

Analysis of the CD200R Family

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*To my son Nazım Erdem Akkaya,
who was born during the preparations of this thesis and has
become the infinite joy of my finite life. I hope, one day, this gives
him inspiration to develop curiosity towards the wonderful world of
science...*

I certify that this thesis is my own work, and materials and sources used in its preparation are acknowledged. I also certify that the work of other students and/or persons has not been copied in part or whole or otherwise plagiarized. This thesis or parts of this thesis have not been submitted for a degree before.

Contents

Abstract.....	i
Acknowledgements.....	ii
Abbreviations.....	iii
Chapter 1: General Introduction.....	1
1.1 Paired Receptors and CD200R Family.....	1
1.1.1 Immune Regulation by Paired Receptors.....	1
The Term Paired Receptors.....	1
Structural Characteristics of Paired Receptors.....	4
<i>C-type lectin domains.....</i>	<i>4</i>
<i>Ig Super Family domains.....</i>	<i>7</i>
1.1.2 CD200R Paired Receptor Family.....	12
CD200 and CD200R.....	12
The CD200-CD200R interaction.....	14
CD200 knock-out mice.....	15
Factors that interfere with the CD200-CD200R interaction.....	16
CD200R Like proteins.....	16
1.2 Co-evolution of immune system and pathogens.....	20
1.2.1 General overview of self defence against pathogens.....	20
The innate immune system.....	20

The adaptive immune system.....	21
1.2.2 Immune evasion mechanisms of microorganisms.....	22
Immune evasion through modulation of Immune regulatory systems.....	23
The central role of MHC-I in immune activation.....	24
Viral strategies for subverting the NKG2 receptor function.....	25
Viruses can benefit from the molecular mimicry between some viral proteins and the host MHC-I.....	30
1.2.3 Evolution of the host's counter defense strategies.....	32
Past Studies on Differential Resistance to CMV.....	32
Pathogen driven evolution of the Ly49H gene.....	34
Studies on Ly49 led to the generation of the paired receptor concept.....	36
1.2.4 Host-pathogen interactions in other paired receptor families.....	38
Nkrp1 Family.....	40
NKG2 Family.....	42
CEACAM Family.....	43
SIGLEC Family.....	45
PIR Family.....	48
CD200R Family.....	49
<i>Viral homologues of CD200.....</i>	52
<i>Conclusions on pathogen driven evolution of the CD200R family.....</i>	55
Aims of this Thesis.....	56
Chapter 2: Materials and methods.....	57
2.1 Reagents and solutions.....	57

2.1.1	General reagents.....	57
2.1.2	Bacterial growth media.....	59
2.1.3	Cell culture media.....	60
2.2	Nucleic acid methods.....	61
2.2.1	PCR.....	61
2.2.2	Nucleic acid isolation.....	62
2.2.3	Agarose gel electrophoresis and gel purification.....	63
2.2.4	Constructs.....	64
	Restriction digestions and ligations.....	64
	DNA sequencing.....	64
	pEF-BOS Mammalian expression vector based constructs.....	65
	Retroviral vector based constructs.....	70
	PEE 14 Based Vectors.....	72
2.3	Cellular Methods.....	72
2.3.1	Transfection.....	72
	Transfection of 293T cells to analyse surface expression.....	73
	Transfection of 293T cells to generate chimeric proteins.....	73

Transfection of CHO K1 cells for generation of large scale	
soluble proteins.....	74
2.3.2 Transduction.....	76
2.3.3 Cell lines.....	78
2.3.4 T cell stimulation model to test antibodies against mCD200R.....	82
2.3.5 RBL degranulation model.....	83
2.3.6 Analysis of cell surface interactions using flow cytometry.....	84
Staining cell surface receptors with antibodies.....	84
Staining cell surface receptors with fluorescent beads coated with	
ligands.....	84
Flow cytometry.....	85
2.3.7 Production of large quantities of antibodies from hybridomas.....	85
2.4 Protein methods.....	86
2.4.1 Biotinylation of soluble proteins.....	86
2.4.2 Inhibiton ELISA for quantification of rCD4 d3-4 containing chimeric soluble	
proteins.....	87
2.4.3 IL-2 ELISA.....	88
2.4.4 β -Hexosaminidase assay.....	89

2.4.5	SDS- PAGE.....	90
2.4.6	Western blot.....	90
2.4.7	Antibody purification using protein G column.....	91
2.4.8	Purification of His tagged soluble recombinant proteins.....	92
	Ni-NTA chromatography.....	92
	Size exclusion chromatography.....	93
2.4.9	Surface Plasmon Resonance (SPR).....	93
	Characterization of antibodies using SPR.....	93
	Measurement of the affinity between rabbit CD200 and CD200R using SPR.....	94
2.5	Methods for screening possible interactions between pathogenic bacteria and various cell surface receptors.....	95
2.5.1	Bacteria strains.....	95
2.5.2	Fluorescent staining of the bacteria.....	95
2.5.3	Analysis of bacterial binding to the magnetic beads coated with the receptors of interest.....	96

Chapter 3: Screening for the heterogeneity in the mouse CD200R family.....97

3.1	Introduction.....	97
3.2	Sequence analysis of CD200RL genes and the PCR strategy used.....	98
3.3	Screen of mice strains for mouse CD200RLa-c genes.....	101
3.4	Characterization of novel members of the CD200R family.....	105
3.5	Analysis of the distribution of CD200RLe.....	106
3.6	D200R is present in all mouse strains in two slightly different allelic variants.....	110
3.7	Discussion.....	112

Chapter 4: Characterization of the rabbit CD200R family and the test of the possible interaction between the rabbit CD200R and M141R (a candidate viral CD200 homologue).....117

4.1	Introduction.....	117
4.2	Characterization of rabbit the CD200R family.....	119
4.3	Characterization of the CD200 CD200R interaction in rabbits.....	120
4.4	Investigation of the interaction between myxoma M141 protein and rabbit CD200R.....	124
4.5	Discussion.....	127

Chapter 5: Screening for possible bacteria-receptor interactions among members of the CD200R family.....	130
5.1 Introduction.....	130
5.2 Development of a screening strategy.....	131
5.3 <i>S. aureus</i> was shown to interact with PIR-A1 but no other receptors tested.....	134
5.4 Discussion.....	138
 Chapter 6: Characterization of novel antibodies against members of the mouse CD200R family.....	 140
6.1 Introduction.....	140
6.2 Generation of stable cell lines expressing members of CD200R family.....	141
6.3 Functional analyses on CD200RL-DAP12 molecules expressed on RBL.2H3 Cell Line.....	142
6.4 Characterization of Antibody Binding Properties for Novel Antibodies.....	144
6.5 OX131 but not OX110 can block CD200-CD200R interaction.....	146
6.6 Discussion.....	151
 CHAPTER 7: General discussion.....	 156
7.1 Concluding Remarks on the evolution of paired receptors.....	156
7.2 Achievements of this study.....	159
7.2.1 Observations on the heterogeneity of the mouse CD200R family.....	160

7.2.2	Novel reagents to study the mouse CD200R family.....	162
7.2.3	Screenings on pathogen binding.....	163
7.3	Unanswered Questions and the future works.....	164
7.4	Conclusions.....	167
	References.....	168

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Abstract

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Analysis of the CD200R Family

Paired receptor families, consisting of multiple genetically and structurally similar but functionally opposite activating and inhibitory cell surface receptors, are among the fine tuners of the immune regulation. Recent studies on the evolutionary origin of these receptor families have suggested links to pathogen driven diversification, according to which activating receptors continuously evolve in order to counterbalance pathogens that try to subvert the immune response by stimulating the inhibitory receptor through their virulence factors.

This thesis is about the CD200R paired receptor family. This family consists of an inhibitory receptor CD200R which is expressed on various leukocytes and delivers inhibitory signals upon engagement with its ligand CD200.

In this study, the possibility that the activating members of the family evolved under pathogen pressure was investigated. Genomic DNA from twenty two different mice strains was screened for the presence of members of CD200R family. The number of activating receptors varied, CD200RLe and CD200RLc were found to be mutually exclusive and three strains possessed previously unknown members of CD200R family.

In addition, the possibility that CD200R family members and other paired receptors interacted directly with bacteria was tested with a new assay but only the interaction of PIR-A1 with *S. aureus* was found as previously reported.

The rabbit CD200R family has been characterized and ligand receptor interaction between rabbit CD200 and rabbit CD200R has been demonstrated. However, no interaction between rabbit CD200R and a candidate viral CD200 homologue, the M141R protein of myxoma viruses, could be shown. This finding suggested a CD200R independent role for M141R molecule and possibly other homologues in pox viruses.

