is less dramatic but will partially downregulate the immune response. This

is accompanied by a complete loss of CD5 expression which is not seen *in vitro*. Evidence here indicates this is a feature of the complex immune response in EAE as I have demonstrated that upon restimulation of pathogenic T cells *ex vivo* there is a downregulation of CD5 expression induced coinciding with an increase in proliferation. It is clear from these results that CD5 is unlikely to be a good therapeutic target in the treatment of autoimmune conditions even with combination therapy using CD6 mAbs.

Chapter 4: The Role of Tyrosine Phosphorylation in CD5 Function.

4.1 Introduction

In studying the role of CD5 in the T cell activation it is important to determine how this receptor signals within T cells. There are clear data demonstrating that CD5 is phosphorylated on tyrosines, serines and threonines upon T cell activation and that multiple CD5 specific signalling pathways can be activated (61, 115). It is known that CD5 associates with CD3 and is recruited to the T cell synapse where it has been shown to exert inhibition (35). Up until now however there has been no clear demonstration of how CD5 tyrosine phosphorylation and signalling in particular affects its ability to regulate signals delivered through the TCR.

4.1.1 Disruption of the CD5 cytoplasmic tail alters CD5 mediated regulation

Disruption of the CD5 cytoplasmic tail has shown the importance of signalling in CD5 regulation (Figure 4.1). Raman *et al.* and Calvo *et al*. published a set of papers showing that S458 and S461 at the C terminus of the CD5 cytoplasmic tail make up a binding and phosphorylation site for the serine threonine kinase Caesin Kinase II (69, 116, 117). This work clearly demonstrates the dependence on these serines for CD5 mediated survival signals delivered following CD5 ligation. CKII activation and cell survival does not occur however when T cells are activated via TCR or in peripheral T cells (69). Other work in the literature has shown CD5 regulation is dependent on tyrosine phosphorylation by specifically mutating them to either alanine or phenylalanine (118, 119). This work looked at the effect of CD5 phosphorylation on the regulation of BCR signalling. Gary-Gouy *et al.* demonstrated a dependence on Y429 (Y1) and Y441 (Y2) for BCR induced calcium flux and Akt localisation to the membrane. The only mutational analysis of tyrosine

phosphorylation carried out on CD5 expressed in T cells indicated a dependence on the Y1 and Y463 (Y3) tyrosine for pervanadate induced CD5 phosphorylation in Jurkats (120). Dennehy *et al*. were however not able to show the effects this had on T cell activation, in particular T cell IL-2 production.

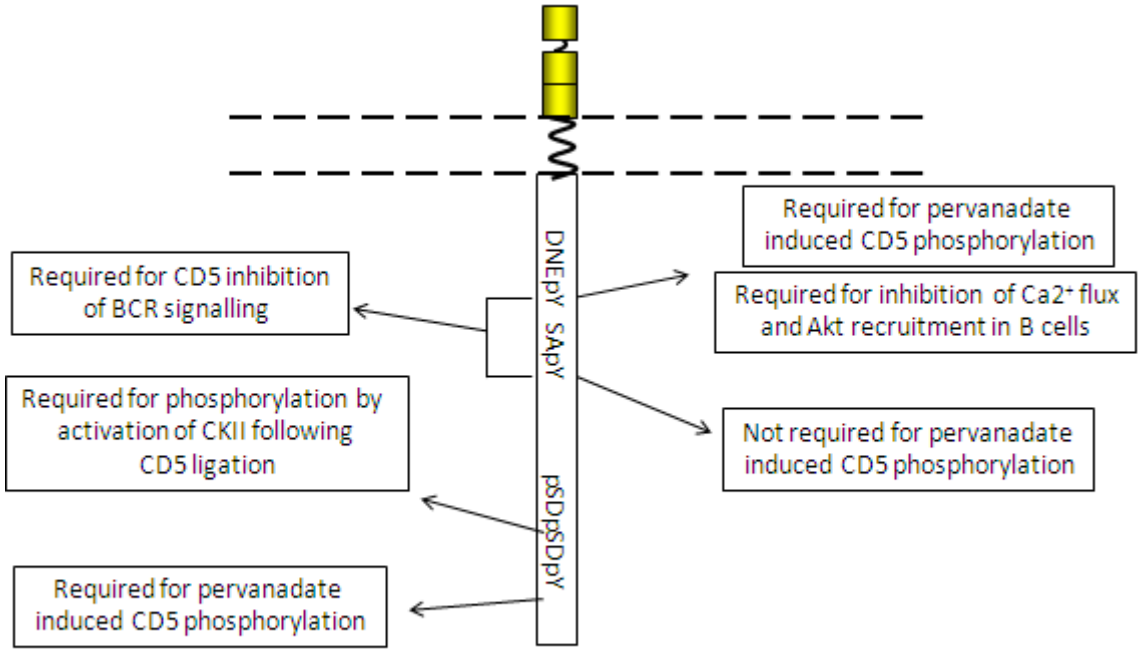


Figure 4.1: A summary of the current knowledge on the requirements of tyrosine and serine phosphorylation of CD5. *Regions of the CD5 cytoplasmic tail which contain phosphorylated tyrosines and serines are denoted. DNEpY contains Y429 is referred to as Y1 in this thesis, SApY contains Y441 which is referred to as Y2. pSDpSDpY contains Y463 which is referred to as Y3.*

4.1.2 T cell activation and IL-2 production

It is known that tyrosine phosphorylation occurs within seconds to minutes of TCR triggering and it is thought that serine/threonine phosphorylation happens later due to serine/threonine kinases dependence on tyrosine phosphorylation for their activation (121, 122). One of the earliest responses to TCR triggering and activation is the production of IL-2 and proliferation. This proliferation will then lead to a differentiation process in which CD4+ T cells will become mature T helper cells and go on to aid antibody production. CD8+ T cells will mature and go on to mediate cellular cytotoxicity of infected cells. The crosstalk between co-receptors and TCR signalling can alter the outcome of TCR triggering. CD28 is known to co-stimulate whilst CTLA-4 inhibits and regulates TCR triggering. As I have discussed it is unclear whether CD5 helps or hinders this process. CD5 mAb ligation can result in an increase in IL-2 production and proliferation in T cells stimulated by CD3 or CD28 (83, 84). As I have demonstrated one must be careful about drawing conclusions about CD5 function based on effects of mAbs unless it is clear how the antibody is operating. There also appears to be crosstalk mediated between CD5 and another co-receptor, CD2, where knocking out both receptors has an additive effect in disrupting positive selection in the thymus (123). Studying CD5 signalling in addition to effects seen through mAb ligation and involvement of other co-receptors may therefore help in determining how CD5 regulates T cell activation.

4.1.3 Aims

There is little work showing the effect of the CD5 cytoplasmic tail on regulation of TCR triggering. As I have discussed CD5 tyrosine phosphorylation has been shown to be important in regulation of BCR triggering (109). Considering that CD5 is highly

expressed on T cells it is therefore sensible to ask how CD5 tyrosine phosphorylation affects TCR induced signals. In light of this, I aim to demonstrate the differential effects of tyrosine phosphorylation on TCR induced IL-2 production using specific point mutations of the tyrosines in CD5. In addition to this I demonstrate that effects of CD2 signalling can affect CD5 regulation of T cell activation.

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4.2 CD5 tyrosine mutants can be stably expressed in a 2B4 mouse T cell hybridoma line

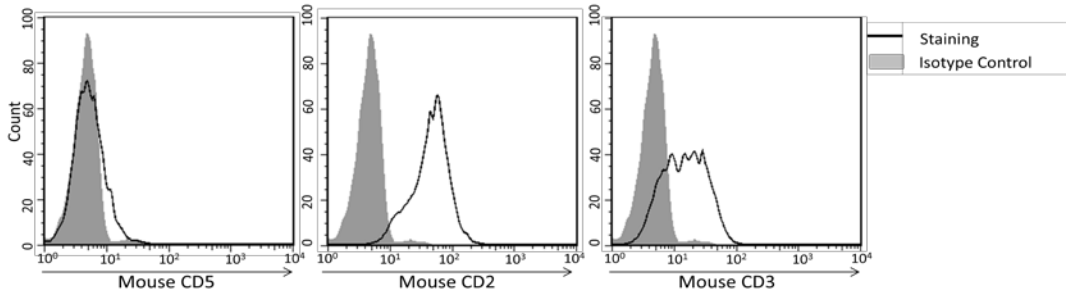
In order to define the role of CD5 tyrosine phosphorylation on the regulation of T cell activation, specific CD5 mutants were generated in which the tyrosines were substituted by phenylalanine. These CD5 mutants are then unable to become phosphorylated on these specific residues. To determine the effect of these CD5 mutants on T cell activation, a system utilising a mouse CD4 T cell hybridoma line (2B4.CD2) which stably expresses CD2 (FACS analysis of expression Figure 4.2.1 A) and the 2B4 TCR was developed (7). Mouse CD2 and the 2B4 TCR recognise mouse CD48 and MCC peptide bound to MHC class II molecule IE^k respectively. 2B4.CD2 cells can be easily transduced and used in antigen specific stimulation assays.

In order to distinguish between endogenous mouse CD5 which is expressed at a low level, human CD5 was transduced into the 2B4.CD2 cells (Figure 4.2.1 A). Human CD5 was mutated on one or more of the three tyrosines in the cytoplasmic tail. There is a high sequence homology between the intracellular region of mouse and human CD5 so this was unlikely to cause any differences in signalling transduction in the mouse T cell line (Figure 4.2.1 B). The three tyrosines Y429, Y441 and Y463 named Y1, Y2 and Y3 starting from the N terminal tyrosine, were mutated to phenylalanine as outlined in Figure 4.2.1 C. One double mutant of both Y1 and Y3 was generated due to the evidence in the literature indicating these tyrosines to be phosphorylated following TCR stimulation and pervanadate treatment (61, 124). Stable cell lines were made as described in the materials and methods and then selected for by the puromycin resistance obtained from the pBABE-puro construct. In addition, sorting of the cell lines for matched CD5 expression levels

was often required in order to achieve a pure population of cells. Figure 4.2.1 D shows representative histograms of CD5 expression levels following transduction.

Despite sorting and selecting these cells, changes in CD5 and CD3 expression in these stable cell lines could be detected over time. Reasons for this could be the effects of cell density on receptor expression or simply unspecific selection of low level expressing clones. In order to determine whether cell density was responsible for this, cells were grown at different concentrations and the CD5 and CD3 expression was measured. Figure 4.2.2 A shows histograms of the CD5 levels on 2B4.CD2 cells expressing CD5WT, CD5Y1F or CD5Y3F grown at increasing concentrations. There is a general trend for a reduction in CD5 expression with increasing cell density in all three cell lines. The greatest CD5 expression level occurred independently of the tyrosine mutations when cells were grown at $5x10^5$cells/ml (Figure 4.2.2 B). Notably the CD3 expression does not change dramatically when cell densities are altered except when CD5 WT expressing 2B4.CD2 cells are grown at $2x10^6$cells/ml. At this cell density the expression of CD3 drops to almost nothing (Figure 4.2.2 C). For the purposes of subsequent experiments high CD5 and CD3 expression were required. Therefore, cells were maintained at $5x10^5$cells/ml in order to maintain the optimal CD5 and CD3 expression. Cells were also reselected if necessary by cell sorting or dynal bead selection.

A



B

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H- VASIILALVLLVVLLVVCGPLAYKKLVKKFRQKKQRQWIGPTGMNQNMSFHRNHTATVRS
M- VASIILTLVLLVVLLAMCGPLVYKKLVKKFRQKKQRQWIGPTGVNQNMSFHRSHTATVRS
   ******:******** :**** **********************:********:*******
                Y1          Y2                      Y3
H- HAENPTASHVDNEYSQPPRNSRLSAYPALEGVLHRSSMQPDNSSDSDYDLHGAQRL*
M- QVENPTASHVDNEYSQPPRNSHLSAYPALEGALHRSSTQPDNSSDSDYDLQVAQRL*
    :********************:*********:***** ************  ****
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C

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429 Y1 Y-F- HAENPTASHVDNEFSQPPRNSRLSAYPALEGVLHRSSMQPDNSSDSDYDLHGAQRL

441 Y2 Y-F- HAENPTASHVDNEYSQPPRNSRLSAFPALEGVLHRSSMQPDNSSDSDYDLHGAQRL

463 Y3 Y-F- HAENPTASHVDNEYSQPPRNSRLSAYPALEGVLHRSSMQPDNSSDSDFDLHGAQRL

  Y1Y3 Y-F- HAENPTASHVDNEFSQPPRNSRLSAYPALEGVLHRSSMQPDNSSDSDFDLHGAQRL
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D

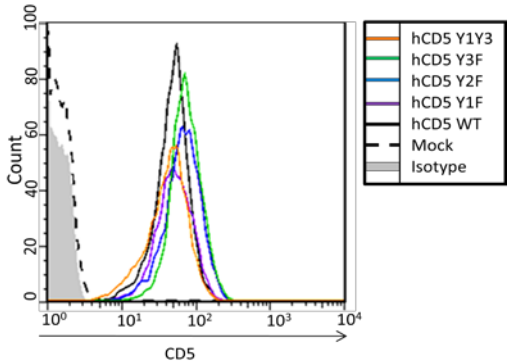
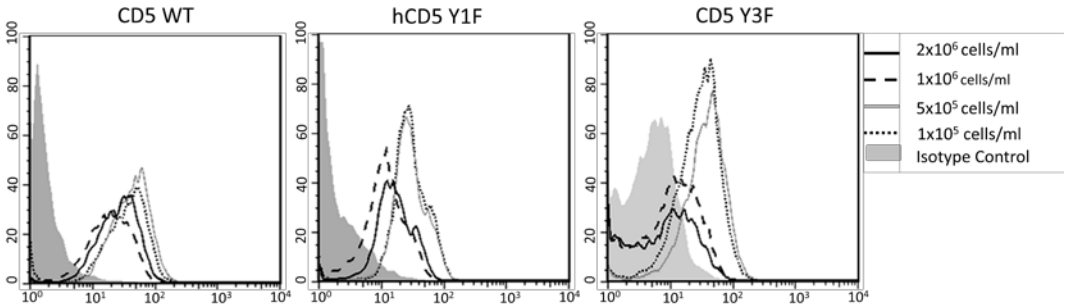


Figure 4.2.1: The 2B4 T cell hybridoma can be stably transduced with CD5 phosphorylation mutants. *A) Histograms showing the expression levels of mouse CD5, CD2 and CD3 on the 2B4.CD2 cell line. B) Amino acid line up of the C terminus of human and mouse CD5 starting from the transmembrane region (underlined). Identical regions shaded grey (*), conservative a.a. differences are indicated (:) and non conservative changes (.). The tyrosines Y1, Y2 and Y3 are highlighted in red. C) A.A line ups of the mutations made in CD5. D) Histograms showing CD5 expression on 2B4.CD2 cells transduced with the CD5 mutant constructs indicated.*

A



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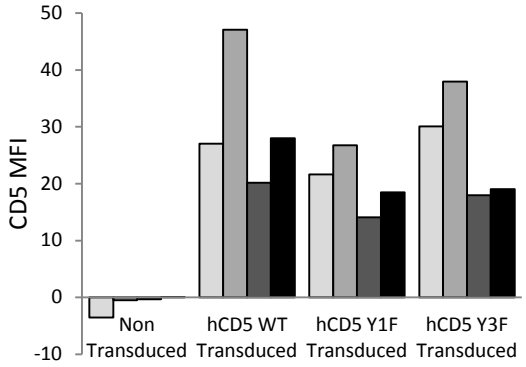

C

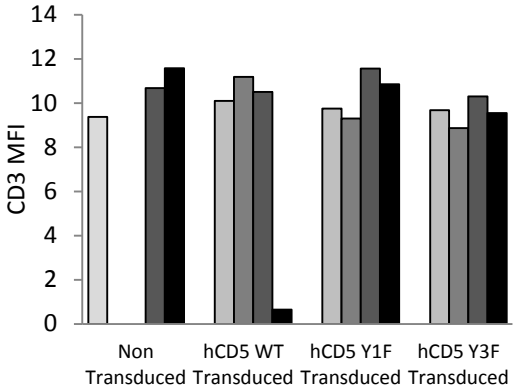

Figure 4.2.2: Optimal expression of CD5 mutants depends on 2B4.CD2 cell density: *A) Histograms showing human CD5 staining on three 2B4.CD2 cell lines seeded at increasing concentrations as indicated and grown for 24 hours. MFI of B) CD5 and C) CD3 expression on the different 2B4.CD2 cells lines grown at different concentrations: □ 1x10^5 cells/ml, ▒ 5x10^5 cell/ml, ■ 1x10^6 cells/ml, ■ 2x10^{6}cells/ml. All FACS plots are gated on live cells.*

4.3 Differential CD5 Tyrosine Phosphorylation affects CD3 induced T cell IL-2 Production

Having made stable T cell lines expressing CD5 with point mutations of the tyrosines, it was then possible to determine the effect this had on T cell activation. In order to dissect the role of these tyrosines during T cell activation, initially these cells were stimulated through the TCR using CD3 mAb coated 96 well plates. 2B4.CD2 CD5 transduced cells were added to wells with increasing amounts of immobilised CD3 mAb and incubated for 24 hours. Supernatants were then collected and IL-2 production was measured by ELISA as a read out of T cell activation. In order to find out if the transduction process had affected the T cells ability to produce IL-2, non transduced 2B4.CD2 cells were compared to mock transduced 2B4.CD2 cells. It was found that they produced similar amounts of IL-2 following CD3 mAb stimulation which reached a plateau between 0.3 and 1 µg/well of CD3 mAb stimulation (results not shown). Mock transduced 2B4.CD2 cells were then used as the control cell line hereafter.