Finally, two novel antibodies (OX131 and OX132) were characterized together with formerly generated antibodies against mouse CD200R family. The binding specificities and their effects on the CD200-CD200R interaction have been shown. This will help usage of these antibodies in various studies on the functionality and distribution of these receptors.

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Abbreviations

APC	Antigen presenting cell
bp	Base pairs
BSA	Bovine serum albumin
CD200RL	CD200 receptor like
CD33r	CD33-related
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CHO	Chinese hamster ovary
clr	C-type lectin like receptor
DAP 10/12	DNAX-activating protein 10/12
DC	Dendritic cell
ddH ₂ O	Double distilled water
DMSO	Dimethyl sulfoxide
Dok 2	Downstream of kinase 2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallizable
FcR	Fc receptor
FcRL	Fc receptor-like
FCS	Fetal Calf Serum
FcεRI	Fc epsilon receptor I
gp	Glycoprotein
gpUL40	Glycoprotein UL40
HA	Hemagglutinin
HBS	HEPES-buffered saline
hCD200R	Human CD200 receptor
hCD200RLa	Human CD200 receptor like a

HCMV	Human Cytomegalovirus
HHV8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICAM	Inter-cellular adhesion molecule 1
IFN- γ	Interferon-gamma
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
ILT2/4	Ig-like transcript 2/4
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
K _D	Dissociation constant
KIR	Killer cell immunoglobulin-like receptor
KO	Knock out
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Lysogeny broth
LSDV	Lumpy skin disease virus
LTR	Long terminal repeats
mAb	Monoclonal antibody
MCMV	Mouse Cytomegalovirus
MHC-I/II	Major histocompatibility complex class I/ II
MOPS	3-(N-morpholino)propanesulfonic acid
MV	Myxoma virus
NK	Natural killer
OMCP	Orthopoxvirus MHC class I-like protein
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PIR	Paired immunoglobulin- like receptor
PTB	Phosphotyrosine-binding domain
RasGAP	Ras GTPase activating protein
RBL	Rat basophilic leukemia
rCD200R	Rat CD200 receptor
rCD4 d3-4	Rat CD4 domains 3 and 4
RPM	Revolutions per minute
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFV	Shope fibroma virus
SH2	Src Homology 2
SHIP	Src-homology 2-containing inositol 5' phosphatase
SIGLEC	Short for sialic acid binding Ig-like lectin
SIRP	Signal regulatory protein
SOC	Super optimal broth with catabolite repression
SPR	Surface plasmon resonance
TAE	Tris-acetate-EDTA
TBE	Tris-borate- EDTA
TBS	Tris-buffered saline
TCR	T cell receptor
TE	Tris- EDTA
TLR	Toll-like receptor
TNF	Tumor necrosis factor
vCD200	Viral CD200 homologue
WT	Wild type
YMTV	Yaba monkey tumor virus

Chapter 1:

General Introduction

1.1 Paired Receptors and CD200R Family

1.1.1 Immune Regulation by Paired Receptors

The Term Paired Receptors

The immune response against a foreign molecule is mediated by a complex network of interactions between various cellular and humoral components of the immune system. By these interactions, the immune system generates an optimal response to eliminate the potentially dangerous non-self elements while sparing host cells and members of commensal flora. This complexity, therefore, requires tight regulation mechanisms and interactions of numerous cell surface receptors with their ligands during cell-cell contacts play a major role in this process. Activating and inhibiting signals generated by these cell surface receptors are integrated and evaluated together within the cell which finally determines the response generated by that cell.

A group of leukocyte cell surface receptors that contribute to the fine tuning of the immune response exist in genetically and structurally similar but oppositely signaling receptor pairs “Paired Receptors” (105). This definition contains receptor groups that 1) are coded by different genes located in the same gene cluster, 2) have significant sequence identity in their extracellular domains, 3) have both activating and inhibitory

members (189). Moreover, in most cases activating and inhibiting receptors are co-expressed on the same immune cell subpopulations (Table 1.1).

Family	Activating	Ligand	Inhibitory	Ligand	Characteristics	References
KIR	KIR2DS1	HLA-C (Weak)	KIR2DL3	HLA-C	IgSF	120, 169
Ly49	Ly49H	M157 (MCMV molecule)	Ly49B	MHC-Class I	C-Type Lectin	1, 36, 40, 48, 66, 97
NKG2	NKG2E	Qa-1 ^b (Weak)	NKG2A	Qa-1 ^b	C-Type Lectin	37, 109, 116
Nkrp1	Nkpr1a	RCTL (RCMV molecule)	Nkrp1b	Clr-b	C-Type Lectin	35,36,85,
CEACAM	CEACAM-3	unknown	CEACAM-1	CEACAM-1,8	IgSF	88,180,181
Clecs	Clec4b1	unknown	Clec4a2	unknown	C-Type Lectin	189
CD200R	CD200RLa	unknown	CD200R	CD200	IgSF	4,173,187
CD300	CD300b	unknown	CD300a	unknown	IgSF	189
SIRP	SIRP β	unknown	SIRP α	CD47	IgSF	13
Siglec	Siglec-14	unknown	Siglec-5	Sialylated Proteins	IgSF	5,45,46
PIR	PIR-A1	MHC Class I(weak)	PIRB	MHC Class I	IgSF	158
PILR	PILRB	unknown	PILRA	CD99 related molecule	IgSF	13, 189
LILR	LILRA5	unknown	LILRB1	MHC Class I	IgSF	189

Table 1.1 Summary of various paired receptor families. Activating and inhibitory refer to the activating and inhibiting examples of that receptor family. IgSF stands for Immunoglobulin superfamily.

Paired receptor families contain inhibitory receptors that have well demonstrated functions in immune regulation. They bind to specific cell surface molecules and transmit

inhibition signals through their long cytoplasmic tails which have motifs to engage with inhibitory downstream elements. Therefore, most of them have a well-studied role in generating a hyporesponsive state against organism's own cells. On the contrary, despite their significant ligand binding domain similarity to their inhibitory pairs, activating receptors either do not bind the ligand of the inhibitory receptor or bind with significantly lower affinity which may not have physiological significance. These activating receptors do not contain long cytoplasmic tails but instead they carry a positively charged amino acid residue in their transmembrane region which lets them engage with transmembrane activating adaptors such as DAP12, DAP10 and FcεR1γ (105). For most cases there are no identified ligands for activating members of paired receptors and therefore, the rationale behind their existence and their interactions with the inhibitory partners are not clear. Therefore, they are either binding to a self- molecule which is expressed under stress conditions, (like atypical stress induced MHC like ligands of NKG2D receptors (38)) or the very low affinity interaction between activating receptor and the ligand of the inhibitory partner has a physiological importance in regulating immune response or they bind with non-self molecules and thus they do not have a natural ligand in the body.

There have been several observations showing that the inhibitory members of some paired receptor families can be targeted by pathogens which try to down-regulate immune response against them (30, 67, 104, 130). In some of these cases activating receptors were shown to play important roles in counterbalancing this pathogen threat by

activating immune responses upon binding to those pathogen derived ligands which originally evolved to bind with inhibitory receptors (9, 130, 145).

The thesis focuses on the CD200R paired receptor family which is described in detail later.

The key questions are: what are the functions of the activating members of the CD200R family and what is driving their evolution? The evidence suggests the evolution is driven by pathogens and the evidence for this is described in detail for some paired receptors in section 1.2.4.

Structural Characteristics of Paired Receptors

C-type lectin domains.

Paired receptors of the immune system structurally fall into two groups. One of them is the C-type lectins superfamily. Paired receptor members of this group include Clecs, Nkrp1s, NKG2s and Ly49s (Table 1.1). C-type lectins consists of a wide range of extracellular metazoan protein domains of between 110 to 140 amino acid residues that were termed C-type because they were able to bind carbohydrates in a calcium dependent manner (58, 192). Later this domain type was shown to be involved in non calcium dependent binding of proteins. Members of this superfamily play a wide range of roles including cell-cell adhesion, binding to pathogen derived ligands, apoptosis, immune regulation, cell trafficking, prevention of autoimmunity or as toxins (98, 192). The first structurally identified member of the family is the rat mannose binding protein (193) and other well-known members include snake venom, NK cell receptors, selectins, anti-

freeze proteins of cold water fish etc. (192). Although C-Type lectins are of metazoan origin, there are examples where non-metazoan pathogens like bacteria and viruses exist. Amongst them bacterial C-type lectins are hijacked host molecules or their imitations (192) and include toxins like pertussis toxin (156) and proaerolysin(134) or outer membrane adhesion molecules such as intimin (117) and invasins (76). Viral C-type lectins are transmembrane proteins or structural components of the envelope and seen in viruses like vaccinia virus, myxoma virus, adenovirus etc. (192).

Structurally, C-type lectin domains contain a double loop formation (loop in a loop) which are stabilized by the disulfide bonds found at bases of the loops and a set of conserved hydrophobic and polar interactions (192). The inner loop is formed by the loop like arrangement of the beta sheets and alpha helices of the domain and it is structurally conserved in all C type lectins. This loop consists of two beta sheets and two alpha helices. The first beta sheet (vertical beta sheet) is formed by the specific alignment of $\beta 1$, $\beta 5$ and $\beta 1'$ strands and the second beta sheet (anti parallel beta sheet) is formed by strands $\beta 2$, $\beta 3$ and $\beta 4$ (193) (Figure 1.1). The second loop, called the long loop is an evolutionary flexible structure which exits and enters the core domain in the same place. It is the second loop, that is responsible for the calcium dependent carbohydrate recognition, and dimerization of some proteins however it is totally absent in a subset of C-type lectins called compact C type lectins (192). $\beta 2$ strand divides the domain into upper and lower lobes. While vertical beta sheet together with the two alpha helices form the lower lobe, anti-parallel beta sheet and the long loop stays in the upper lobe (193) (Figure 1.1). There

are four important cysteine residues that form conserved disulfide bonds (C1-4). C1-C4 bond links $\beta 5$ and $\alpha 1$ and maintain the stability of the first (whole domain) loop. C2-C4 bond links $\beta 3$ and $\beta 5$ and is required for the formation of the second loop (192) (Figure 1.1).

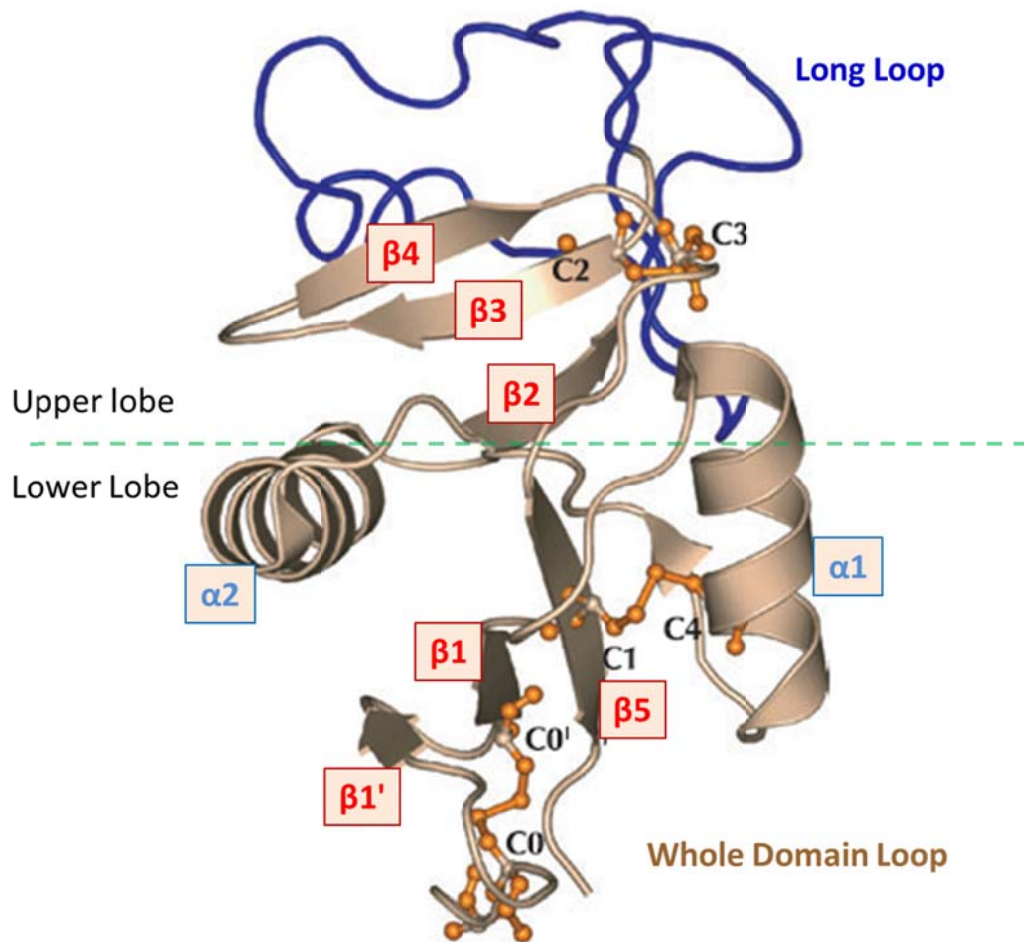


Figure 1.1 Cartoon representation of a typical C type lectin. Long loop is shown blue and the whole domain loop is shown grey. Positions of alpha and beta strands are indicated boxed. Orange bricks represent the places of disulphide bonds. C0-4 represent the cysteine residues. Adapted from the structure of 1k9i (DC sign) C0-C0' bond is specific for this C-type lectin (192).

Ig Super Family domains.

The second structural group seen in a wide range of paired receptors is the immunoglobulin Super Family (IgSF) domains. PIRs, CD200Rs, SIRPs, KIRs, CEACAMs, Siglecs, ILTs and PILRs are amongst the paired receptor families that contain IgSF domains. Members of this superfamily play important roles in immune regulation, cell-cell communication, self- nonself discrimination, defense against pathogens and cell adhesion (2). The prototype and the first member of this family is the immunoglobulin whose Fab fragment structure was identified approximately 40 years ago (136). Later on, many cell surface receptors and soluble molecules of the immune system have been shown to have partial amino acid sequence identity as well as tertiary structure similarity to the immunoglobulin molecule and thus, they were included in this family (2, 12). Today, with the addition of new members outside the immune system that share the same basic properties, this superfamily is considered as one of the largest in the vertebrate genome. The criterion for including a protein in this superfamily is the presence of one or more Ig-like domains that are homologous to either variable (V) or constant (C) domain of a typical immunoglobulin(2). The minimal degree of accepted sequence homology is 15% and these conserved sequences are responsible for the generation of a globular structure called the Ig-fold (2, 12). An Ig fold is a sandwich like arrangement of two beta sheets, each formed by three to five anti parallel beta strands (Figure 1.2). Each of these beta strands consists of five to ten amino acid residues. The inward looking residues are typically hydrophobic and the outward looking ones are hydrophilic. This arrangement is the major contributor

of the stability of the fold. Additionally, most Ig-like domains contain conserved cysteine residues which form intra-chain disulphide bonds to generate 55-70 amino acid long loops (2). The two types of Ig folds V and C differ from each other by the shorter length of the latter which is due to the lack of C' and C'' beta strands (12). Later on, with the discovery of new IgSF members, a third type (C2 or H) was introduced to describe the Ig-like domains that are similar to C in terms of size and similar to V in terms of amino acid sequence and after the introduction of C2 type, C type has been called as C1 (12, 186).

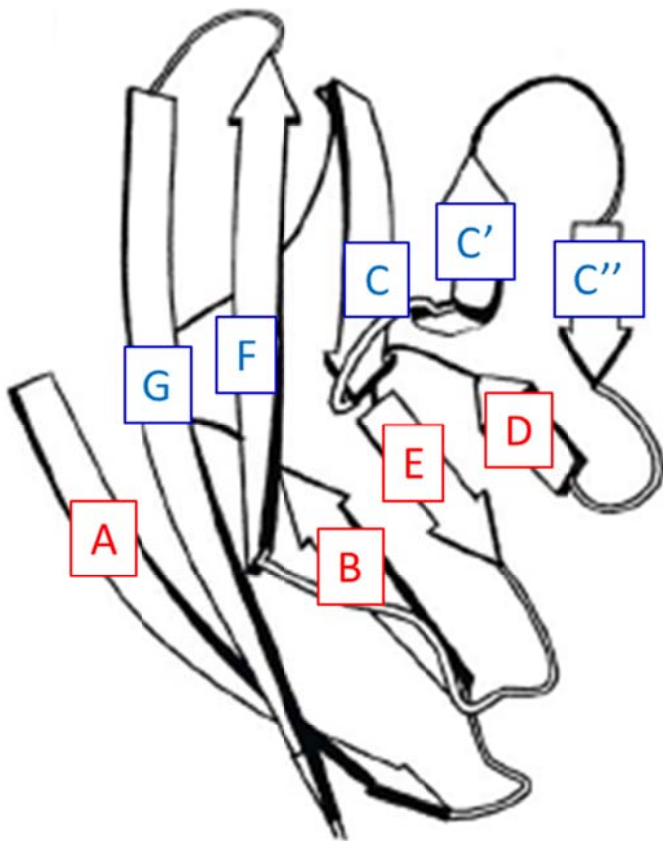


Figure 1.2 Typical organization of an Ig fold. The beta strands are shown as broad arrows and the loops are shown as lines joining them. The letter code of each beta strand is shown boxed. Strands that are forming the same beta sheet are shown with the same color (red or blue).