2B4.CD2 cells expressing hCD5WT (2B4.CD5WT) produced less overall IL-2 following CD3 stimulation when compared to Mock 2B4.CD2 cells (Figure 4.3.1 A). This demonstrated an inhibitory effect of CD5WT expression. When hCD5Y2F and hCD5Y3F expressing 2B4.CD2 cells were stimulated with CD3 there was less IL-2 produced than Mock 2B4.CD2 cells. However, more IL-2 was produced by these two mutants than the 2B4.CD5WT cells. This shows CD5Y2F and CD5 Y3F mutations partially relieve the inhibition exerted by CD5WT. If CD5 is mutated on the 1st tyrosine however the inhibition of IL-2 production is greater. This indicates a possible positive role for the phosphorylation of CD5 Y1. Both the 1st and the 3rd tyrosines are mutated in order to

determine which of the two is dominant. It appears the inhibitory effect of the 3rd tyrosine out competes the positive effect of the 1st tyrosine because the IL-2 production is equivalent to that of the CD5Y3F expressing cells. These results are representative of 4 separate experiments.

Due to the variability in levels of expression of CD5, the expression of hCD5 was analysed following these stimulations. In addition, the expression levels of other receptors which contribute to the activation of these 2B4.CD2 T cells, such as CD3, CD2 and CD5, were analysed before any concrete conclusions were drawn from these results. Flow cytometric analysis was carried out to determine CD3, CD2 and CD5 expression on these cells following the stimulation with immobilised CD3 mAb (Figure 4.3.1 B). The levels of expression of all three receptors, CD3, CD2 and CD5 are similar between different mutant cell lines following no stimulation. There were differences in levels of CD5 and CD2 expression following CD3 mAb stimulation between the different mutant cell lines. In particular the CD2 expression appears to increase following stimulation on most of the cell lines (right hand panels of Figure 4.3.1 B).

CD5 and CD3 expression levels were compared before and after stimulation by CD3 mAb and results are expressed as mean fluorescence intensity subtracted from the MFI of isotype control antibodies (Figure 4.3.1 C and D). All the cell lines have similar levels of CD5 expression before and after stimulation apart from those transduced with the 3rd tyrosine mutant (Figure 4.3.1 C). These CD5Y3F cells have higher baseline CD5 expression than the other cell lines despite cell sorting and CD5 expression increased further after CD3 mAb stimulation. The CD3 expression is similar for all the cell lines

except the CD5WT transduced cells (Figure 4.3.1 D). These have slightly higher levels of CD3 expression showing that the CD5WT inhibition of TCR activation in fact is quite potent. The CD3 expression drops after the CD3 mAb stimulation in Mock 2B4.CD2 cells and 2B4.CD5WT cells. This downmodulation of CD3 however does not occur in any of the mutant cell lines (Figure 4.3.1 D). There is in fact an increase in CD3 expression following stimulation of 2B4.CD2 cells expressing CD5 Y3F or CD5 Y1Y3.

Taken together these results show a differential effect of phosphorylation of the three tyrosines in the CD5 cytoplasmic tail on CD3 mAb induced T cell IL-2 production. The phosphorylation of Y1 contributes to a positive effect of CD5 on T cell IL-2 production and Y2 and Y3 contribute to inhibition of IL-2 production mediated by CD5. In addition to this there is a lack of CD3 modulation from the surface of the cells in all three of these tyrosine mutants.

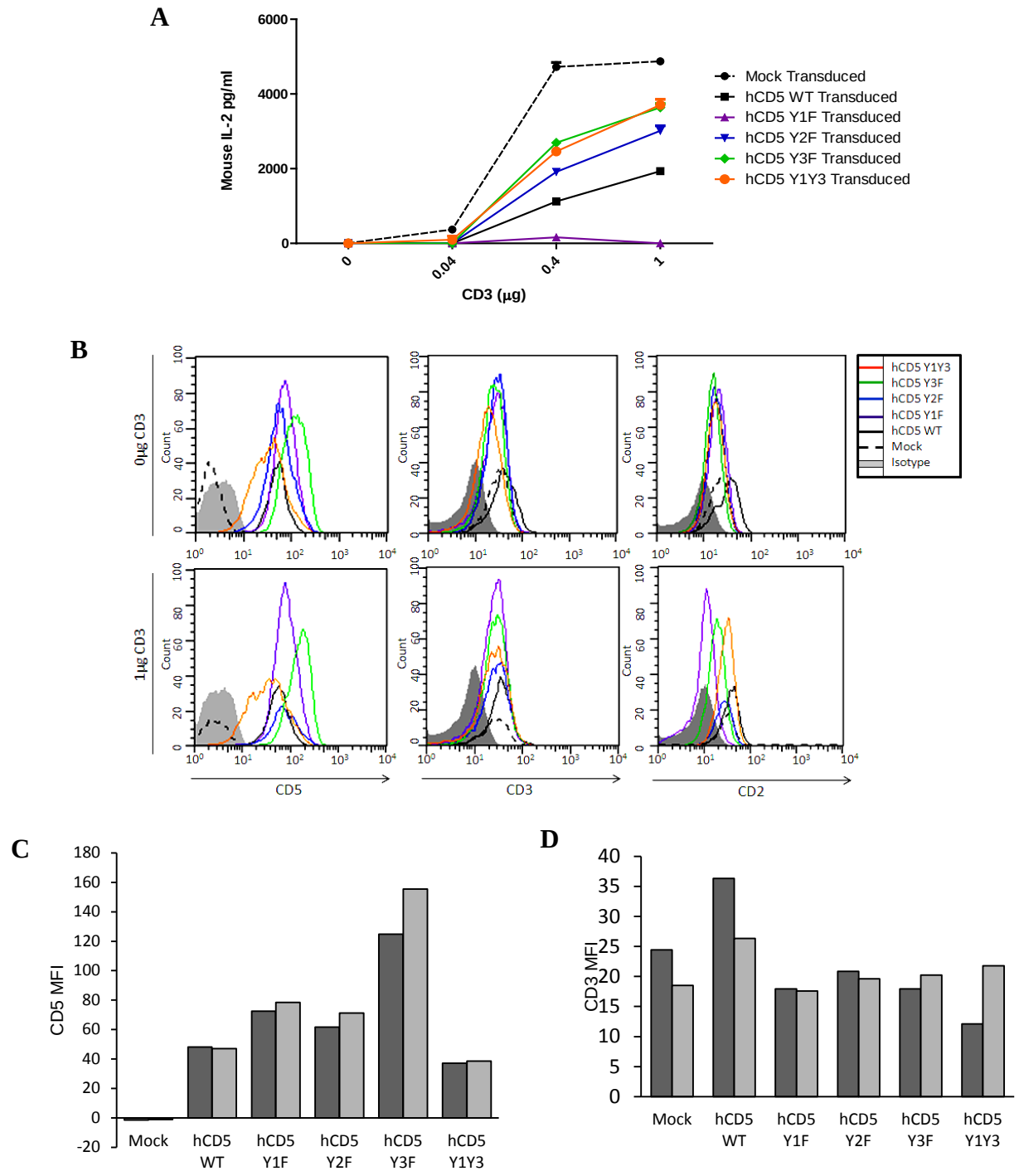

***Figure 4.3.1: Differential CD5 Tyrosine Phosphorylation affects CD3 induced T cell IL-2* Production.** *A) IL-2 production from T cell hybridoma cell lines transduced as indicated in legend following increasing amounts of CD3 mAb stimulation. Cells were plated out at 5 x 10^5cell/well and supernatants and cells were harvested for ELISA and staining respectively following 24 hours stimulation. Error bars represent SEM of triplicate wells. B) Histograms showing CD5, CD3 and CD2 expression harvested as described in A following stimulation with 0µg CD3 and 1µg CD3 stimulation of the different cell lines indicated. MFI of C) CD5 and D) CD3 highlighting the change in expression levels on the cells with:■ 0µg and □ 1µg of CD3 stimulation.*

The data here indicates CD5 phosphorylation may be important in antibody induced CD3 downmodulation. This downmodulation of CD3, however, was not followed by a downregulation of CD5 expression from the surface. If CD5 is aiding CD3 downmodulation one would assume that it too would be removed from the surface. In order to investigate this further, confocal microscopy was used to view the synapse between CHO IE^k mCD48 cells and 2B4.CD2 hCD5WT cells. Following the addition of MCC peptide at a concentration of 1 µM to the CHO IE^k mCD48, 2B4.CD2 hCD5 WT cells where spun onto the peptide loaded CHO IE^k mCD48 cells and stimulated for 30 minutes at 37 °C. Following fixation and staining the cells were visualised using confocal microscopy as described in Chapter 2. Figure 4.3.2 shows DAPI staining (left panel) showing both the CHO IE^k mCD48 cell (top right) and 2B4.CD2 hCD5 WT (bottom middle). The next panel to the right shows hCD5 staining (red) highlighting hCD5 is dispersed throughout the 2B4.CD2 hCD5 WT cell with some concentrated at the cell-cell interface. The next panel to the right shows CD3 staining (green) indicating CD3 localisation on the 2B4.CD2 hCD5 WT cell at the cell-cell interface. The last panel shows the merge of the three stains with an arrow indicating the immune synapse and yellow showing colocalised CD5 and CD3 staining. Altogether these preliminary results show that whilst some CD5 colocalises in the synapse with CD3, there is a large percentage dispersed elsewhere on the cell. These data indicate that CD5 could therefore be mediating CD3 downmodulation whilst remaining expressed at a stable level on the cell surface.

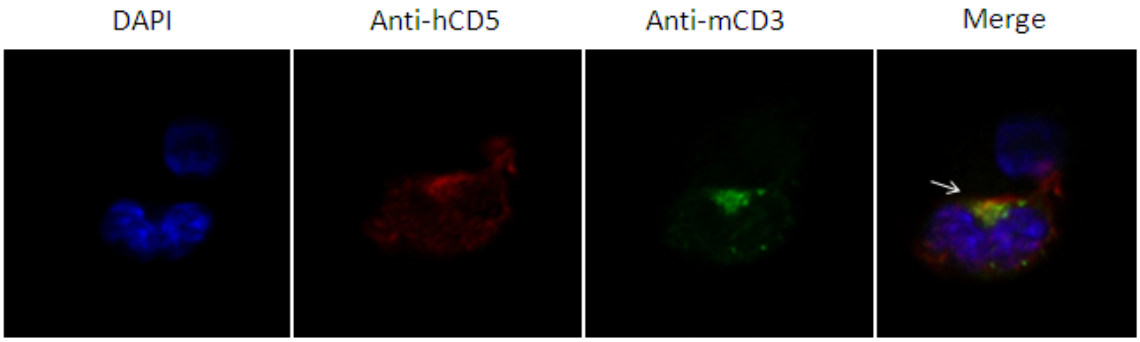

Figure 4.3.2: CD5 colocalises with CD3 in the T cell synapse between CHO IE^{k}mCD48 cells and 2B4.CD2 CD5WT cells. *CHO IE^k cells were loaded with 1µM of MCC peptide and incubated with 2B4.CD2 CD5WT cells for 30 mins and stained with hCD5 mAb (red), mCD3 mAb (green) and the nucleus stained with DAPI. The cell with DAPI alone is the CHO IE^k cell. Arrow indicates the T cell synapse. (preliminary data)*

4.4 Differential CD5 Tyrosine Phosphorylation affects antigen induced T cell IL-2 Production

I have shown that CD5 phosphorylation affects CD3 induced T cell activation. In order to determine whether these same effects can be seen in a more physiological situation, antigen specific stimulation using antigen presenting cells was carried out. To determine whether CD5 phosphorylation is important in the CD5 regulation of antigen specific T cell activation, the hamster ovarian cell line (CHO) which is stably transfected with MHC class II molecule IE^k was used as a pseudo antigen presentation cell (105). This MHCII molecule presents a peptide from moth cytochrome c (MCC) which is recognised by the 2B4 TCR expressed on the 2B4.CD2 transduced with the CD5 mutants. To provide a more physiological stimulation the accessory molecule CD48 was also stably expressed in the CHO IE^k cells. CD48 has been shown to bind to CD2 expressed on the 2B4.CD2 cells and enhance activation (105). Levels of expression of IE^k and mCD48 on the CHO cells can be seen in the histograms in Figure 4.4 A. The CD48 negative CHO IE^k cell line did not induce high amounts IL-2 production from the 2B4.CD2 cells and resulted in inconsistent results and were therefore not used (results not shown).

CHO IE^k mCD48 cells were plated out and loaded with increasing concentrations of MCC peptide. 2B4.CD2 cells were then added to this and incubated for 24 hours. Supernatants were removed and assayed for IL-2 production by ELISA as a read out of T cell activation. FACS analysis of the cell lines was carried out before the stimulation in order to ensure matched expression levels of hCD5, mCD3, mCD2 and mCD5 in the different CD5 mutant 2B4.CD2 cell lines (Figure 4.4 B). Following antigen specific stimulation of 2B4.CD2 cells there was no consistent inhibition of IL-2 production exerted by expression

of CD5WT (Figure 4.4 C). There was however an increase in IL-2 production seen from the CD5Y3F expressing 2B4.CD2 cells and again a decrease in IL-2 production from the CD5Y1F expressing 2B4.CD2 cells when compared to Mock 2B4.CD2 cells or 2B4.CD5WT cells. Results shown here are representative of 4 experiments.

Altogether these data show the same trend for inhibition of antigen specific T cell activation mediated through the 3rd tyrosine and activation of T cell activation mediated through the 1st tyrosine in the CD5 cytoplasmic tail.