IgSF family members of leukocyte surface receptors differ enormously in the number of Ig-like domains they contain. While some like CD8, CD28, CD47, Thy-1 contain only one domain NCAM, PDGFR contain five and the most exceptional member sialoadhesin contains seventeen Ig-like domains (2, 12). However it is noteworthy that approximately half of these receptors are two domains long which gives them advantage to pair with other two domain molecules to fit the roughly four domain intercellular distance formed between an effector cell and an antigen presenting cell (12, 184) (Figure 1.3).

Most (>90%) IgSF molecules are integral cell surface molecules having the Ig-like domains in the extracellular region which is followed by a hydrophobic transmembrane region and a highly divergent intracellular tail. While almost all IgSF members are devoid of an intrinsic enzymatic activity, the intracellular tails are frequently associated with adapters that recruit kinases or phosphatases (2).

IgSF molecules can interact with a range of different molecules, such as members of IgSF or other protein superfamilies or carbohydrates as seen in Siglecs (12). However, the most frequently observed interactions are between members of IgSF (Figure 1.3). These interactions can be either homophilic or heterophilic and can involve V or C domains of the molecule. Although the immunoglobulin molecule is the prototype of this family its interactions (antibody –antigen interactions) with the ligands can be up to 1000 fold stronger than interaction of many other IgSF molecules (12). This is the main difficulty in

identification of ligands for these molecules and therefore, instead of using monomeric proteins with such low affinities multimeric forms of these proteins are recombinantly generated when searching for ligands (12). These multimerization techniques include usage of Fc fusion molecules, tetramers or binding molecules onto beads (12, 23, 178).

Glycosylation is another commonly found feature of IgSF molecules. These residues can be as big as a domain itself and hence are expected to play important roles in the function of the molecule. The contributions of glycosylation to the function of IgSF molecules include generation of restricted mobility for optimal receptor-ligand interaction, protection from proteolysis in regions of continuous T-cell antigen presenting cell interaction, facilitating their interactions with carbohydrate binding molecules such as siglecs and other specific roles such as in loading of antigens onto MHC molecules (46, 49, 142). However it should be noted that in most cases, IgSF members can properly fold or interact with their protein ligands in the absence of sugars which indicate that sugars minimally effect the folding or the ligand receptor interactions for many IgSF members (12).

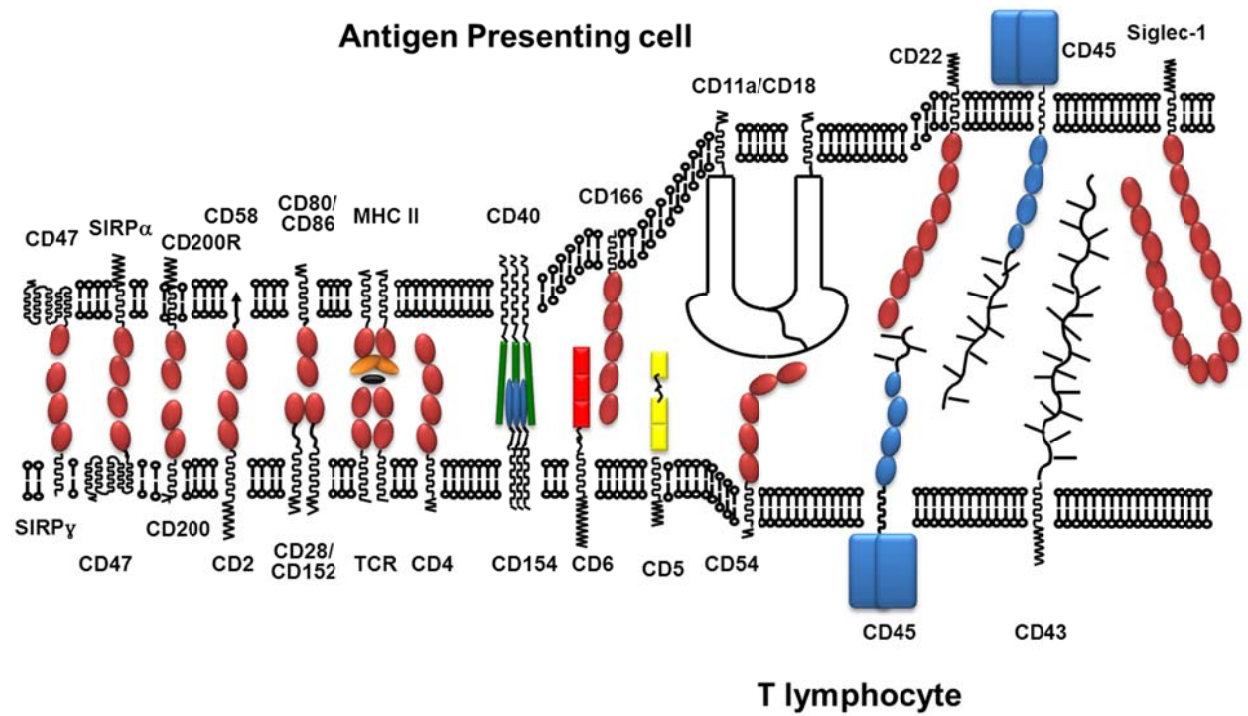


Figure 1.3 Schematic representation of cell-surface molecular interactions, occurring during T-cell Antigen presenting cell contact. IgSF molecules are shown as red ovals. Relative sizes of domains are based on structural data.

1.1.2 CD200R Paired Receptor Family

CD200 and CD200R

CD200R was first identified in 2000 as a rat macrophage cell surface receptor which can bind with OX2 (also known as CD200) (another cell surface molecule with a wide tissue distribution which had been known for more than 20 years) (188). This first characterization was rapidly followed by identification of the CD200R family in mice and human (187). Finally, a recent study demonstrated existence of this receptor family in chicken (168) which highlights the importance of this evolutionary conserved receptor family.

CD200R is a type I transmembrane glycoprotein with two extracellular IgSF domains. The membrane distal domain is homologous to the variable (V) and the membrane proximal domain is homologous to the second constant (C2) domains of an immunoglobulin. It is expressed on various leukocyte subsets with minor differences in expression levels between different species (Table 1.2).

	Mouse CD200R	Human CD200R
T cells	moderate	moderate
Th1 clone	none	low
Th2 clone	low	moderate
B cells	low	low
NK cells	none	none
NKT cells	low	low
Monocytes	high	moderate
Granulocytes	high	high
Macrophages	high	moderate
Mast Cells	moderate	low
Dendritic Cells	low	high

Table 1.2 Expression levels of mouse and human CD200R on various leukocytes (This data is based on a previous publication (187) and levels (high-moderate-low) correspond to fold differences in mRNA level expression of these molecules as described in that publication.

CD200R binds to its natural ligand CD200 which is another cell surface protein containing two IgSF domains. They interact through the GFCC' faces, formed by the specific alignment of the beta strands encoded with these letters, on their membrane distal domains and this creates a 4 Ig domain distance between participating cells which fits the conserved intercellular distance during interactions of many IgSF molecules (79). The CD200 gene is located 0.43 to 0.52 Mega bases away from the CD200R family gene cluster in mouse and human genome respectively and it is possible that receptor and ligand were generated by gene duplication and their interaction derived from a homotypic one (79, 187). However, CD200 expression is not restricted to cells of immune system only. Instead, it is expressed on a wide range of cells including vascular endothelium, trophoblasts, dying corpus luteum, follicular dendritic cells, activated T cells, B cells,

smooth muscles, neurons, thymocytes, keratinocytes and glomerulus (11, 25, 140, 182).

This unusual tissue distribution is conserved between species.