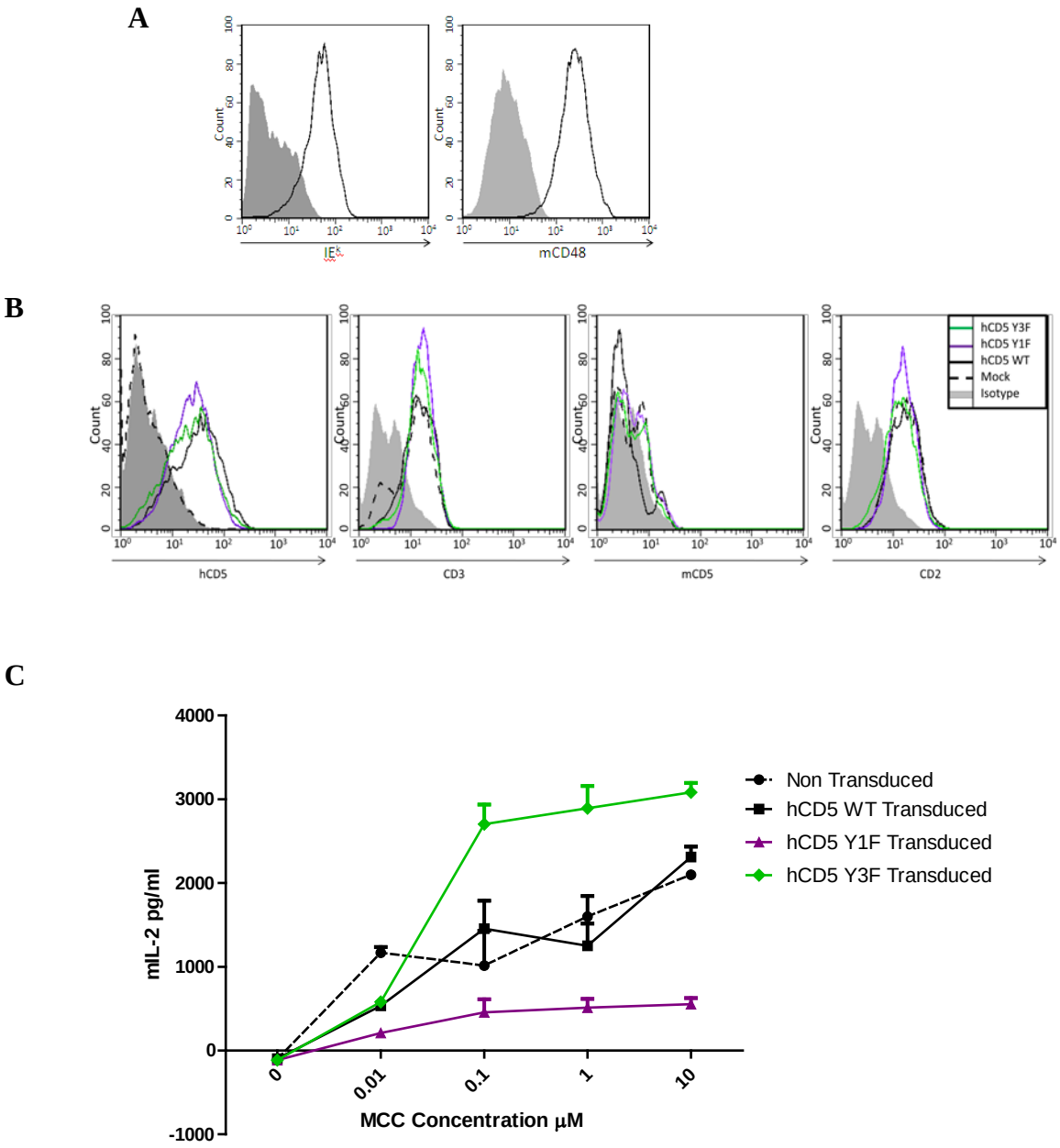

Figure 4.4: Phosphorylation of tyrosines in CD5 is important for CD5 regulation of antigen specific responses. *A) Histograms showing the mCD48 and MHC levels of the CHO IE^k mCD48 cells. B) Histograms showing the levels of hCD5, mCD3, mCD2 and mCD5 before stimulation of the indicated cell lines. C) IL-2 production from T cell lines indicated following stimulation by CHO IE^k mCD48 presenting increasing amounts of MCC peptide. Error bars represent SEM of results carried out in triplicate*

4.5 CD2 masks the positive signal transduced by CD5 tyrosine phosphorylation

With the addition of CD2 costimulation, the effects of CD5 regulation of T cell activation are less potent (Figure 4.4 C). These data indicate that there may be cross talk between the costimulatory signals delivered by CD2 to regulate TCR activation, and the signals delivered by CD5. The 2B4 T cell hybridoma cells used stably express high amount of mouse CD2 (7). Another 2B4 T cell hybridoma exists which stably expresses mouse CD2 without its cytoplasmic region and therefore is unable to signal (2B4.CD2Δ) (7). These cells are refractory to TCR stimulation as indicated by the lower amount of IL-2 production following CD3 mAb stimulation (Figure 4.5 A). This shows the positive effect of CD2 signalling on TCR activation.

This 2B4.CD2Δ cell line was then transduced with hCD5WT, hCD5Y1F and hCD5Y3F (Figure 4.5 B). In order to define the effect that these double receptor mutants have on T cell activation, the cells were first of all stimulated with plate bound CD3mAb. IL-2 was assayed by ELISA as a read out of T cell activation (Figure 4.5 C). Strikingly hCD5WT expression in these 2B4.CD2Δ cells appears to partially recover IL-2 production (Figure 4.5 C). Mutation of the 3rd tyrosine has no effect on this IL-2 production compared to CD5WT expressing 2B4.CD2Δ cells. However this CD5WT induced IL-2 production from the 2B4.CD2Δ cells appears to be dependent on the 1st tyrosine. In the hCD5Y1F cells there is little IL-2 production following stimulation.

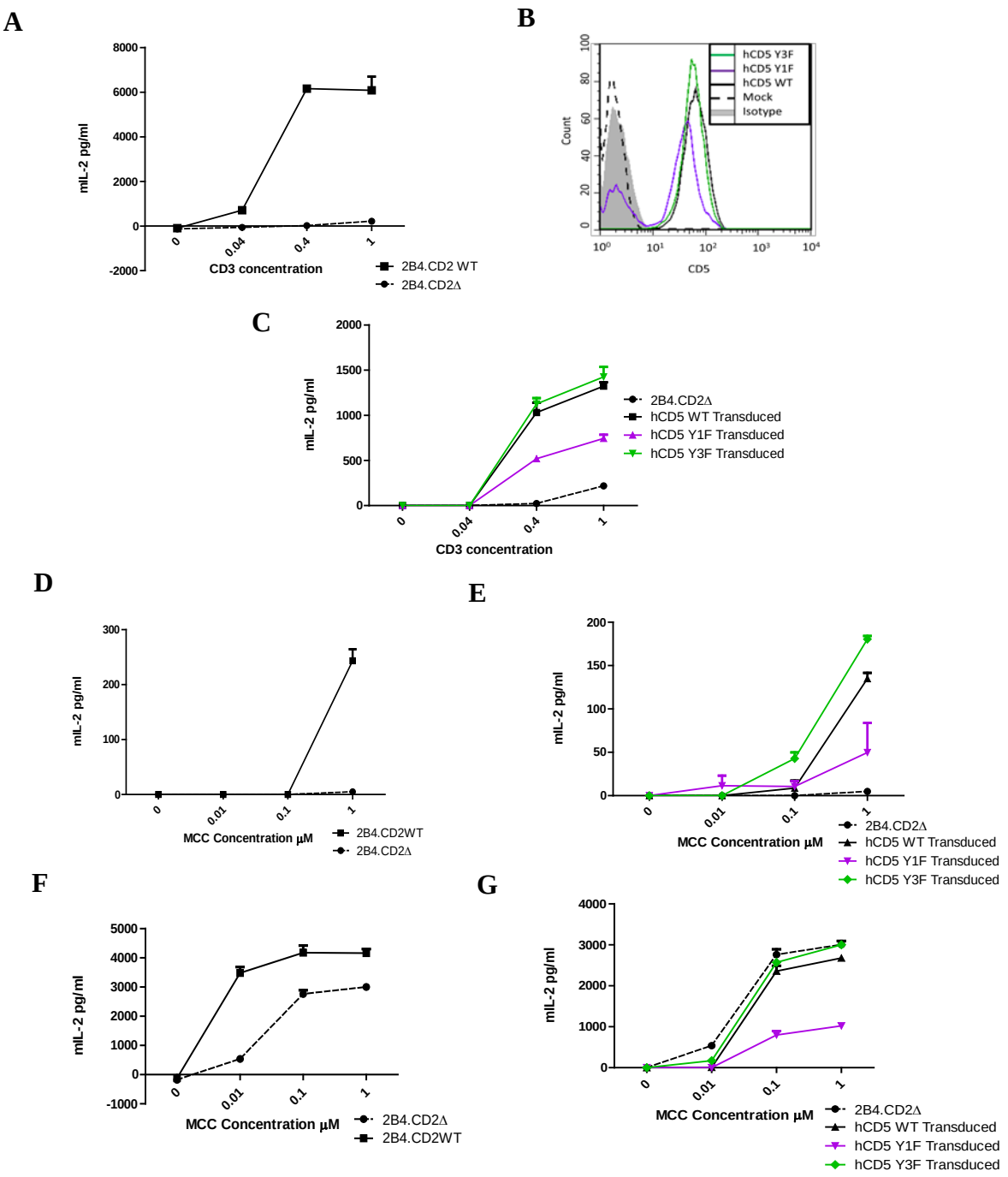

Figure 4.5: CD2 masks positive signalling delivered by CD5 tyrosine phosphorylation. *IL-2 production from 2B4.CD2 WT and 2B4.CD2 Δ plated out at 5 x 10^5cell/well, stimulated for 24 hours with A) plate bound CD3 or D) CHO IE_K cells loaded with MCC peptide or F) CHO IE_K mCD48+ cells loaded with MCC peptide. B) Histograms showing CD5 expression levels on 2B4.CD2Δ cells following transduction. IL-2 production from 2B4.CD2Δ transduced as indicated stimulated with C) plate bound CD3 or E) CHO IE_K cells loaded with MCC peptide or G) CHO IE_K mCD48+ cells loaded with MCC peptide. Error bars represent SEM of results carried out in triplicate.*

To determine the effect of antigen specific stimulation and CD2 ligation on this positive effect of CD5 phosphorylation 2B4.CD2Δ transduced cells were stimulated with CHO IE^k cells with and without mCD48. As with CD3 mAb stimulation, mCD48 negative CHO IE^k stimulation showed the 2B4.CD2Δ cells were refractory to TCR stimulation (Figure 4.5 D). Stimulation of 2B4.CD2Δ cells transduced with hCD5 mutants by mCD48 negative CHO IE^k cells show a similar pattern to the CD3 mAb stimulated cells 2B4.CD2Δ cells (Figure 4.5 E). While 2B4.CD2Δ cells do not respond to antigen specific stimulation, 2B4.CD2Δ cells with hCD5WT and hCD5Y3F will respond and produce a moderate level of IL-2. 2B4.CD2Δ cells with hCD5 Y1F cells produce less IL-2 to antigen stimulation demonstrating a partial requirement for Y1 tyrosine phosphorylation in a positive regulation by CD5 in cells in which CD2 is unable to signal.

When the ligand for CD2, CD48, is present on the CHO cells however the 2B4.CD2Δ cells are more responsive to antigen stimulation although still to a lesser extent than 2B4.CD2WT cells (Figure 4.5 F). This could be simply an adhesion effect of the CD2 and CD48 enabling strong binding for TCR and peptide MHC. However it is possible there is a small level of endogenous CD2WT present in these 2B4.CD2Δ cells. If this low level of endogenous CD2 WT is engaged it could be enough to aid T cell activation. Given this stimulation by CHO IE^k mCD48 cells of 2B4.CD2Δ cells, the positive effect of hCD5WT presence in the cells is not apparent (Figure 4.5 G). This may be due to a masking of CD5 positive signalling by CD2 ligation. Interestingly CD5 inhibition mediated by phosphorylation of Y3 is not apparent in these cells. CD5Y1F cells still produce less IL-2 regardless of the stimulation showing a strong dependence on this tyrosine for CD5 positive regulation of TCR signals. Altogether these results demonstrate a positive effect

of CD5 when there is a lack of costimulation. This is dependent on phosphorylation of the first tyrosine in the CD5 tail.

4.6 C-terminal serine phosphorylation of CD5 is required for CD5 inhibition of T cell activation

There is evidence showing CD5 is also capable of being phosphorylated on serines (62). Mutating tyrosines in the cytoplasmic tail of CD5 could be altering the level of serine phosphorylation due to the close proximity of serines to the tyrosines Y1 and Y3. The C-terminus of CD5 has been proposed to interact with CKII which can promote cell survival and may alter the outcome of CD5 regulation response to T cell activation (88). Therefore specific mutations of serines with and without mutation of Y3 in the C terminus of CD5 were transduced into 2B4.CD2 cells in order to determine whether this altered the effect this tyrosine has on CD5 regulation of T cell activation. Preliminary data using 2B4.CD2 cells transduced with human CD5 mutated on S458A and S461A with and without Y3F mutation show a similar trend for increased IL-2 production (Figure 4.6). This data shows no dramatic difference when the Y3F is mutated or not in either of the serine mutations. Further work will have to be carried out, however, in order to further define this effect.

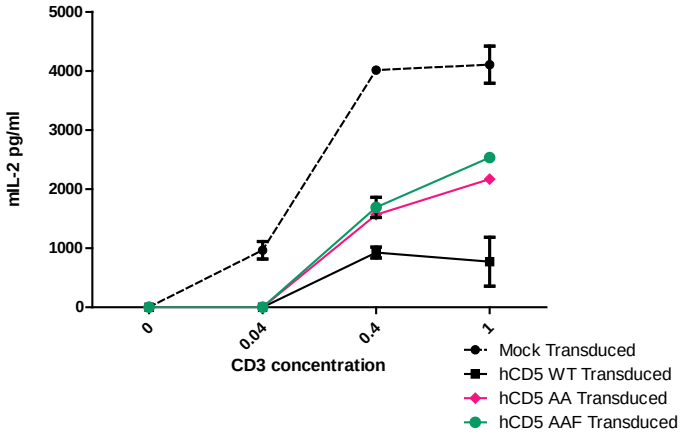

Figure 4.6: C-terminal serine phosphorylation is required for CD5 inhibition of T cell activation. *IL-2 production from 2B4.CD2 cells transduced with hCD5 mutants of S459A and S461A (CD5AA) and hCD5 mutants of S459A. S461A and Y3F (CD5 AAF) stimulated with plate bound CD3 for 24 hours.*

4.7 Discussion

Up until now there has been very little understanding about how tyrosine phosphorylation of CD5 contributes to CD5 regulation of T cell activation in the periphery. I have demonstrated here for the first time a differential contribution of the phosphorylation of the first, second and third tyrosine to CD5 regulation of T cell activation. Phosphorylation of the first tyrosine in CD5 appears to mediate a positive effect on T cell activation. Mutation of Y1 results in a reduction of IL-2 production following TCR stimulation mediated by CD3 ligation or antigen specific stimulation of 2B4.CD2 cells when compared to CD5WT transduced cells. This positive contribution to T cell activation mediated by CD5 becomes apparent when CD2 signalling is stopped. CD2 is known to be synergistic to CD3 induced T cell activation and this is dependent on its cytoplasmic domain (6, 125). In cells with a signalling null CD2, CD5 expression alone partially rescues IL-2 production following TCR stimulation. This positive effect on T cell activation was observed early on when studying the role of CD5. Colligation of CD5 along with CD3 or CD28 resulted in increased IL-2 production and T cell proliferation (83). Until now however it was not clear whether this was due to specific CD5 signalling. In addition the potential for crosstalk between CD2 and CD5 was first observed in the double CD5 and CD2 knock out where positive selection in T cell development was affected (123). Evidence in this thesis indicates that this crosstalk may be mediated via signalling of the two receptors. Further work needs to be carried out in order to further define this.

Countering this positive effect on T cell activation, phosphorylation of the second and third tyrosines appears to contribute to CD5 inhibition of T cell activation. Mutation of either of these tyrosines to phenylalanine results in an increase in IL-2 production

following CD3 triggering or antigen specific stimulation when compared to CD5WT transduced cells. CD5 inhibition of TCR transduced signals has been well established in thymocytes following the finding that in CD5 KO mice, thymocytes are hyper-responsive to TCR stimulation (10, 77, 78). Here I have clearly demonstrated a dependence on tyrosine phosphorylation of Y2 and Y3 for CD5 inhibition of T cell activation. In addition to this it was not clear from the literature that the second tyrosine is ever phosphorylated following T cell stimulation, however these experiments point to a role for Y2 tyrosine phosphorylation in CD5 inhibition of TCR signalling.

The signalling mechanisms underlying this differential effect of tyrosine phosphorylation still remains to be determined. There are a number of events that could result from mutation of these tyrosines. The most direct of these would be stopping recruitment of signalling proteins which interact with phosphorylated tyrosine such as SH2 domain containing proteins. In addition a lack of phosphorylation could cause a differential recruitment of proteins which are unable to bind to phosphorylated tyrosines. One such reported interaction is that of AP2. This has been shown to interact to the unphosphorylated Y1 (71). This would result in a downregulation of CD5 expression. However considering that there is no dramatic loss of CD5 expression in the CD5Y1F expressing cells when compared to CD5WT or CD5Y3F cells this is unlikely to be the case.

Another effect that these mutants could be having is altering the level of serine phosphorylation. Considering the close proximity of serines to the tyrosines Y1 and Y3, lack of tyrosine phosphorylation could promote serine phosphorylation by making the

serines more accessible for phosphorylation. This could then result in recruitment and activation of CKII which has been shown to promote survival (88). Preliminary evidence of analysis of cell death following stimulation in these cells however did not show dramatic differences in apoptosis. Considering these are tumour cell lines however analysis of apoptosis is unlikely to be physiological and would have to be repeated with primary cells. In considering the crosstalk between serine and tyrosine phosphorylation especially of the C terminal region where this interaction with CKII has been reported (88) differential phosphorylation of this region between serines and tyrosines can be analysed through combinations of specific mutations. Preliminary data shown here using 2B4.CD2 hCD5 SA-SA and hCD5 S-A S-A Y3F mutants shows similar trend for increased IL-2 production (Figure 4.6). No dramatic difference in IL-2 production was observed when the Y3F is mutated or not in either of the serine mutations. Further work will have to be carried out in order to further define this effect.

In carrying out these experiments demonstration of the levels of expression of CD5 and also contributing receptors was carried out. This was important in order to ensure correct interpretation of the results. Differences in IL-2 production could be a result of different amounts of receptors on the surface of the cell. Levels of expression are often overlooked in these assays and can easily account for differences seen in results (116). In studying the levels of expression of CD3 before and after CD3 stimulation of the 2B4.CD2, CD3 downmodulation was observed in both the mock transduced and CD5WT transduced cells. It is known that CD3 downmodulation occurs following T cell activation and it is thought this is the primary mechanism used to prevent aberrant signalling through the TCR (126-128). This CD3 modulation is thought to occur by recruitment of CD3 and the TCR to the

central synapse where it is then ubiquitinated by Cbl-c and targeted for degradation. Interestingly CD5 phosphorylation appears to aid this downmodulation because in the three CD5 phosphorylation mutants there is no observable CD3 modulation from the surface and in fact an enhancement of CD3 expression is seen in the case of the double CD5 Y1Y3 mutant. One could argue if CD5 was contributing to CD3 modulation that its expression would also drop. This downregulation of CD5 expression was not observed here. However, further investigation of these cells using confocal microscopy of the synapse has shown that whilst some CD5 resides in the synapse with all of the CD3, there is a large percentage dispersed elsewhere on the cell (preliminary results Figure 4.3.2). The molecular mechanism underlying CD5 regulation of CD3 downmodulation remains to be determined but could underlie the molecular mechanism of CD5 regulation of T cell activation.

In addition to analysis of the role of tyrosines, this work has also highlighted the role of CD5 expression on T cell activation. Plate bound CD3 stimulation can deliver a strong signal to T cells (129). These 2B4.CD2 cells are stimulated efficiently by plate bound CD3ε mAb to produce around 6000-8000pg/ml of IL-2. In this setting where a strong signal is being delivered through the TCR via ligation by CD3 mAb, CD5 expression strongly inhibits the resulting IL-2 production. Antigen specific stimulation using the CHO IE^k mCD48 cells as an antigen presenting cell, appears to deliver a weaker signal to the 2B4.CD2 cells, inducing IL-2 production which plateaus at between 4000 and 5000 pg/ml. In this setting CD5 expression alone does not dramatically inhibit T cell activation which may be due to the effect of introducing CD2 ligation. These differences may

explain some of the contradictory results in the literature due to the different methods used for T cell activation (10, 130, 131).

Direct interpretation of previous work in the literature based on the results I have found here would not be possible considering most of the experiments carried out in the literature involve ligating CD5 directly. This ligation of CD5 may in fact alter its natural localisation and therefore alter how it regulates T cell activation. It may be the case that CD5 works to regulate both strong and weak signals. If the signals are too strong, CD5 will inhibit T cell activation and if the signal is too weak CD5 may aid T cell activation. Here I have demonstrated the later effect when TCR signalling is impaired by defective coreceptor signalling, CD5 appears to partially aid T cell activation. Forcing the system with antibodies would encourage this effect as is seen in the literature (132).

Altogether this work has demonstrates the importance of tyrosine phosphorylation in CD5 regulation of T cell activation and that differential phosphorylation may be able to account for CD5 being both inhibitory and stimulatory to T cell activation.

Chapter 5: Direct and in direct interactions of the CD5 and CD6 cytoplasmic tails

5.1 Introduction

I have shown that the tyrosine phosphorylation of CD5 is important for CD5 mediated regulation of T cell activation. There is an extensive literature showing that CD5 can associate with a wide range of signalling proteins (detailed in figure 5.1 B) (62, 64, 65, 67, 68, 70-73, 117, 133). Much of these data were obtained through immunoprecipitation of CD5 associated complexes following CD5 ligation by mAb and yeast-2-hybrid screens. It is therefore not clear which of these signalling proteins mediate direct interactions with the phosphorylated tyrosines in the CD5 tail *in vivo*. Elucidating these direct interactions, and determining those which are indirect, will go towards a greater understanding for the molecular mechanisms underlying CD5 regulation of T cell activation.

Phosphorylated tyrosines can bind to proteins via their Src homology 2 (SH2) domains. SH2 domain containing proteins are involved in a variety of different signalling pathways including activating kinases, which will induce calcium flux and IL-2 production, and inhibitory phosphatases, which will inhibit these pathways thereby reducing IL-2 production. In order to determine how CD5Y1 phosphorylation can enhance TCR induced IL-2 production whilst CD5Y2 and CD5Y3 phosphorylation can inhibit TCR induced IL-2 production it is important to consider the direct interactions of these phosphorylated tyrosines (see results Chapter 4).

5.1.1 The organisation of tyrosines in CD5 cytoplasmic tail

The intracellular region of CD5 is a short sequence in relation to that of CD6, and contains four tyrosines. These tyrosines have been described as making up various motifs which

could potentially interact with a variety of different signalling proteins. The amino acid line ups in figure 5.1 A details the regions of CD5 which mimic various motifs in CD6, FcγRIIb, CD3ζ and CKII phosphorylation sites, SH3 binding sites and the Cbl C binding site. Of note, parts of the CD5 cytoplasmic sequence show homology to the related SRCR protein CD6 which is coexpressed on T cells along with CD5 (Figure 5.1 A). The motif surrounding the 3rd tyrosines in both CD5 and CD6 are serine rich and may make up an important binding domain which has yet to be described for cell surface receptors.

Well characterised tyrosine based motifs are also present in CD5. The Y0 has been described as an Immunoreceptor Tyrosine-based Inhibitory (ITIM) like motif which is made up of (Ile/Val/Leu/Ser) - X - Y - X - X - (Leu/Val) consensus sequence (73). This has a binding capacity for the negative regulating tyrosine phosphatases SHP1, SHP2 and SHIP (Figure 5.1 A and B). This region in the CD5 cytoplasmic tail has been shown in one instance to associate with SHP-1 (73). However this has been disputed by a subsequent paper (119) and showed no direct binding to CD5 phosphorylated peptides (unpublished work Moses and Brown). The motif surrounding Y2, SAYPAL, has been described as a second putative ITIM (119). The regions surrounding Y1 and Y2 are considered to belong to an Immunoreceptor Tyrosine-based Activation (ITAM) like motif which is made up of (Y-X-X (L/I) X(6-8)Y – X-X (L/I) consensus sequence. The spacing of the two phosphorylated tyrosines constitutes binding sites for tandem SH2 domains. ITAMs recruit kinases such as ZAP-70, which interacts with ITAMs in the CD3 ζ chain of the TCR complex (Figure 5.1 A) (64). In addition to SH2 domain binding capacity, there are regions which could have SH3 domain binding capacity in the tail of CD5 (Figure 5.1 A). SH3 binding sites are reported to consist of proline rich regions with the consensus

site X-P-P-X-P but have also been reported to bind the consensus site R-X-X-K which is present in CD5 (Figure 5.1A) (134, 135). There are also a number of serines in the CD5 cytoplasmic tail which make up putative phosphorylation sites for serine/threonine kinases such as Caesin Kinase II (Figure 5.1A) (62). A consensus binding site for the E3 ubiquitin ligase Cbl C (DNEY) which is known to downregulate the TCR complex following TCR triggering, is also found in the CD5 cytoplasmic tail (136, 137). In addition, motif predictor databases (138-140) identify a number of other SH2 domain containing proteins which could potentially bind to regions in the CD5 cytoplasmic tail. These include RASA1, CRK, CRKL, NCK, SYK kinase and BLNK. Non SH2 binding motifs identified by these searches also highlighted Cyclin 1, PKA phosphorylation sites, PDZ 3 interaction, 14-3-3 and WW repeat domain binding sequence. However, none of these have been identified in experiments.

5.1.2 CD5 can be both tyrosine and serine/threonine phosphorylated

In order for a lot of these interactions to take place, namely ones mediated by SH2 domains, tyrosines must become phosphorylated. Out of the three tyrosines, Y1, Y2 and Y3, it is only the first and the last (Y1 and Y3) which have been reported to become phosphorylated following TCR triggering (124), and mutation of these tyrosines has opposing effects on TCR induced IL-2 production (see Chapter 4). Another tyrosine, Y378 (Y0), has been predicted to be embedded in the transmembrane region as it occurs before a triple lysine doublet anchoring sequences and therefore is unlikely to be accessible to kinases (Figure 5.1A) (118). A number of kinases have been reported to associate with CD5 and mediate its subsequent phosphorylation. Raab *et al*. and others have shown the Lck Src family kinase can mediate CD5 phosphorylation and can also

associate with CD5 (65, 141). Another reported CD5 association was with the kinase ZAP-70 and the CD3 zeta chain, which CD5 is able to specifically pull down (64). This interaction could also be responsible for TCR mediated CD5 tyrosine phosphorylation. In addition CD5 can be phosphorylated by Fyn, Lyn and ITK kinases *in vitro* (66, 76, 142). Along with tyrosine phosphorylation there are also a number of serines which are available for phosphorylation. Casein kinase II, a serine/threonine kinase has been shown to associated with CD5 and appears to be responsible for serine phosphorylation of CD5 which may be important in late CD5 signalling (62).

5.1.3 CD5 can associate with a wide range of TCR induced signalling components

Figure 5.1 B highlights the proteins that have been found to associate with various regions in the cytoplasmic tail of CD5. These proteins can be grouped into kinases, phosphatases and other inhibitory like signalling proteins, lipid metabolism proteins and cytoskeletal regulators. Proteins containing SH2 domains have been shown to associate with CD5 via immunoprecipitation such as the regulatory subunit p85 of PI-3K which leads to PKC activation and lipid metabolism (67). Members of this signalling pathway have been implicated in CD5 associations, including VAV1 and Rac1 which also contain SH2 domains (68). PLCγ1 activation is thought to be enhanced following CD5 stimulation and has the potential to interact via its SH2 domains (143). In addition to SH2 domains other proteins have been identified to interact with non phosphorylated regions or to S/T phosphorylated regions in CD5. The adaptor AP2 which can bind to non phosphorylated Yxx(/θ) motifs where θ represents a hydrophobic residue, has been shown to interact with Y1 in CD5 through a yeast 2 hybrid screen (71). This adaptor is involved in receptor down regulation. Another signalling component known to be responsible for TCR

downregulation, the E3 ubiquitin ligase Cbl-C, has also been implicated in mediated CD5 down regulation and may also interact with CD5 directly (72).

A

```
CD5            - VASIILALVL LVVLLVVCGP LAYKKLVKKF RQKKQRQWIG PTGMNQNMSF HRNHTATVRS HAENPTASHV DNEYSQPPRN SHLSAYPALE GALHRSSMQP DNSSDSDYDL HGAQRL
CD6            - ------------------------------------------------RIKG KYALPVMVNH QHLPTTIPAG SNSYQPVPIT IPKEVFMLPI QVQAPPPEDS DSGSDSDYEH YDFSAQ
FcγRIIb ITIM   - ----------------AENT ITYSLLKHP-------------------------------------------------------A ENTITYSLLK HP
CD3ζ ITAM      - ------------------------------------------------------------------RRKNPQEGLYNELQKD KMAEAYSEIG
CK2 phospho site- ---------------------------------------------------------------------------------SXXS--------------------SXXS
SH3 binding    - --------------------------------RXXK-----------------------------------------------XPPXP
Cbl-C binding   - ------------------------------------------------------------------------------DNEY
```

B

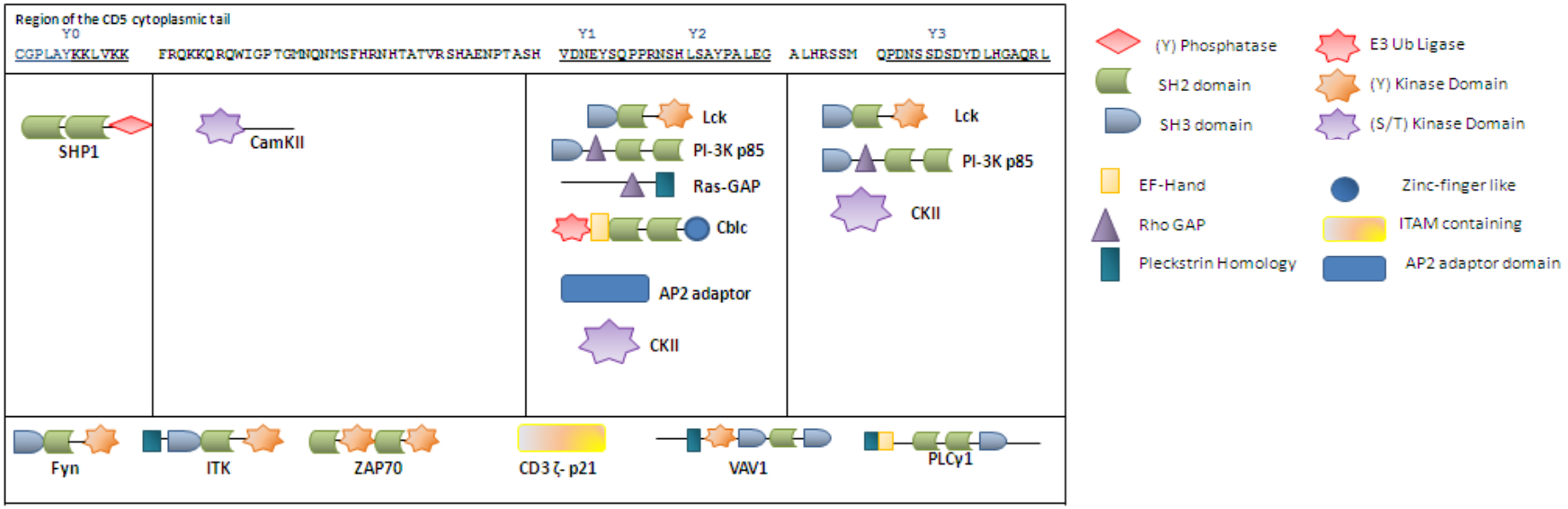

Figure 5.1: A) Line up of tyrosines in the CD5 intracellular region. B) Reported interactions of the CD5 intracellular region. *Diagram illustrating the different domains of signalling proteins reported to interact with the indicated regions of the CD5 cytoplasmic tail. The key to the left defines the domain types. The last row represents signalling proteins shown to interact with CD5 as a whole.*

5.1.4 Aims

With so many candidates that have been identified as associating with CD5, determining which of these mediate direct interactions with CD5 phosphorylated tyrosines may provide a molecular mechanism underlying CD5 regulation of T cell activation. In order to measure direct interactions, surface plasmon resonance can be used to determine the binding affinities of some of the CD5 association candidates postulated in the literature. Following this a hierarchy of binding affinities can be created which may highlight the most likely direct interactions mediated by phosphorylated tyrosines in CD5. In addition mass spectrometry of CD5 immunoprecipitation is a non biased approach to look at the associated proteome of CD5 in order to gain a more global picture of both direct and indirect CD5 associations.