The CD200-CD200R interaction

CD200 binds to CD200R with low to moderate affinity of K_D : 0.5 μ M, 2.5 μ M, 4 μ M for human, rat and mouse respectively (80, 187, 188). Nevertheless, this interaction is strong enough to generate inhibitory signals which involve phosphorylation of tyrosine residues in its cytoplasmic tail. Interestingly, the cytoplasmic tail of CD200R does not contain the Immunoreceptor-tyrosine based inhibitory motif (ITIMs) (consensus sequence: V/L/I/SxYxxV/L/I) which is commonly seen in most other inhibitory receptors (47). Instead, CD200R in mammals contain a motif (NPxY) for phosphotyrosine binding domains (PTB). In this way, CD200R can recruit Dok2 to link with other downstream inhibitory molecules such as RasGAP to down-regulate cellular activity (122). In addition to the recruitment of inhibitory downstream signaling elements, Dok2 has recently been shown to recruit another molecule Dok1 which generates a negative feedback loop on inhibitory actions of Dok2 by recruiting crk and crkL (123). This well characterized, unusual downstream signal generated by the CD200R-CD200 interaction is an alternative strategy to ITIM mediated recruitment of SH2 domain containing phosphatases, seen in most other inhibitory receptors. However, it is also noteworthy that CD200R in chicken contains an ITIM domain instead of a motif for PTB domains which may indicate that either mammalian CD200R has evolved from an ITIM containing receptor or the ancestral NPXY containing receptor evolved to an ITIM containing one in chicken (168).

CD200 knock-out mice

CD200 knock-out mice have been used to assess the *in vivo* effects of the CD200-CD200R interaction. These mice have normal life spans and can breed normally. Although they show enlarged lymph nodes, expanded macrophage, dendritic cell and microglia populations indicative of increased cellular activation, the knock-out mice do not spontaneously develop any signs of immunopathology (84). However, when these mice were challenged with experimental settings of infections, trauma or autoimmune conditions, the responses dramatically differed from normal mice. For example, CD200 knock-out mice developed a significantly earlier onset and accelerated response when subjected to facial nerve transection and an increased tendency to develop autoimmune diseases was noted in collagen induced arthritis, experimental autoimmune encephalomyelitis and experimental autoimmune uveoretinitis models (21, 84). Moreover, syngeneic skin grafts, taken from CD200 knock-out mice and transplanted in to normal mice, were rapidly infiltrated by inflammatory cells from the recipient mice which led to the loss of hair on grafted skin, although the graft survived otherwise (140). Lack of CD200 was also tested using the same knock-out mice under infectious settings. In one observation, CD200 knock-out mice developed fatal lung inflammation upon infection with influenza virus (symptoms could be reversed by administration of stimulatory antibodies against CD200R or CD200 Fc molecules) (143, 154). However, an exaggerated inflammatory response induced by lack of CD200 was found to be beneficial in another infection setting where CD200 knock-out mice had increased toxoplasma clearance rate

compared to the wild type mice (51). In summary, various studies on CD200 knock-out mice suggest that the lack of CD200 can be compensated under normal conditions but the immune regulatory role of CD200-CD200R is clearly visible under stress conditions such as induction of autoimmunity, trauma or infection.

Factors that interfere with the CD200-CD200R interaction

The immune inhibition generated by signaling pathways upon the CD200-CD200R interaction is also targeted by various pathogens and neoplasms which try to evade the immune response. Over expression of CD200 resulting in better escape from immune responses has been demonstrated in neoplastic cells of colon, prostate, ovarian, breast, renal cancers as well as melanoma, subtypes of lymphoma, leukemia and some central nervous system tumors (42, 57, 93, 151). The immune suppressive effects of this strategy have been characterized in many studies which highlight the importance of developing targeted therapies on the CD200-CD200R interaction in these neoplasms. Pathogens use a similar strategy to evade from immune surveillance as well. Viral acquisition of host CD200 has been noted for a variety of herpes and pox viruses which is discussed in section 1.2.2.

CD200R Like proteins

The characterization of CD200R in human and mice genomes also showed that these receptor genes were not alone but instead encoded within a cluster of related similar genes (187). In C57BL/6 mouse genome there are three more CD200 Receptor like genes

(mCD200RLa-c or mCD200R2-4) (Figure 1.4), while there is one extra gene in human genome (hCD200RLa). In addition, a fourth gene CD200RLd was identified in mouse genome. But its sequence was partial and thus it is most probably a pseudo gene. CD200 Receptor Like (CD200RL) genes have significant protein level sequence identity to CD200R but they only have a very short cytoplasmic tail which has not been shown to link any signalling molecules (Figure 1.4) (Table 1.5). Instead, the mouse CD200RL's were shown to be capable of pairing with an activating adapter (DAP12) through the lysine residue they carry in their trans-membrane region (173, 187). Although hCD200RLa also contains the key lysine residue in the transmembrane region and therefore is expected to pair with DAP12, previous investigations failed to get cell surface expression of this molecule either with DAP12 or with any other adaptor molecules. Therefore, there is a possibility that this gene is a pseudo gene as well. Despite their high structural similarity to CD200R, CD200RL proteins in mouse do not bind with CD200 with significant affinity. Two of these molecules mCD200RLa and mCD200RLc bound to CD200 with a barely detectable affinity ($K_D > 500 \mu\text{M}$ which is more than 100 fold weaker than the mCD200-mCD200R interaction) in biochemical assays but, although not tested previously, it is unlikely to have physiological consequences (80).

mCD200RLa was found to be expressed on surfaces of monocytes, macrophages, dendritic cells NK cells, NKT cells, basophils and mast cells (both resting and activated but higher on resting) and Th2 subset of CD4+T cells (low). mCD200RLb is expressed on dendritic cells (low), Th2 subset of CD4+ T cells (higher), basophils and mast cells (both resting and activated but higher on activated). There are no expression data for other CD200R like

proteins (187). A recent study also showed that mCD200RLa and mCD200RLb expressions were seen on basophils after helminthic infestation with (*N.brasiliensis*) but not in healthy mice (173).

mCD200RLb is a unique member of this family in several ways. Firstly, it is the most distantly related molecule to CD200R. While other CD200RL's have more than 80% identity to CD200R, this molecule only has 39% (Table 3.3). mCD200RLb is by far the least glycosylated member of the family with only two glycosylation sites compared to at least seven sites in other mCD200RL's and mCD200RLb is also the only homodimeric molecule in the family (99). Lastly, it has eight splice variants some of which can get expressed on cell surface (99). These interesting observations may indicate a distinct role for this molecule.

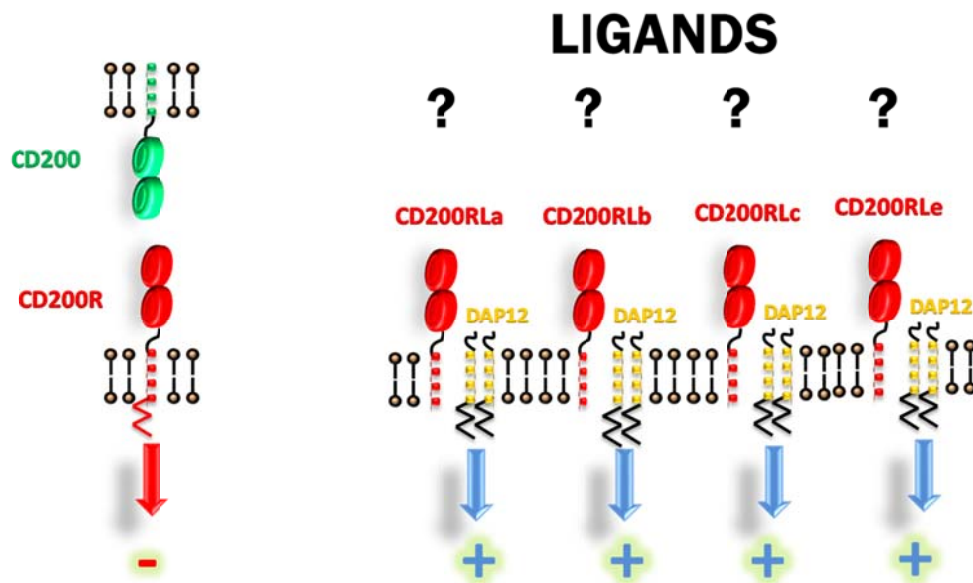


Figure 1.4 Schematic representation of the mouse CD200R family

A few years after the characterization of CD200R family in C57BL/6 mouse, an additional sequence was observed in the cDNA from two other mouse strains (CD1 and NOD1). This sequence was named as mCD200RLe or mCD200R5 (173). This molecule also shares the transmembrane and cytoplasmic domain properties seen in other CD200RL's and pairs with DAP12, however no ligand or surface expression data have been identified for this allele yet. What is particularly interesting about this new allele is its differential expression in different mouse strains. This may refer to a wider heterogeneity in CD200R family, like that seen in Ly49 receptors of NK cells and if that is the case, that observation may suggest an evolutionary pressure (most probably a pathogen) that has been reshaping this family.