5.2 CD5 phospho-peptides bind SH2 domains with weak affinity

It is not clear which one of the many identified signalling proteins directly interacts with CD5. Many of these proteins contain SH2 domains which are known to bind to phosphorylated tyrosines e.g. Lck, Fyn, ZAP-70 and PI-3K p85. To elucidate if any of these candidates mediates a direct interaction with tyrosine phosphorylated CD5, surface plasmon resonance was used to identify and create a hierarchy of binding affinities. Tyrosine phosphorylated peptides were designed previously in this laboratory (N.J. Hassan and M.H. Brown) to contain part of the CD5 cytoplasmic region which contains the pseudo ITAM and the region containing third phosphorylated tyrosine. Subsequently I designed another peptide containing the first phosphorylated tyrosine alone based on the sequence used by Dennehy *et al*. and unphosphorylated peptides of Y1 and Y3 (120). These phospho-peptides are outlined in Figure 5.2 A. The peptides were biotinylated to enable immobilisation to a streptavidin coated BIACore™ or ProteOn™ CM5 chip.

Serial dilutions of SH2 domains were run over these peptides and response units representing the amount of bound material on the chip was recorded. The response units obtained were subtracted from the negative control flow cell and plotted against the SH2 domain protein concentration in μM. The K_D of binding was then determined by applying a non-linear one site specific binding curve to the data and was measured as the half maximal binding. This calculation was only carried out if binding saturation was achieved at an appropriate amount of response units relating to the stoichiometry of binding material. Curve fits with an R^2 value of more than 0.9 are reported. Where possible a positive control peptide was used to ensure the proteins run over the chips were not

denatured. In all of the experiments carried out, a monoclonal anti-phosphotyrosine antibody was run over the chip to ensure peptides remained phosphorylated during the experiment and were immobilised at similar levels in response units (Results not shown).

Figure 5.2 B-G show representative binding curves of interactions with CD5 phosphorylated peptides which were run with an unrelated phospho-peptide. The kinases Lck, Fyn and ZAP-70 showed no binding to the CD5 phosphorylated peptides when compared to phospho-peptides, CD244 ITSM1, CD244 ITSM1 and CD3 zeta ITAM1 respectively (Figure 5.2 B, C and D). The Itk SH2 domain bound to both CD5 Y1Y2 and Y3 phospho-peptides with around 10 μM affinity, 10-fold weaker than another phospho-peptide (Figure 5.2 E). This binding is not seen when Itk with both its SH2 and SH3 domain were run over these peptides (data not shown). The E3 ubiquitin ligase Cbl-c showed no measurable affinity to CD5 peptides, despite the region surrounding Y1 having the consensus sequence required for binding (136). A positive control peptide from ZAP70 which has recently been crystallised with Cbl-c PTB domain bound with a weak but measureable affinity of greater than 10 μM which has not been previously measured (137) (Figure 5.2 F). This demonstrated that the Cbl-c protein was not denatured. PLCγ1 N+C SH2 domains bound to the CD5 doubly phosphorylated peptide Y1Y2 with around 1 μM affinity and to the Y3 phospho-peptide with an affinity of 3 μM (Figure 5.2 G). Again these affinities were 10 fold lower than those measured for the control phospho-peptide.

Table 5.2 details all the interactions measured along with any measurable K_D. It is clear from the numbers that none of the signalling components showed binding for the singly

phosphorylated Y1 peptide. As I have discussed, it was possible to gain binding K_{Ds} for the doubly phosphorylated Y1Y2 pseudo-ITAM peptide and the singly phosphorylated Y3 peptide. The Y3 phospho-peptide bound the p85 subunit of PI-3-Kinase with a 10 µM affinity which again was 10 fold lower than that of a doubly phosphorylated CD5 Y1Y2 peptide. The well established interaction of p85 for CD28 was measured at between 0.6 and 0.08 µM affinity (144). Altogether these data indicate that CD5 phosphorylated peptides bind weakly to certain SH2 domains namely ITK kinases and PLCγ1 and p85 PI-3K.

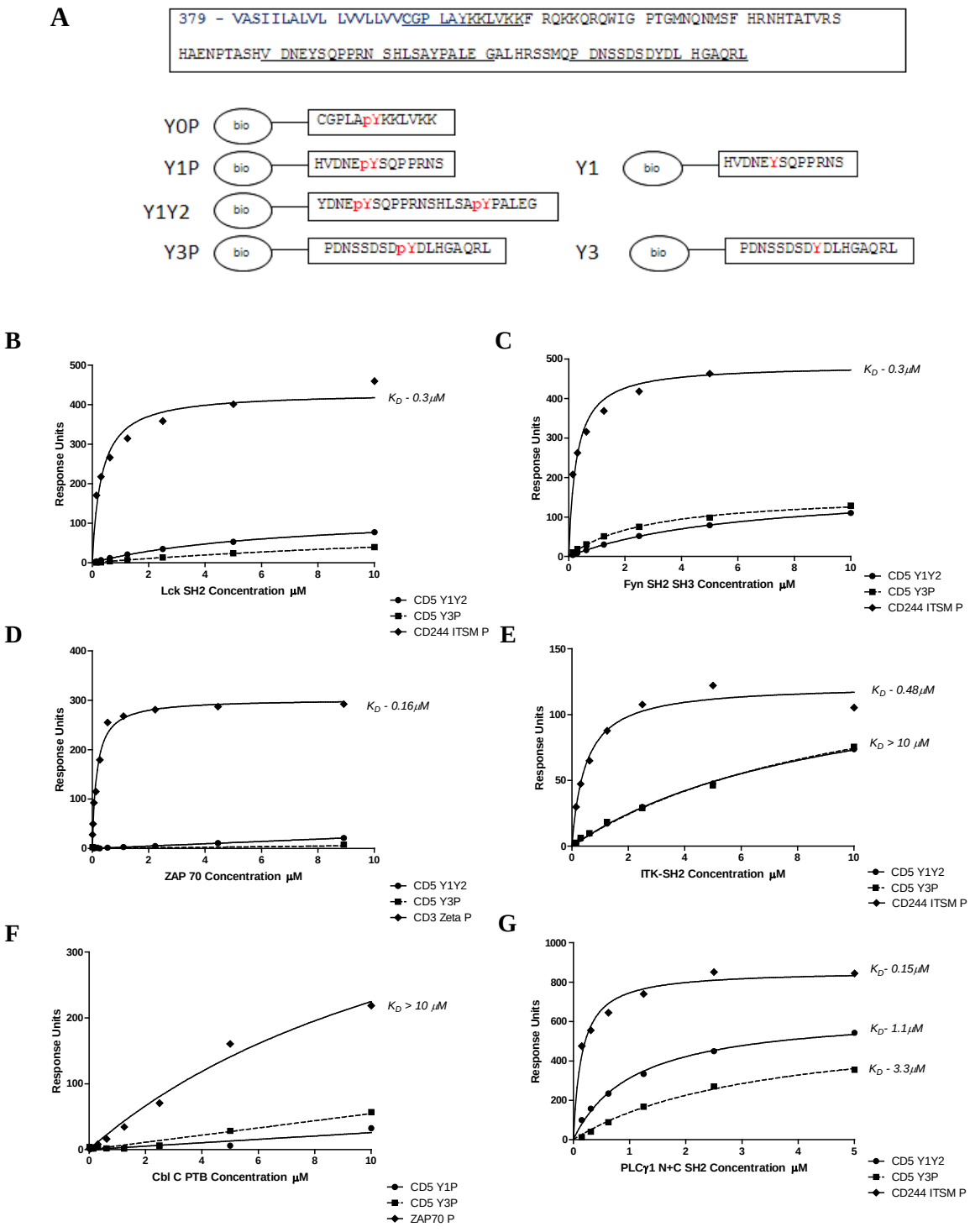

Figure 5.2: CD5 phosphorylated peptides bind with moderate affinity to ITK and PLCγ 1a SH2 domains but not to Cbl C SH2 domain. *Graphs representing the response units recorded against increasing concentrations of SH2 domain containing proteins B) Lck, C)Fyn, D) ZAP 70, E) ITK, F) Cbl C and G) PLCγ 1 N+C run over the CD5 and control phosphorylated peptides indicated. Affinities were measured using one site specific binding calibration curve and calculating the half maximal binding K_D.*

Function	Protein and Domain	CD5 Y1P	CD5 Y1Y2	CD5 Y3P	Positive Control	Positive control peptide
Kinases	Lck SH2	N/A	No Binding	No Binding	0.3	CD244
	Lck SH2 SH3	N/A	No Binding	No Binding	N/A	N/A
	Fyn SH2 SH3	N/A	No Binding	No Binding	0.1	CD6 Y3Y4
	Zap70 SH2	N/A	No binding	No binding	0.2	Zeta
	ITK SH2	N/A	>10	>10	0.5	CD244
	ITK SH2 SH3	N/A	No Binding	No Binding	5.4	CD6 Y3Y4
Ubiquitin E3 Ligase	Cbl-C	No Binding	N/A	No Binding	>10	Zap70
Lipid Metabolism	PI3K p85	N/A	No Binding	10.5	0.9	CD6 Y3Y4
	PLCγ1N+C	N/A	1.1	3.3	0.2	CD244
Inhibitory Kinase	CSK	No binding	N/A	No binding	0.5	Positive control peptide

Table 5.2: Table summarising measured interactions of CD5 phosphorylated peptides with proteins. *Pink boxes highlight non binding interactions. Yellow boxes highlight those interactions for which a K^D was measured. Grey boxes indicate control or unrelated phospho-peptides used to test for denatured protein. N/A refers to those interactions which were not measured.*

5.3 Binding characteristics of conserved phosphorylated tyrosine motif in CD5 and CD6

In studying the cytoplasmic domain of CD5, it is important to note the C-terminal sequence, which contains a number of serines and the Y3 tyrosine, is highly conserved across many species (see Chapter 1). Functionally this region has been identified in this thesis and by others to be important in CD5 regulation of T cell activation (69). Interestingly, the sequence denoting SDSDY in the C terminal end of CD5 is also present in the related CD6 (Figure 5.1 A). I have shown that the CD5 phospho-peptide containing this sequence binds to SH2 domains with affinities which are an order of magnitude lower than that of other phospho-peptides. In CD6 there is another tyrosine in close proximity to the SDSDY which has been shown to increase the binding capacity of this region in CD6 for the SH2 domain of SLP-76 by 10 fold (39). The lack of a second tyrosine may account for the low affinity measurements for SH2 domains of the CD5 Y3 phospho-peptide.

To study the binding characteristics of this conserved tyrosine motif further, peptides from CD6 containing the 3rd (Y486) and then the 4th tyrosine (Y489), alone and together (Figure 5.3 A), designed previously in this laboratory (N.J. Hassan and M.H. Brown) were used. These were measured for binding to a number of the SH2 domain containing proteins alongside the CD5 phospho-peptide Y3. Studying this motif in more depth may help understanding of the functional effects following mutation of CD5Y3 and help elucidate a molecular mechanism underlying the role of CD5 in T cell activation.

The tyrosine kinase Fyn SH2 SH3 domain does not interact with the appropriate stoichiometry seen with the unrelated CD244 ITSM1 peptide, with either singly phosphorylated CD6 peptides Y3 and Y4 or the doubly phosphorylated CD6 peptide Y3Y4 (Figure 5.3 B). Like with the interaction of SLP76 SH2 domain with CD6 Y3Y4, the Lck SH2 domain and Itk SH2 domain bound to the doubly phosphorylated CD6 peptides bind with a 10 fold higher affinity than to the singly phosphorylated peptides (Figure 5.3 C and D). The addition of SH3 domains to either Lck or Itk negated the binding capacity to the singly phosphorylated peptides of CD6 Y3 or CD6 Y4 (Table 5.3). Lck SH2SH3 and Itk SH2SH3 domains were still able to bind to the doubly phosphorylated peptide CD6 Y3Y4 but with an almost 10 fold lower affinity than the SH2 domains alone (Table 5.3). This is unsurprising considering SH3 domains are thought to confer further specificity of the SH2 domains (134) and can alter the binding capacity of the SH2 domains if they are bound (Clarkson unpublished).

Table 5.3 compares these affinity measurements with that of CD5 Y3. What is clear is the affinity of CD5 Y3 for Itk and p85 PI-3K is equivalent to those measured with either of the singly phosphorylated CD6 peptides. There appears to be a specificity of PLCγ1 SH2 domain for CD5 Y3 which showed no binding to any of the CD6 phospho-peptides. Conversely there is specificity of Lck SH2 domain for CD6 Y3Y4 as this showed no binding to any CD5 phospho-peptides. These data indicate a possible cooperativity between Y3 and Y4 in CD6 which may account for the low affinity binding seen with the equivalent motif in CD5. In addition this highlights a specificity of the PLCγ1 SH2 domains for the C terminal tyrosine of CD5.

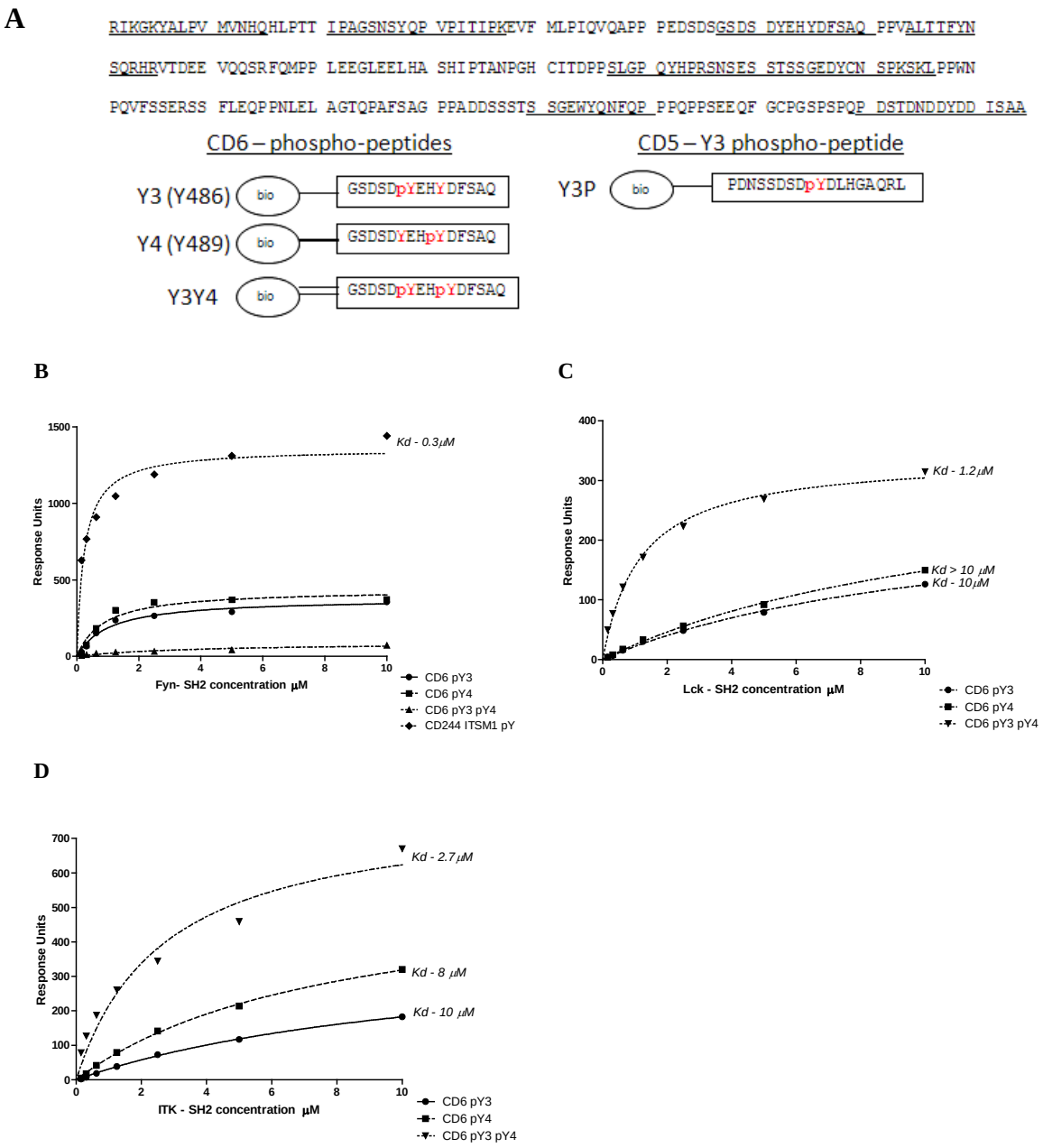

Figure 5.3: Cooperative binding and specificity of tyrosine phosphorylated CD6 peptides. *A) Amino acid sequence of the cytoplasmic region of CD6 and representation of CD6 phospho-peptides alongside the homologous CD5 phospho-peptide. Graphs representing the response units recorded against increasing concentrations of SH2 domain containing proteins B) Fyn, C) Lck and D) Itk run over the CD6 and control phosphorylated peptides indicated. Affinities were measured using one site specific binding calibration curve and calculating the half maximal binding* K_D.

Function	Protein	CD5 Y3P	CD6 pY3	CD6 pY4	CD6 pY3 pY4	CD244 ITSM 1 yP
	Fyn SH2 SH3	No Binding	N/A	N/A	0.08	0.3
	Lck SH2	No Binding	10	>10	1.2	0.3
	Lck SH2 SH3	No Binding	No Binding	No Binding	22.04	No Binding
	ITK SH2	10.7	10	8	2.7	0.5
	ITK SH2 SH3	No Binding	No Binding	No Binding	5.4	No Binding
Lipid metabolism	PLCγ1N+C SH2	3.3	No Binding	No Binding	No Binding	0.1
	PI3K p85 SH2	10.4	6.1	11.4	No Binding	No Binding
Cytoskeletal regulation	VAV1	No Binding	14.1	No Binding	0.9	No Binding

Table 5.3: Comparison of binding affinities of CD5 Y3P and CD6 Y3P, Y4P and Y3Y4PP. *Pink boxes highlight non binding interactions. Yellow boxes highlight those interactions for which a K^D was measured. N/A refers to those interactions which were not measured.*

5.4 Mass spectrometry can be used to identify specific interactions with phosphorylated peptides.

Including direct interactions, indirect associations of the CD5 cytoplasmic tail may also help in the understanding of the molecular mechanisms underlying CD5 regulation of T cell activation. Previous work which identified associations of the CD5 cytoplasmic tail were mostly determined through the use of immunoprecipitation and western blotting (69, 72, 73). This is a biased approach to determine protein associations because it depends on deciding which proteins are blotted for. In order to take a more unbiased approach, mass spectrometry can be used to identify associated proteomes of particular proteins (145, 146). By using CD5 phospho-peptides as bait it may also be possible to determine associations of the different regions in the cytoplasmic tail of CD5. In order to define an appropriate protocol, the known interaction of the CD6 C terminal tyrosine Y662 with SLP76 was used by way of a positive control (39).

First of all the CD6 phosphorylated peptide published to bind to SLP 76 SH2 domain along with the doubly phosphorylated CD6 Y3Y4 peptide were bound to streptavidin coated dynal beads. These were added to pervanadate treated lysates of primary human T cells which had been stimulated with PHA and IL-2 for 7 days. Proteins were then eluted from the beads and trypsin digested before running the mass spectrometer using a 30 min gradient on the HPLC. Protein identifications (I.D's) were then determined and validated using the Central Proteomics Facility Pipeline (CPFP) (147). This will assign a false discovery rate to the protein and peptide identifications if a big enough data set is collected. These results were then analysed further on protein centre software and filtered for protein I.D.s with 2 or more peptides assigned. For each protein I.D. the peptide

probability level was recorded with 1.00 being 100% probability of accuracy. UNIQ highlights the number of unique peptides sequenced for each I.D. and the % highlights the percentage of the protein covered by these peptide sequences.

Results detailed in table 5.4 highlighting that the only non background binding protein I.D. identified in 3 independent experiments was in fact SLP 76. In each case 3 unique peptides were identified with a peptide probability score of 1.0 and a percent coverage of the protein of around 7 %. In one experiment a known SLP-76 associated protein named GRB-2 was identified with a PP of 1.0, 2 unique peptides covering 11% of the protein. It is possible that GRB-2 may be involved in this CD6 mediated pathway however more work would have to be carried out in order to explore this. The other protein hits identified are commonly found in mass spectrometry samples and it is therefore difficult to determine whether they are specifically binding to this CD6 phospho-peptide. These are referred to as background binding proteins. The CD6 Y3Y4 peptide did not pull down anything other than these background binding proteins.

			Expt 1			Expt 2			Expt 3		
Protein	**GENE**	**No. of Expt**	**PP**	**UNIQ**	**%**	**PP**	**UNIQ**	**%**	**PP**	**UNIQ**	**%**
SLP 76	SLP76	3	1	3	6.75	1	3	8.82	1	3	7.13
GRB2-related protein 2	GRB-2	1	-	-	-	-	-	-	1	2	11.82
Albumin	ALB	2	1	5	6.86	-	-	-	0.99	2	4.15
Calreticulin	CALR	1	-	-	-	1	2	6.24	-	-	-
NEDD8	CAND1	1	-	-	-	1	3	3.74	-	-	-
T-complex protein 1	CCT2	1	-	-	-	1	2	6.92	-	-	-
Cyclin-dependent kinase 6	CDK6	1	-	-	-	1	2	8.28	-	-	-
PIH1 domain-containing protein 1	PIH1D1	1	-	-	-	-	-	-	1	2	10.34
Transitional endoplasmic reticulum ATPase	VCP	1	-	-	-	1	4	9.31	-	-	-
WD repeat-containing protein 1	WDR1	1	-	-	-	1	2	5.78	-	-	-
Methylosome protein 50	WDR77	1	-	-	-	1	2	6.14	-	-	-
14-3-3 protein	YWHAZ	1	-	-	-	1	2	15.1	-	-	-
Actin, cytoplasmic 1	ACTB	1	-	-	-	-	-	-	1	6	25.87
ATP synthase subunit alpha, mitochondrial	ATP5A1	1	-	-	-	1	2	6.15	-	-	-
similar to Calnexin	CANX	1	-	-	-	1	2	4.94	-	-	-
Cofilin-1	CFL1	1	-	-	-	1	2	25.3	-	-	-
Clathrin heavy chain 1	CLTC	1	-	-	-	1	7	7.46	-	-	-
Cytoplasmic dynein 1	DYNC1H1	1	-	-	-	1	3	1.05	-	-	-
Fatty acid synthase	FASN	1	-	-	-	1	17	11.67	-	-	-
Hornerin	HRNR	1	-	-	-	-	-	-	1	2	3.54
NADP	IDH2	1	-	-	-	1	4	11.73	-	-	-
Nascent polypeptide-associated	NACA	1	0.9979	4	23.26	-	-	-	-	-	-
Protein disulfide-isomerase	P4HB	1	-	-	-	1	2	4.92	-	-	-

Table 5.4: Mass Spectrometry data from CD6 Y662 phospho-peptide immunoprecipitation. *The peptide probability (PP)1 refers to 100% probable, number of unique peptides identified for each protein (UNIQ), and the % coverage of the final protein sequence (%).*

5.5 CD5 tyrosine phosphorylated peptides do not pull down SH2 domain containing proteins.

Having shown that this method can be used to identify the association of SLP-76 with the published CD6 phospho-peptide, CD6 Y662 (39), CD5 phospho-peptides were used to I.P. proteins from lysates prepared from primary human T blasts. Given that only weak interactions of around 10 µM were found from CD5 phospho-peptides, using this method may highlight proteins which interact with CD5 with a higher affinity. SLP-76 binds to CD6 Y662 with a 0.5 µM affinity which is apparently strong enough to be maintained throughout the protocol. In light of this it is no surprise that no proteins other than background binding proteins were identified in a pull down of the doubly phosphorylated peptide, CD5 Y1Y2. The singly phosphorylated peptides, CD5 Y1 and CD5 Y3 also did not pull down anything other than background binding proteins.

Table 5.5 highlights the protein hits from the singly phosphorylated peptides, CD5 Y1 and CD5 Y3, and their non phosphorylated equivalents. It is clear that these peptides did not pull down proteins containing SH2 domains. An abundant protein S100 A7, A8 and A9 which binds calcium was pulled down with CD5 Y1 and Y3 non phosphorylated peptides and was not found in the phosphorylated equivalent. These three isoforms were identified with 2-3 unique peptides covering around 20 % of the protein with a PP of 1.0. This protein family is involved in calcium signalling like Calmodulin. These particular isoforms have been shown to inhibit Caesin Kinase, a known CD5 associated kinase (88). Interestingly Calmodulin-5 is also present in the pull down with CD5 Y3 with 2 unique peptides, 31 % protein coverage and a PP of 1.0. A calmodulin associated protein calmodulin dependent kinase II has previously been found to associate with CD5 and

therefore may be important (70). 14-3-3, a known regulator of Akt function and an important adaptor in T cell signalling was identified in the pull down with CD5 Y3 (non phosphorylated) with 2 different isoforms of 2-4 unique peptides, 7-30 % coverage and a PP of 1.0. All of these proteins are however very abundant and are commonly found unspecifically in mass spectrometry samples. It is therefore difficult to determine whether these are in fact specific for these CD5 phospho-peptides. Interestingly IL-1R antagonist was identified in CD5 Y3 (non phosphorylated) with 2 peptides, 19 % coverage and a PP of 1.0. This is an extracellular protein however and this is unlikely to be of physiologically relevant interaction.

Given that the protein hits found in these CD5 phospho-peptide pull downs were of abundant proteins, it is important to determine whether these are specific for CD5 and not just background. The Pipeline software has a method of analysing mass spectrometry data by using spectral quantification to compare samples and determine whether there is more of a certain protein in one sample versus the other (SINQ label free quantification) (148). If the relative ratio of protein is higher in the sample of interest by more than 2 fold it is more likely to be specific and not background. Figure 5.5 shows the comparison between the CD5 Y3 phosphorylated and non phosphorylated peptide samples. From these data it is clear that there is an enrichment of the S100 protein in the non phosphorylated CD5 Y3 pull down when compared to its phosphorylated equivalent. It is therefore possible that this is a real association of this CD5 Y3 peptide. There were too few proteins identified in the CD5 Y1 phosphorylated or non phosphorylated pull downs and therefore this enrichment quantification could not be carried out in this case.

Altogether these results indicate no strong candidates which associate to the different tyrosine phosphorylated regions of CD5. Interestingly there were more interacting candidates seen with the non phosphorylated regions, especially the CD5 Y3 peptide given a possible interaction with S100 protein. The specificity of this interaction, however, is yet to be determined.

			Expt - Y1P			Expt - Y3P			Expt - Y1			Expt - Y3		
Protein	**GENE**	**No. of Expt**	**PP**	**Uniq**	**%**	**PP**	**Uniq**	**%**	**PP**	**Uniq**	**%**	**PP**	**Uniq**	**%**
Actin	ACTG1	3	-	-	-	1	2	14.65	1	2	14.65	-	-	-
Prolactin-inducible protein	PIP	3	-	-	-	1	2	13.7	1	3	23.97	1	2	13.7
Dermcidin	DCD	2	-	-	-	1	2	25.45	1	3	25.45	-	-	-
Calcium binding	S100A7	1	-	-	-	-	-	-	1	2	22.77	-	-	-
Calcium binding	S100A9	1	-	-	-	-	-	-	-	-	-	1	2	24.56
Calcium binding	S100A8	1	-	-	-	-	-	-	-	-	-	1	3	32.26
14-3-3	YWHAE	1	-	-	-	-	-	-	-	-	-	1	2	7.06
14-3-3	YWHAZ	1	-	-	-	-	-	-	-	-	-	1	4	35.12
Interleukin-1 receptor antagonist	IL1RN	1	-	-	-	-	-	-	-	-	-	1	2	19.21
Calmodulin-like 5	CALML5	1	-	-	-	-	-	-	-	-	-	1	2	31.51
Protein disulfide-isomerase	P4HB	1	-	-	-	-	-	-	-	-	-	1	2	3.94
Thioredoxin	TXN	1	-	-	-	-	-	-	-	-	-	1	2	22.86
Tubulin	TUBA1C	1	-	-	-	0.5	6	26.9	-	-	-	-	-	-
Alpha-2-macroglobulin-like protein 1	A2ML1	1	-	-	-	-	-	-	-	-	-	0.99	3	2.61
Desmoglein-1	DSG1	1	-	-	-	-	-	-	-	-	-	1	3	4.67
Isoform 1 of Beta-enolase	ENO3	1	-	-	-	-	-	-	-	-	-	0.99	2	7.6

Table 5.5: Mass spectrometry data of CD5 phosphorylated and un phosphorylated peptide immunoprecipitation. *The peptide probability (PP), 1 refers to 100% probable, number of unique peptides identified for each protein (UNIQ), and the % coverage of the final protein sequence (%).*