1.2 Co-evolution of immune system and pathogens

1.2.1 General overview of self defence against pathogens

A major goal of the immune system is to protect the host from attacks by pathogens. This involves protection against bacterial, viral, fungal and protozoal threats. As the pathogenicity of each microorganism is mediated by different virulence factors, the immune system needs to offer a wide range of protection to cover all these different intrusion mechanisms. The innate and adaptive immune responses therefore work in coordination to overcome different microbial invasion tactics.

The innate immune system

Innate immunity mainly focuses on the common strategies that are shared between various pathogens and offers a non-specific protection against all invaders using those strategies (3). This provides a general first stage protection against many different organisms. Physical barriers like epithelium, stomach acidity, mucous layer e.g. to prevent microbial entry (56); complement activation through cell wall components of bacteria (146), recognition of various non-self pathogen molecules on microbes by pattern recognition receptors preceding phagocytosis and intracellular lysis by macrophages (128); detection and elimination of viral infected host cells by NK cells (172); and generation of inflammation to boost the immune response at the site of infection are amongst many strategies used by innate immune system. Although these protection

strategies are able to wipe out the vast majority of threats even before they actively progress at the desired site of disease, they are not always capable of overcoming very specialized pathogen invasion tactics.

The adaptive immune system

In such cases innate immunity requires assistance from the highly specialized and pathogen specific adaptive immune system. This comprehensive type of immune system is only seen in vertebrates whereas the innate immune system is seen in similar forms throughout the animal kingdom (113). Instead of the germ line encoded receptors of innate immune system, specific only against common microbial molecules, the adaptive immune system contains receptors that can undergo genetic re-arrangements to achieve specificity against millions of different non-self molecules (162, 172). These re-arranged receptors (TCR for T cells and antibodies for B cells) are found on the surfaces of the adaptive immune system cells. Naive T and B cells are found at a low frequency in the body whereas after the introduction of epitopes they are specific for, they rapidly increase in number by clonal expansion.

The adaptive immune system is limited in two ways: Firstly, the process of clonal expansion, where a very small sub population of naive lymphocytes grow into abundant effector cells, requires a gap time of typically 4-7 days which might be too long to combat

some types of infections (119). Secondly, the optimal response against a pathogen needs to be directed according to the nature of the microorganism which cannot be identified by the adaptive immune response (119). Those two major weaknesses of the adaptive immune system make the coordination between adaptive immunity and innate immunity even more important.

This comprehensive nature of the immune system is maintained and controlled by a complex network of regulation and surveillance mechanisms. It is therefore down to this comprehensive machinery to keep cells optimally responsive to pathogens whereas non-responsive to molecules of host and commensal flora.

1.2.2 Immune evasion mechanisms of microorganisms

The immune system uses a variety of different mechanisms with numerous detectors and effectors to achieve perfection in the combat against microbes. However, pathogens continuously evolve different strategies to subvert the host immune response directed against them to enhance the survival of the microorganism. Three of the biggest global infectious threats for humans are HIV, malaria and tuberculosis. Each belongs to a different pathogen group (virus, protozoa and bacteria respectively). They are responsible for 3-6 million world-wide deaths per year (127). This huge death toll implies highly effective immune evasion strategies of these microorganisms. Some evasion strategies are specific to one group of pathogens like the blockade of TAP transporter by viruses;

while some others are shared between different groups of pathogens such as antigenic variation seen in almost all groups (2). These strategies can target innate or adaptive immune responses (Table 1.3). Although examples exist for both categories, strategies targeting innate immunity are more prevalent. This is attributed to a more central role of innate immunity in the immune response as it is the first immune response a pathogen encounters and it is also responsible for triggering the adaptive immune system. Thus disabling innate immunity with various strategies might offer a pathogen a dual advantage as it includes secondary blockade of adaptive immunity (75).

Immune evasion through modulation of Immune regulatory systems

The immune response in vertebrates is shaped by a highly complex machinery, which consists of a wide range of innate and adaptive immune system elements, their signaling and effector pathways and the very complex web of crosstalk between them. The main goal for that complexity is to generate the appropriate immune response, at the required localization and for the duration of infection while sparing the host tissues at the site of immune activation. Regulation of this complexity can only be maintained by a set of immune regulatory mechanisms which in general act to “fine-tune” the immune response (13). This fine tuning involves recruitment and interaction of various activating and inhibitory receptors with their ligands in synapse regions between different immune cells or between an immune cell and another antigen presenting cell. The outcome of these multiple opposing signaling events carried into both cells of the synapse, determine the optimal level of response (105, 189).

The central role of MHC-I in immune activation

The antigen presentation system, involving MHC-I and II molecules, is central to the adaptive immune response. Therefore, it is a target for many pathogens to increase their virulence.

There is growing evidence that pathogens develop virulence factors to hijack critical immune regulation mechanisms and manipulate the final immune response towards their advantage. The first examples of this evasion strategy were characterized while investigating the roles of NK cells in the elimination of virus infected cells of the host. NK cells harbor a wide range of inhibitory and activating cell surface receptors. Activating receptors target pathogenic patterns, tumor specific molecules, stress induced molecules or even some normal self-molecules whereas the inhibitory receptors generally target MHC I molecules (36, 106, 155, 189). Therefore, while NK cells up-regulate their cytotoxic effectors towards abnormal or infected cells which bear activating ligands, they remain silent against normal cells of host which have no activating ligands but have MHC I expression on their surface (106).

The detection of MHC-I by inhibitory NK cell receptors provides a mechanism for recognizing virus infected or transformed cells which reduce their MHC expression to evade from CD8+ T cell cytotoxicity. Stimulation of NK cytotoxicity due to lack of interaction between MHC-I and inhibitory NK receptor is called “missing self” (91, 114)

and provides an additional defense layer. Inhibitory Ly49 and KIR as well as CD94/NKG2A, NKG2D and ILT2 (Ig-like transcript 2) are some of the receptors that mediate the “missing self” activity of NK cells (70, 116). Although the cooperative work of innate and adaptive immune responses, as explained above, looks effective for viral elimination, these microbes rapidly develop a variety of additional virulence factors to overcome host defenses. Human CMV viruses target a weak point in NK and CD8+ T cell communication, which is the differential stimulation of each cell type by a different group of MHC-I molecules. While antigen presentation function to T-cells is carried out by classical MHC-I molecules (HLA-A and B), it is the non-classical MHC-I group (HLA-C and E) that binds the inhibitory NKG2A/CD94 receptors to deliver “self” recognition and keeps them silent (116).

Viral strategies for subverting the NKG2 receptor function

Human cytomegalovirus (HCMV) US2 and US11 proteins specifically bind to classical MHC-I proteins to facilitate their rapid degradation whereas they do not bind to non-classical MHC-I proteins (Table 1.3). Therefore, these molecules selectively extinguish antigen presentation to T cells while maintaining self-detection by NK cells (71). An enhancement to this strategy involves another HCMV protein, gpUL40, which selectively up-regulates HLA-E to ensure NK cell silence while down-regulation of classical MHC-I molecules is being carried out by other viral proteins (160). Even though the exact molecular mechanisms remain unknown, a similar evasion mechanism seems to exist for mouse cytomegalovirus (MCMV), where virus rapidly up-regulates expression of a non-classical

MHC-I (Qa-1^b) in infected salivary gland cells to occupy inhibitory NKG2A/CD94 receptors of CD8+T cells (37). Differential down-regulation of classical MHC-I types is an evasion strategy, also used by viruses other than CMV. Kaposi Sarcoma associated Herpes Virus (KSHV) encodes a multifunctional protein (K5) which is responsible for the down regulation of two important ligands (ICAM-1 and B7-2) for NK cell cytotoxicity in addition to selective suppression of classical MHC-I molecules (HLA-A&B) in infected cells (86, 87). Moreover, HIV nef protein was shown to act in a discriminative manner in down-regulation of only classical MHC-I molecules to avoid both NK and CD8+T cell responses (41). Independent re-emergence of selective MHC-I down-regulation in various virus types, as exemplified above, shows the importance of breaking NK and T cell co-operation in order to increase virus survival.