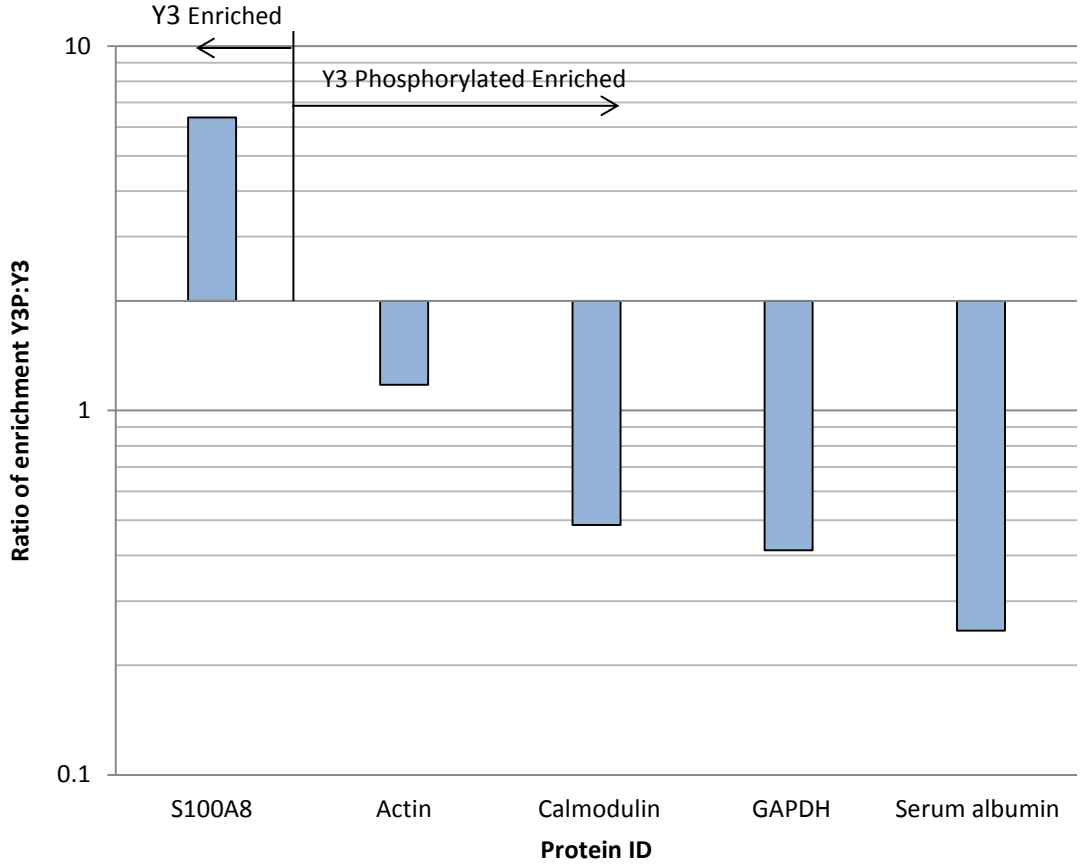

Figure 5.5: SINQ enrichment quantification of Y3P versus the Y3 peptides. *Ratio of enrichment in pull downs of CD5 Y3 phospho-peptide (CD5 Y3P) versus CD5 Y3 non phosphorylated peptide (CD5 Y3). Ratio is in a log scale with the cut off at 2 fold enrichment in one sample.*

5.6 Intact phosphorylated CD5 can associate with SHP1 and CD45 as identified by mass spectrometry

Considering the CD5 phospho-peptides did not manage to immunoprecipitate any likely signalling candidates, it is possible that the phosphorylated peptides used do not encompass the binding domains required. Previous work in the literature used immunoprecipitation of the whole CD5 receptor in order to identify associated signalling proteins (67). Therefore it may be possible to identify novel interactions through immunoprecipitation and mass spectrometry of the whole CD5 receptor.

CD5 was immunoprecipitated from lysates prepared from non pervanadate stimulated and pervanadate stimulated primary human T cells. These samples were then trypsin digested and run on the mass spectrometry with a 2 hour gradient. In order to confirm the experimental procedure was successful in each case, Table 5.6.1 highlights the success of the immunoprecipitation showing the level of CD5 protein that was identified. In the 7 pull down samples run on the mass spectrometer, CD5 was identified with between 2 and 16 unique peptides, covering 4-32 % of the protein with a PP of 1.0. The samples in which more unique peptides were identified indicated there was more CD5 protein present in the immunoprecipitation which therefore would imply more CD5 associated proteins would be present in the sample. This was indeed the case with one sample in which 16 unique peptides for CD5 were identified, 131 specific protein I.D.s were found. If this is compared to a sample in which 4 unique peptides of CD5 were found, only 13 specific proteins I.D.s were found and all of these were background binding proteins.

Given that the immunoprecipitations were carried out on lysates prepared from pervanadate treated cells one could assume this would result in tyrosine phosphorylation of CD5. It is normally difficult to see phosphorylated peptides on the mass spectrometer without prior purification with anti-phosphotyrosine antibodies or titanium oxide. However, in one of the samples run, a peptide containing CD5 Y1 has been identified as being phosphorylated (SHAENPTASHVDNEYSQPPR). The underlined tyrosine was identified as being phosphorylated and was validated using ModLS on the pipeline software with a Post Translational Modification (PTM) score of 14 (147). Considering this is a low score which would normally be expected to be above 20, it could be mistaking the phospho-serine for a phospho-tyrosine considering the two amino acids are next to each other (DNEYS). Therefore it should not be assumed which of these two amino acid residues are phosphorylated in this case.

Experiment – CD5 IP protein ID	Uniq	PP	%	Modifications		Other Protein hits found
Mouse T cell Hybridoma	16	1	28.3	N/A		No
Pervanadate treated PHA expanded primary hu T cells	8	1	18.4	N/A		Yes
Non treated PHA expanded primary hu T cells	14	1	31.7	N/A		Yes
Non treated PHA expanded primary hu T cells	4	1	11.5	N/A		Yes
Pervanadate treated PHA expanded primary hu T cells	6	1	13.7	N/A		Yes
Non treated PHA expanded primary hu T cells – PFA crosslinked	2	1	3.8	N/A		No
Pervanadate treated PHA expanded primary hu T cells – PFA crosslinked	6	1	16.8	PY	DNEpYS	Yes

Table 5.6.1: Identified CD5 from CD5 I.P.s. *The experiments listed show the cells from which lysates were prepared. The details of the CD5 protein hits identified following CD5 I.P. from these lysates are detailed here showing the number of unique peptides, peptide probability score (PP), the % coverage and any modification identified. Also listed is whether there were any other proteins found in the samples other than background binding proteins.*

Analysis of the associated proteins found by the mass spectrometer was then carried out. CD5 was immunoprecipitated from lysates prepared from the 2B4.CD2 cells that had been used in previous IL-2 production experiments (see Chapter 4). As I have discussed CD5 was identified from this sample (Table 5.6.1) but few other proteins were identified other than background binding proteins such as Keratin (results not shown).

CD5 was also immunoprecipitated from lysates prepared from proliferating primary human T blasts which had been pervanadate treated to ensure phosphorylation of proteins. In the pervanadate stimulated samples no proteins other than background binding proteins were identified along with CD5. The non stimulated samples however contained a number of possibly relevant proteins. One of these was the SH2 domain containing tyrosine phosphatase SHP-1 Table 5.6.2. SHP-1 was identified with 2 unique peptides covering 5 % of the protein with PP 0.99. In the same sample, the receptor phosphatase CD45 was also present with 3 unique peptides covering 2 % of the protein with a PP of 1.0. In addition the S100 A7 and A9 proteins and 14-3-3 was identified in this sample but as I have discussed it is not possible to determine whether these are specific due to their abundance. The S100 proteins were identified with 2-3 unique peptides covering 12-24 % of the protein with a PP of 1.0. 14-3-3 isoforms were identified with 2 to 4 unique peptides covering 7 – 24 % of the protein with a PP of 1.0. MHCI HLA and MICB proteins were also identified with an average of 3 unique peptides covering 14 % of the protein with a PP of 1.0. Caspase 14 was also identified with 2 unique peptides covering 8% of the protein with a PP of 1.0. It is important to note that while these are possibly relevant findings all of these proteins were only identified in one out of the 7 experiments carried out highlighting the inconsistency of this method.

SINQ quantification was carried out comparing the control immunoprecipitation with the CD5 immunoprecipitation from lysates prepared from non pervanadate treated primary T blasts in order to show whether 14-3-3 or S100 had a higher specificity for CD5 than for the control I.P. In this case there is an enrichment of the 14-3-3 present which may indicate a specificity for CD5 (Figure 5.6.2). However, alternate analysis of this interaction would need to be carried out in order to confirm this finding.

Altogether these data show that mass spectrometry analysis of immunoprecipitates of whole CD5 identified few proteins involved in T cell activation. The results gained from this would need to be validated by western blotting of immunoprecipitates along with affinity binding measurements using SPR. This highlights that this is not an appropriate technique to measure interactions of the CD5 cytoplasmic domain.

			Non Stimulated Expt 1			Non Stimulated Expt 2		
Protein	**GENE**	**No. of Expt**	**PP**	**Uniq**	**%**	**PP**	**Uniq**	**%**
CD5	CD5	2	1	14	31.72	1	4	11.52
SHP-1	PTPN6	1	0.9996	2	5.21	-	-	-
CD45	PTPRC	1	1	3	2.62	-	-	-
Calcium Binding	S100A7	1	1	3	12.87	-	-	-
WD Repeat Protein	WDR1	1	1	2	15.38	-	-	-
Calcium Binding	S100A9	1	1	2	24.56	-	-	-
Clathrin	CLTCL1	1	1	2	1.34	-	-	-
14-3-3	YWHAE	1	1	2	7.06	-	-	-
14-3-3	YWHAZ	1	1	4	24.4	-	-	-
Caspase-14	CASP14	1	1	2	8.68	-	-	-
HLA	HLA-A	1	1	4	16.17	-	-	-
HLA	HLA-B	1	1	3	14.05	-	-	-
HLA	HLA-C	1	1	4	17.76	-	-	-
HLA	HLA-H	1	1	2	10.22	-	-	-
MHC class I	MICB	1	1	2	10.73	-	-	-
Cathepsin D	CTSD	1	1	2	6.31	-	-	-
Prolactin-inducible protein	PIP	1	1	2	15.07	-	-	-
Dermcidin	DCD	1	1	5	35.45	-	-	-
Cofilin-1	CFL1	1	1	3	31.93	-	-	-
Filamin-A	FLNA	1	0.9945	2	1.44	-	-	-
D-3-phosphoglycerate dehydrogenase	PHGDH	1	1	3	6.19	-	-	-
Secretoglobin	SCGB1D2	1	0.9997	2	22.22	-	-	-
Actin	ACTB	2	-	-	-	1	6	20.53
Beta-actin	ACTBL2	1	-	-	-	0.9933	2	9.04
Tubulin	TUBA1C	2	-	-	-	0.9989	2	10.74

Table 5.6.2: Proteins identified from a CD5 I.P. of lysates prepared from non treated primary human T blasts. *Proteins are shown with name and gene name along with the number of experiments it was found in. For each protein I.D. unique peptide numbers, peptide probability and the % coverage is listed.*

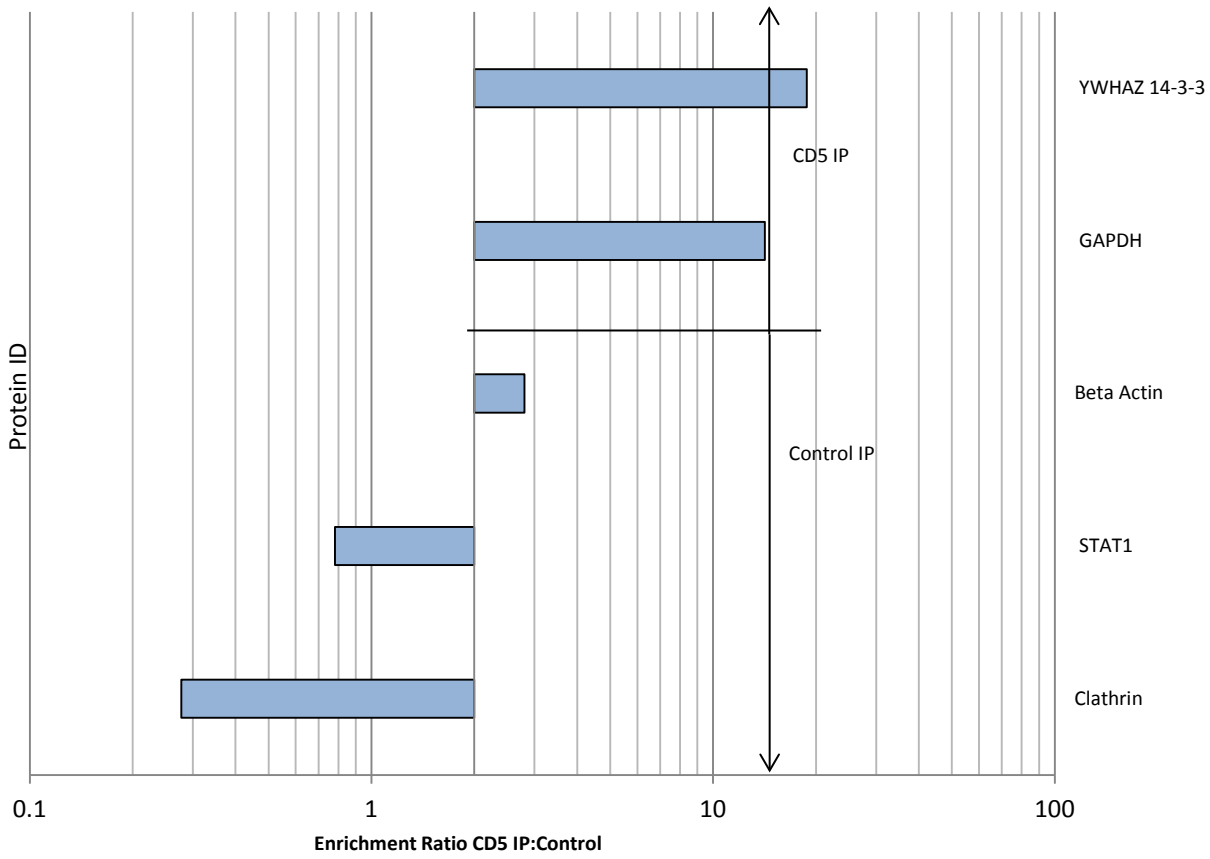

Figure 5.6.2: SINQ enrichment quantification of CD5 IP versus the control IP. *Ratio of enrichment in control I.P. versus CD5 I.P. from lysates prepared from non treated primary human T blasts. The ratio is in a log scale with the cut off at 2 fold enrichment in one sample.*

5.7 Discussion

I have demonstrated here that CD5 tyrosine phosphorylated peptides do not interact with a high affinity with primary CD5 associated candidates from the literature. Surface plasmon resonance analysis has shown around 10 fold lower binding affinities of CD5 for the SH2 domains of PLCγ1, PI-3K p85 and Itk when compared to the binding to unrelated phospho-peptides (Figure 5.7). PI-3K p85 and Itk have been reported to associate with and phosphorylate CD5 respectively and it is possible that these mediate weak but direct interactions with the CD5 cytoplasmic tail (67, 68, 76). I have also shown here that there is no measurable binding of CD5 phospho-peptides to Cbl-C. Cbl-C has been reported to be responsible for CD5 ubiquitination and has been shown to associate with CD5 following pervanadate stimulation or CD5 crosslinking in thymocytes (72). This interaction however appears to be indirect.

The addition of the SH3 domain to the SH2 domain of Itk negated any binding to CD5 phospho-peptides making it unlikely to be a physiological interaction. The SH3 domain of Itk has been shown to have a high specificity for regions containing the consensus sequence GWYSKPPPPIP which may explain why this interaction did not occur with CD5 (149). Interestingly there was an affinity for Itk SH2SH3 domains for the double phosphorylated CD6 Y3Y4 peptide with a K_D of 5.4 µM. This peptide contains no such consensus sequence either but there is evidence in the literature showing CD6 can be immunoprecipitated with Itk along with Lck which greatly increases CD5 phosphorylation (76). Further evidence found here for this cooperation between CD5 and CD6, is the preferential binding of Lck SH2 SH3 domains to the CD6 Y3Y4 peptide (Table 5.3). It

was not possible to gain an affinity of the Lck SH2 domain for any of the CD5 peptides despite evidence to the contrary in the literature showing an association of this kinase with CD5 (65, 141). Lck has been indicated as the kinase responsible for the phosphorylation of CD5. The lack of a direct interaction of CD5 with Lck does not rule this kinase out as being responsible for CD5 phosphorylation. Considering the evidence I have shown here it is possible that CD6 directly interacts with both Lck and Itk kinases, albeit with a modest affinity when compared to other SH2 mediated affinities which are normally in the sub μM range, thereby recruiting them to aid CD5 phosphorylation. Added to this is the fact that this particular region in CD6 is the same distance from the membrane as the C terminal of CD5. Altogether this has provided a molecular mechanism to explain cooperativity and crosstalk between these two related receptors.