Selective modulation of MHC-I expression is not the only viral evasion strategy targeting inhibitory NK receptors. Interfering with the balance between opposing signals of activating and inhibitory receptors could shift the final outcome towards a silenced NK cell (Table 1.3). A clear demonstration of this strategy can be seen by analyzing the function of the m152 virulence gene of murine CMV. This gene is one of the three genes (m04, m06, m152) whose products (gp34, gp38 and gp40 respectively) interfere with functional antigen presentation in viral infected cells (92, 138). The strategies used by these genes include binding with surface MHC-I to prevent its interaction with TCR (m04), targeting cytoplasmic MHC-I to lysosomes (m06) and sequestering pre-formed MHC in pre-golgi cellular compartments (m152). These three genes work in a synergistic manner to ensure

evasion from CD8⁺ T cells. But, additional mechanisms to evade NK cell immunity are required. Although the m04 gene, by only interfering with TCR-MHC-I interaction with no decrease in MHC-I expression, offers a promising escape route from NK cell cytotoxicity (92), recent research showed that it is not sufficient (97) (to be discussed in section 1.2.4). Besides, the role of m04 in this three gene cooperation, seems to be dealing with only those very few MHC-I molecules that have managed to escape from the control of m06 and m152 genes which would still mean a decrease in total surface MHC-I and therefore increased susceptibility to NK cytotoxicity. This weakness is overcome via a novel strategy offered by m152 gene. Apart from down-regulation of MHC-I expression, this gene can also suppress surface expression of RAE-1 in infected cells (101, 115). RAE-1 together with H-60 and MULT-1 are high affinity ligands of activating NKG2D receptors on mouse cells and activating signals they generate through NKG2D can be strong enough to override NK cell inhibition maintained by numerous inhibitory receptors (31, 38). These ligands show some level of sequence identity with classical MHC-I molecules and therefore are considered as non-classical MHC molecules. They are up-regulated on the surface of host cells only under abnormal conditions such as stress, infection and transformation to attract NK cytotoxicity. Therefore, down-regulation of RAE-1 expression by m152 gene gives virus a remarkable survival advantage (115). However, a successful viral evasion requires silencers of other NKG2D ligands as well. This requirement is met by two recently characterized MCMV genes. The m155 gene acts as a silencer of H-60, while m138 offers a wider range of ligand down-regulation including MULT-1, H60 and (ϵ) subset of RAE-1 (6, 78, 108). Looking at this complex harmony of genes, it is clear that down-regulation of

both MHC-I and virus induced ligands are strictly regulated at different levels, examples of which are also seen in HCMV viruses.

A totally independent and unique immune evasion mechanism targeting NKG2D signaling exists among pox viruses (Table 1.3). Various members of pox viruses, including monkey-pox, cow-pox and contain an MHC-1 homolog called Orthopox MHC Class I like Protein (OMCP). This soluble protein which is secreted by infected cells and binds with high affinity to NKG2D receptors, was shown to competitively inhibit binding of natural ligands and dramatically antagonize NKG2D signaling. (29). The role of OCMP as an immune evasion protein is also in correlation with the recently identified key regulatory roles of NKG2D mediated signals against pox virus infections (63). In addition to its well documented role as an NKG2D antagonist, it was observed that this molecule is capable of interacting with B cells and macrophages (both cells were shown to lack NKG2D expression) in an independent manner. The target of OCMP on B cells was identified to be FcR like 5 (FCRL-5) receptor which is an immune-modulator that may act as activating or inhibitory in different settings (28). However, functional data on the outcome of OCMP-FCRL5 interaction have yet to be discovered. Lastly, the binding partner of OCMP on macrophages that are naturally expressing neither NKG2D nor FCRL5 might suggest a third receptor.

Pathogen	Virulence Factor	Mechanism of immune evasion	References
HCMV	US2	-Binds and degrades classical MHC Class I molecules involved in antigen presenting	71
	US11		
	UL40	-Facilitates expression of non-classical MHC Class I molecules involved in self detection by NK cell receptors	160
	UL18	-MHC Class I analog (decoy ligand for NKG2A, ILT-2&4)	16,24
	UL16	-Down-regulation of virus induced ligands (MICB e.g.) of activating NKG2D receptors	31
MCMV	gp34	-Binds MHC Class I on cell surface to prevent its interaction with TCR	92,97
	gp38	-Targets cytoplasmic MHC Class I to lysosomes for degradation	92
	gp40	-Sequesters preformed MHC Class I in pre golgi compartments to prevent its surface expression	92,101
		-Prevents surface expression of RAE-1 (a virus induced MHC like molecule which stimulates activating NKG2D receptors on NK cells)	115
	m155 gene product	-Prevents surface expression of H-60 (a virus induced MHC like molecule which stimulates activating NKG2D receptors on NK cells)	78
	m138 gene product	-Prevents surface expressions of MULT-1, H60 and (ε) subset of RAE-1 (virus induced MHC like molecules which stimulate activating NKG2D receptors on NK cells)	6,108
	m144 gene product	-MHC Class I homolog with unknown functionality	17
	m157 gene product	-Structural analog of MHC- Class I (decoy ligand of Ly49I inhibitory NK cell receptor)	9,177
Pox Viruses	OMCP	-Soluble antagonist of NKG2D activating NK cell receptors	29
		-Binds FCRL-5 receptor on B cells to interfere with its regulatory function	28
		-Binds macrophages through an unknown mechanism and carries out an unknown function	29
	MC80R	-MHC Class I analog with unknown function	148

Table 1.3 Summary of MHC-I related viral evasion strategies

Viruses can benefit from the molecular mimicry between some viral proteins and the host MHC-I

The first example of molecular mimicry between some viral molecules and host MHC-I was UL18 which was identified as a MHC-I heavy chain homologue in HCMV (16). UL18 was shown not to be essential for successful viral replication which was strongly suggesting UL18 to be acting as a virulence factor (24). But as it could bind β 2-microglobulin, one of the domains forming MHC-I, it was suggested to have a role such as distorting the MHC-I complex in host cells (16, 24); however following studies proved otherwise (109). Instead, this non-functional MHC-I homologue is responsible for engaging with inhibitory NKG2A/CD94 receptor complex on NK cells to compensate for the virus induced down-regulation of host MHC-I (109). Besides, the same molecule (UL18) can also engage with ILT-2 and ILT-4, other inhibitory receptors detecting MHC-I. These two molecules are expressed on a wide range of leukocyte subsets and therefore targeting them provides a more general immune suppression against virus infected cells (44).

Thus, using acquired viral homologues of MHC-I is an alternative evasion mechanism to the selective down-regulation of classical MHC-I molecules. Screening of genomes of other CMV for MHC-I homologues led to identification of m144 and r144 in mouse and rat CMV respectively (17, 65). Although these two homologues act to down-regulate NK cytotoxicity in similar ways to UL18, specific inhibitory receptors that interact with r144

and m144 remain unknown (17, 65). UL-18, which has less sequence identity when compared to the m144 and r144, is located in a different position in CMV genome (17). UL-18 gene generates a more MHC-I like molecule that can complex with β 2-microglobulin and can even bind and present endogenous peptides (the physiological role is not clear), whilst mouse and rat 144 genes generate a significantly shorter protein without such capabilities (62). These findings strongly suggest that UL-18 and (m-r) 144 genes were independently acquired in CMV genome. Therefore, each receptor might be engaged in different mechanisms to suppress NK cell cytotoxicity which renders investigation of a matching NK receptor for (m-r) 144 proteins even more difficult.

Viral acquisition of host MHC-I homologs is not restricted to herpes viruses. Genome wide studies showed that Molluscum Contagiosum Virus (MCV), a pox virus, contains an MHC-I homolog (MC80R) (148). This is another heavy chain homolog, capable of pairing with host β 2-microglobulin. This complex was shown to be trapped in the endoplasmic reticulum without surface expression. This correlates with the acquired loss of β 2-microglobulin in MCV infected patients suggesting a MHC-I down-regulating role for this molecule.

However, considering the high affinity of host MHC-I heavy chain to β 2-microglobulin and presence of normal MHC-I expression in MC80R transfected cells, this does not seem to be the case (148). Therefore, for the time being, MC80R must still remain among the viral homologs of MHC-I whose functionality is unidentified.

1.2.3 Evolution of the host's counter defense strategies

Considering the ability of pathogens to evolve numerous virulence factors, many of which act in coordination to meet every known challenge the immune system makes against them, it seems difficult to imagine how the host can still survive and maintain a critical balance with these pathogens. The better understanding of how the immune system has evolved to react against that many different immune evasion strategies could be made easier by comparing the differences between resistant and susceptible strains of the same host in terms of dealing with a known pathogen. The investigation of CMV resistance between different strains of mice has, in this manner, provided great insights on host-pathogen evolution.