The strongest affinity of an SH2 domain for CD5 was measured with PLCγ1 which would out-compete binding of the p85 PI-3K SH2 domain. There is evidence showing ligation of CD5 can augment the activity of PLCγ1 and therefore enhance T cell activation (143). The SH2 domain in PLCγ1 had a higher affinity by 3 fold for the CD5 Y1Y2 peptide than for the CD5 Y3 peptide. Considering that mutation of CD5 Y1 results in a decrease in IL-2 production (see Chapter 4) the evidence shown here indicates that this may be mediated through a direct interaction with PLCγ1. Further investigation to show this is the case however would be required. The affinity for PLCγ1 SH2 domain binding to CD5 phospho-tyrosines is weak when compared against those of positive controls which were all around the 0.5 μM range. It could be argued therefore that if there was a limited amount of PLCγ1 around for binding then it would not bind to CD5 preferentially.

The overall lack of high binding affinities to tyrosine phosphorylated CD5 peptides found here could also be as a result of the peptides not containing the correct region to mediate binding or may not have the correct modifications. It could be argued that the proximity of CD5 to the TCR may mean that CD5 does not require high affinity interactions. Overall the direct interactions of the CD5 cytoplasmic tail appear to mediate weak affinity binding to SH2 domains and will be transient in their nature.

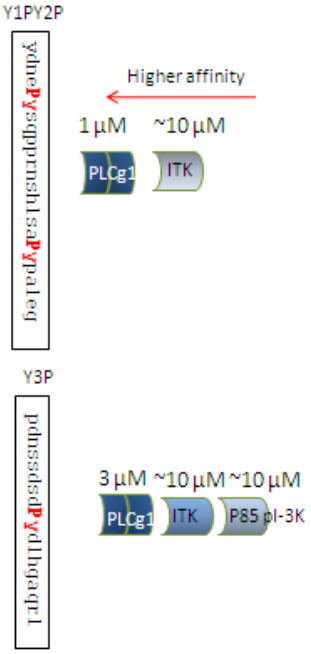

Figure 5.7: Diagram highlighting the binding hierarchy of SH2 domains to regions in CD5 cytoplasmic tail.

I have demonstrated here that the use of mass spectrometry to analyse direct and indirect associations with both tyrosine phosphorylated peptides of CD5 and the whole receptor is not an appropriate technique for identifying novel interactions. The interaction of CD6 Y662 with the adaptor SLP-76 which has been measured to bind with a 0.5 µM affinity at

37 degrees is a strong interaction and therefore was able to be identified clearly using this technique at 4 degrees. In fact it was possible to identify a novel interaction of PLCγ1 using mass spectrometry of immunoprecipitations of an adaptor Eat-2 in this laboratory (150). Again this interaction was measured to be of a high K^D of 0.04 μM.

There is some indication from the data I have shown here, however, of possible additional candidates which may be associated with CD5. This was found interestingly with non phosphorylated peptides and non pervanadate stimulated T blasts. This may point to an inhibition of recruitment mediated by tyrosine phosphorylation however this is simply speculation. The S100 A7, A8 and A9 came up in the Y3 non phosphorylated samples and were not found in the phosphorylated equivalent. As I previously discussed, these particular isoforms have been shown to inhibit Caesin Kinase for which there is evidence for its phosphorylation and binding to CD5 (69). Considering that these proteins are present in a lot of mass spectrometry samples however it is important to validate these findings through other techniques. 14-3-3 was also identified in many of the CD5 samples and appeared to be enriched in some. This protein is known to interact with a wide variety of proteins and is involved in most parts of T and B cell signalling in particular apoptosis, cell cycle and the stress response. There is only part of a consensus binding site for 14-3-3 (RSXpSXP) in the CD5 cytoplasmic tail (SXP) however, so this is unlikely to be a direct interaction. Considering how ubiquitous this protein is would mean it is not possible to define whether this is in fact a specific association with CD5. The phosphatase SHP-1 that has been shown to associate with CD5 Y0, which is buried in the transmembrane region, was identified from an immunoprecipitation of the whole CD5 receptor from lysates prepared from non stimulated T blasts (73). Considering this was not reproduced however

this may highlight the variability that can occur with immunoprecipitation, added to the fact no one has validated this result (72, 118).

Altogether the evidence here highlights that this technique is not appropriate for a receptor such as CD5 which appears to mediate weak and transient interactions through its cytoplasmic tail. This method may not be sensitive enough to identify these types of interactions. These data do agree with the binding affinity analysis that was carried out here in that only weak binding was measured showing that for this method of identification, a sub micro molar affinity may be required in order to maintain the binding during the protocol.

Altogether this data indicate CD5 positive signalling may be mediated through an interaction with PLCγ1 and that CD6 may aid CD5 phosphorylation by direct recruitment of Itk and Lck kinases. Further investigation must be carried out however to test this. Evidence of how CD5 Y2 and Y3 mediate CD5 negative signalling however remains to be determined. What is clear is that CD5 mediated interactions are weak and probably transient and are most likely to involve interaction with domains other than SH2 or SH3 domains. The work here has shown the possibility for further investigation into interactions with the S100 proteins. The results here also show the importance of defining the timing and location of tyrosine and serine phosphorylation. Understanding this may help in further defining the direct and indirect associations of the CD5 cytoplasmic tail.

Chapter 6: General Discussion

6.1 Summary of key findings

In this thesis I demonstrate that OX19, a rat CD5 mAb, will block extracellular engagement of CD5 *in vitro* resulting in an increase in proliferation following T cell activation. In demonstrating the mode of action of OX19 I show that CD5 engagement will inhibit T cell activation following stimulation *in vitro*. I also show, in the absence of T cell activation CD5 can be crosslinked by OX19 to induce a low level proliferation. I clearly demonstrate that OX19 does not modulate CD5 from the surface *in vitro* contrary to its modulatory activity *in vivo*. I also demonstrate that following a secondary stimulation of antigen specific T cells *ex vivo* CD5 is downregulated from the surface coinciding with an increased proliferative response to antigenic stimulation which indicates timing and location may be important for CD5 inhibition of proliferation. CD5 may be downregulated in order to allow T cell proliferation to occur.

Another key finding in this thesis is that differential phosphorylation of tyrosines in CD5 can account for positive and negative signalling by CD5 during T cell activation. I show that CD5 Y1 is required for positive signalling which becomes more apparent in the absence of CD2 mediated costimulation. CD5 Y3 and Y2 are required for the CD5 inhibition of T cell activation. These have been shown by looking directly at the effects of CD5 phosphorylation on T cell activation either through CD3 stimulation or antigen specific stimulation.

Phosphorylation of these tyrosines is also necessary for CD5 regulation of CD3 downmodulation following TCR triggering which may point to a possible molecular

mechanism for CD5 mediated regulation. CD5 may control CD3 expression on the surface in order to control T cell activation.

In carrying out this study I have also shown the importance of considering the signalling effects of other receptors. Removing CD2 signalling allowed a better understanding of the differential results seen and the true effect of CD5 signalling. The evidence here has highlighted the importance of considering the effects of receptor crosstalk in T cell activation and that this can in fact lead to a better understanding of a receptor's role in T cell activation.

The later part of this thesis demonstrated that the CD5 cytoplasmic tail mediates weak interactions with SH2 domain containing proteins via its phosphorylated tyrosines and that these weak and possibly transient interactions were not detectable by mass spectrometry. Creating a hierarchy of these interactions however did highlight a novel interaction of PLCγ1 which bound to the positive CD5 Y1 with a 1 μM affinity, three fold higher than that of the inhibitory CD5 Y3. In addition I demonstrate evidence for a possible molecular mechanism to explain increased phosphorylation of CD5 in the presence of CD6 (76). The region in the cytoplasmic tail in CD6, CD6Y3Y4, which lies at the same distance from the membrane as the C-terminus of CD5, will bind to Itk and Lck. Given CD5 and CD6 are often coexpressed this would allow phosphorylation of CD5 by these two kinases.

6.2 Manipulation of CD5 – the possibility of therapeutics

There have been many attempts made to create an antibody therapy that could treat autoimmune conditions such as multiple sclerosis and rheumatoid arthritis, and also cancers such as BCL-L. A lot of these have been designed to specifically remove or inhibit the pathogenic T cells causing the disease. The main molecules targeted in these therapies have been ones associated with the activation of T cells such as CD3, CD4 and other receptors associated with the TCR complex including CD5 and CD6. CD6 mAbs have been used successfully in depleting donor bone marrow in order to prevent graft rejection (100). Another successful antibody treatment has been a humanised antibody Campath-1H which recognises CD52, a receptor expressed on lymphocytes (151). This receptor is expressed on a subset of BCL-L patients and therefore can be used to specifically target and remove these cancerous cells. Like CD52, CD5 is also highly expressed on a subset of BCL-L cells and so such a therapy may also prove effective (90). However, CD5 mAb treatment of BCL-L has been unsuccessful in preliminary experiments showing a high variability between patient subsets (152). In addition, as I and others have demonstrated the use of CD5 mAbs to treat autoimmune conditions has produced variable results. One antibody, OX19, will inhibit EAE if given before and not after disease induction and will enhance a similar disease, EAN, if given during the height of disease response (93, 94, 112). The unpredictable nature of CD5 means that it is unlikely to be a good therapeutic target for antibody based therapies. Understanding how this receptor functions on a molecular basis however could give insight into other small drug therapies which could be used to manipulate CD5 and make it behave in a way which promotes recovery.

6.3 Positive and negative signalling – a new paradigm for dual functional receptors.

There are a number of receptors expressed on T cells which apparently mediate both a negative and positive regulation of T cell activation, including CD2, CD5 and CD45. It has been proposed that this could be due to receptor localisation and sequestration of signalling from the TCR or by inhibition of one signalling pathway in order to let another signalling pathway become active (18). Evidence I have shown here demonstrates that the dual function of CD5 can be due to both positive and negative signalling mediated by the receptor. Mechanistically, the differential tyrosine phosphorylation of tyrosines in CD5 could create docking sites for different signalling proteins and these could in turn compete for signalling. Following TCR triggering, receptors including CD5 are recruited to the TCR allowing proximal kinases to phosphorylate tyrosines. Following this phosphorylation, recruitment of SH2 domain containing proteins such as PLCγ1 to the tail of CD5 Y1 will then promote T cell activation. The only other evidence involving this tyrosine in the literature demonstrates it is phosphorylated following TCR stimulation and that it is involved in CD5 inhibition of BCR induced calcium flux and Akt recruitment (118-120). This apparent contradiction could simply be a result of the differences in BCR and TCR signalling. Inhibition by CD5, mediated by CD5 Y3 could be a result of recruitment of phosphatases or E3 ubiquitin ligases, although there is no evidence for this. Another possibility is that phosphorylation of Y2 and Y3 could change CD5 localisation and therefore sequester positive signalling molecules such as PLCγ1 from the TCR. The latter is less likely due to the low affinities of binding which are mediated by this region on CD5. CD5 may not be able to compete for PLCγ1 binding to adaptors such as LAT which bind with 100-fold higher affinity (153).

It is important to consider the significance of serine phosphorylation in this dual activity of CD5 which has shown previously to be important in CD5 mediated signalling (117). It is striking that the tyrosines in CD5 are in close proximity to multiple serine residues. Given this proximity it is unlikely that both serines and tyrosines would be phosphorylated at the same time, and therefore there may be an interplay between these two phosphorylation events adding further regulation by this receptor.

Altogether the results in this thesis have given a good grounding for looking at the signalling of dual functioning receptors in order to understand the mechanisms underlying the balance between inhibition and activation. Mechanistically for CD5 this appears to be a result of differential phosphorylation of the 3 tyrosines in the CD5 tail which can result in both activation and inhibition mediated by CD5. Defining these mechanisms clearly may allow more accurate and effective design of therapeutic agents in autoimmunity and cancer.

6.4 Cooperation of receptors

As I have discussed the localisation of receptors on the T cell surface is very important in their function (Chapter 1). I have proposed a molecular mechanism involving the proximity of CD6 and CD5 which may be account for CD5 tyrosine phosphorylation through recruitment of kinases to CD6 (Chapter 5). Given this theory, if CD6 is not expressed or co localised with CD5, CD5 may then not become highly tyrosine phosphorylated thereby changing its regulatory role on T cell activation. This in turn may result in increased serine phosphorylation of CD5.

I have also demonstrated how the signalling of other co-receptors such as CD2 can mask those effects of CD5 signalling (Chapter 4). Signalling through CD2 in the 2B4 cell line studied in this thesis results in enhanced T cell activation. Removal of this strong stimulus results in a dramatic loss in T cell IL-2 production which can be partially rescued by over expression of CD5.

Further analysis of this cooperation between receptors, and the tight regulation of expression of CD5, may help define the role of CD5 in T cell activation. Depending on where and when CD5 is expressed could potentially result in a different overall function of the receptor. For example, CD5 is strongly inhibitory to BCR signalling in immature B cells (118). Immature B cells will undergo apoptosis in response to BCR triggering whereas mature B cells will undergo proliferation in response to BCR triggering (reviewed (154)). Therefore CD5 expression in immature B cells may promote survival and CD5 expression on mature B cells may promote apoptosis (54, 109, 155). Moreover CD5 is

only expressed in mice on B-1a cells which are an unusual subset of B cells that reside in the peritoneum. These are thought to consist of a regulatory cell type, which produces high amounts of IL-10 (156-158). It has therefore been proposed that CD5 in fact promotes B cell regulation through increased IL-10 production (159, 160). In T cells the study of how CD5 regulates different T cell subsets has not been studied but may account for the differences seen in CD5 function. The only differences measured have been the role of CD5 in regulatory T cells in which CD5 appears to have an inhibitory role (89). It is therefore possible that the functional outcome of CD5 regulation will be different depending on the 'hard-wiring' of the cell in question, along with its localisation and expression levels of other receptors such as CD2.

6.5 Future work

In light of the key findings uncovered in this thesis and given the theories that I have proposed, future work on the role of CD5 in T cell activation and beyond are as follows.

1. Cooperation of the receptors and their localisation can be analysed through imaging of the synapse using the cell lines I have made. This will help define how CD5 phosphorylation and CD2 signalling may affect CD3, CD5 and CD2 localisation. This can be expanded to include CD6. Preliminary data show this is indeed possible to image using these cells and will be an interesting line of investigation (see Discussion in Chapter 4).

2. Analysis of serine phosphorylation and how this affects tyrosine phosphorylation of CD5. Preliminary data shows serine phosphorylation does have a functional effect on T cell activation (see Discussion Chapter 4).

3. Further characterisation of CD5 tyrosine and serine phosphorylation using lentivirus transduction in vivo. This will then allow dissection of CD5 function at different stages of T cell differentiation and effects on apoptosis.

4. Further dissection of the role of CD5 phosphorylation in CD3 modulation by blocking function of Cbl and AP2 in order to define the signalling responsible.

5. Interplay between serine and tyrosine phosphorylation. Using surface Plasmon resonance to detect changes in receptor phosphorylation levels using a non specific

phospho-agent could be done to determine whether phosphorylation of either tyrosine can affect phosphorylation of serines and vice versa.

6. Due to the apparently transient nature of CD5 associations, protocols concentrating on difference in timings and differentiation states of the cell may be more appropriate. Also through the use of specific inhibitors with the tyrosine mutants I have developed may be more insightful.

In carrying out these experiments further insight into the specific molecular mechanisms underlying the dual signalling capacity of CD5, and cooperativity with coreceptors on T cells, may be uncovered and help bring the findings in this thesis further towards a defining role of CD5 in the regulation of T cell activation.

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