Past Studies on Differential Resistance to CMV

The first observation of variation in susceptibility to CMV infection among different mice strains dates back to 1974. That study showed that the CBA strain was up to 10 fold more resistant to MCMV (but not to HSV) compared to C57BL/6 and the resistance could be inherited in a dominant fashion (147). Analysis of mice from other strains attributed this difference to presence or absence of specific MHC-I genes. According to that study, resistant strains (C3H and CBA) both possessed the H2^K allele while more susceptible strains such as C57BL/6 and BALB/c contained H2^B, H2^D or other unknown MHC genes and this susceptibility was significantly lost in H2^K containing congenic mice of susceptible background. This clearly demonstrated the key role of H2^K in resistance to CMV infections (39). This observation was later emphasized as the key resistance factor especially for the

Ma/My mouse strain (54), whose NK cells were shown to be responsive to the combination of H2^K and viral gp38 on infected cells (discussed later).

Although the link between H2^K and MCMV resistance was very clear, follow on studies using F1 hybrids of susceptible (C57BL/6 & BALB/c) and resistant (C3H) strains showed that while introduction of H2^K allele in BALB/c mice increased CMV resistance by two fold, the increase in C57BL/6 background was by 8-10 fold. This differential increase in the resistance against CMV infection between two mice strains suggested the existence of non H2 genes which were acting in coordination with the H2 type to generate resistance (74). The resistance to CMV was shown to be mediated mainly by NK cells (149) and this resistance could be transferred by adoptive transfer of NK cells between different mice strains (26). The genomic localization of the non-H2 genes involved in NK cell mediated resistance against MCMV was characterized later and named as *cmv-1*(144). Analysis of this locus together with susceptibility studies on various NK cell subsets from different strains led to the identification of the exact gene (Ly49H) responsible for MCMV resistance (22, 48, 66). At the time of this identification, Ly49H had only recently been characterized in NK cells as the second DAP12 associated activating Ly49 receptor with unknown functionality (72). The link between this activating receptor and resistance to MCMV infection was later revealed by the discovery of a MCMV encoded protein (m157) with ability to bind to Ly49H (9, 153). Although this viral protein had no amino acid sequence similarity to any molecule of known function, tertiary structure predictions suggested a resemblance to MHC-I molecules and thus, this viral molecule probably had evolved as a

decoy ligand in order to bind with an inhibitory NK receptor (like UL-18) to mask the virus mediated down-regulation of MHC-I on infected cells. Indeed, in the MCMV susceptible mouse strain 129/J, activating Ly49H is absent and instead m157 can bind with the inhibitory Ly49I and achieve the desired immunosuppression. On the other hand, the resistant strain C57BL/6 contains a variant form of Ly49I which does not bind with m157 and additionally, this strain possesses an activating Ly49H as a counter defense mechanism to detect this viral escape strategy and respond to it (9).

Pathogen driven evolution of the Ly49H gene

In the light of these observations, a likely evolutionary explanation for the cascade of events could be as follows: After acquisition of m157 by MCMV, the virus started to benefit from this molecule by pairing it with the inhibitory Ly49I^{129/J} (the Ly49I type in susceptible mouse) receptor on NK cells. Under this pathogen-mediated evolutionary pressure, some mice evolved a variant form of the ancestral Ly49I which is fully functional in binding with the host MHC-I but devoid of m157 binding ability and that neutralized the immunevasive advantage of m157 (Ly49I^{C57BL/6}). On the other hand, independent gene duplications and mutations generated another copy of Ly49I from the ancestral gene which in contrast retained the ability to bind with m157 but not with host MHC-I. At the same time, this gene went through a gene conversion event with the activating Ly49D gene resulting in the acquisition of exons coding trans-membrane and intracellular regions of that activating gene. That transformed this Ly49I variant into activating Ly49H which provided extra resistance against MCMV (7, 8). Although it is not possible to know the

exact sequence of events, the above hypothesis suggesting evolution of Ly49H under pathogen pressure was supported by several later studies. The two key studies verifying m157 as the main evolutionary drive behind formation of Ly49H are: one the observation that Ly49H KO mice had no detectable abnormalities other than a significant increase in MCMV susceptibility (40) and secondly the identification of escape mutants in virus m157 occurring after being passaged in a Ly49H(+) host but not in the Ly49H(-) (69, 177).

Escape mutants which either down-regulate m157 expression or generate variant forms that do not pair with Ly49H are rapidly detectable within a few passages in Ly49H(+) and exclusively on m157 allele but none of the neighbors of the same genetic family (m158, m159). Although this clearly indicates the evolutionary pressure the immune system exerts over the virus, one question still remains unanswered: Why does the virus keep this m157 allele in the first place? It could be due to the ability of this molecule to pair with Ly49I, the inhibitory receptor, which is maintained in the genome of susceptible mice (129/J). However, as Ly49I is expressed only on 10% of the NK cells in the 129/J mouse, it does not look likely to be the only reason. It is possible that either m157 is capable of binding to a yet unidentified inhibitory receptor with greater expression compared to Ly49I among wild mice or it exerts a dual function like m152 which makes this gene beneficial despite the obvious disadvantage against Ly49H. A more complicated explanation is that the virus gets a paradoxical benefit by stimulating host immunity. By doing this the virus keeps the host alive and achieves its goal of transforming the infection into latency. This way, virus can spread much more efficiently via transient reactivations

whereas an acute infection only allows a very short time for viral spread before the host dies (69). This paradoxical approach may also help our understanding of the maintenance of Ly49I^{129/j} in the mice genome which has proven to be vulnerable against this viral attack compared to its alternative Ly49I^{C57BL/6}. This susceptible allele might very well have a use in limiting the CMV spread through mice population at the cost of sacrificing the one infected host.

Studies on Ly49 led to the generation of the paired receptor concept.

The role of Ly49-MCMV interaction has given insight into the host-pathogen co-evolution and the role of other activating receptors that are very similar to their inhibitory counterparts in terms of their extracellular regions but signaling in opposite direction. These genetically linked activating and inhibitory receptors are now thought to have evolved from the same ancestor under different evolutionary pressures and are classified as “paired receptors” (105). Inhibitory receptors have long intracellular tails which engage with inhibitory downstream signaling elements; whereas activating ones have a short non-functional tail but they pair with a transmembrane adaptor in order to signal. Genetic analyses on the Ly49 family, showed that inhibitory receptors can convert into their activating pairs by two simple changes: one is the insertion of a premature stop codon to remove the long inhibitory tail and the second is the insertion of a charged amino acid in the transmembrane region, which enables the association of adaptors that contain a matching opposite charged residue at the same place (7).

Phylogenetic analyses showed that inhibitory receptors evolved from a common ancestral receptor around 100 million years ago and diverged by natural selection (for both KIR and Ly49 families). On the contrary, there is no common ancestor for activating receptors suggesting that these receptors evolved independently but in parallel by modifications described above for each order of animal. Therefore, once an activating receptor evolved from its inhibitory counterpart, others quickly followed by combining different extracellular regions with the same signaling domain by gene conversion duplication and recombination (1). This study also showed that these activating receptors are generated periodically most probably under pathogen pressure and rapidly diverged for a while due to their benefit against that threat, but once the pathogen threat is diminished, they are rapidly eliminated from the genome due to other disadvantages they bring such as higher susceptibility to autoimmunity. Therefore, activating receptors are short lived and are generated and eliminated in an episodic pattern over and over rendering the oldest example being younger than 13 and 18 million years old for KIR and Ly49 respectively (1). These active evolutionary dynamics of activating receptors are also the main reasons behind the heterogeneity in numbers and types of receptors between individuals of the same population.

The clear link between m157 and Ly49H suggested that other activating Ly49 receptors have pathogen derived ligands as well; however so far Ly49P is the only other activating Ly49 receptor associated with an increased resistance to CMV infection. This receptor activates NK cells upon detection of gp38 protein (virulence factor encoded by m04 gene

that interferes with the MHC-I-TCR interaction) when it is exclusively bound to H2^K molecule on the surface of infected cell. Additionally, significant resistance requires homozygosity for both H2^K and Ly49P and this mechanism provides the explanation of H2^K mediated MCMV resistance seen in the Ma/My mouse despite not having Ly49H (53, 97).

1.2.4 Host-pathogen interactions in other paired receptor families

Paired receptors that have activating and inhibiting members encoded within the same gene cluster are seen among many cell types of the immune system. Despite the differences in their structure, ligands, cellular distribution and signaling pathways these families engage, similar pathogen driven evolution mechanisms may have played roles in the formation of their activating members (Table 1.4).

Receptor Family	Receptor Name	Host	Function	Pathogen Ligand	Outcome	Reference
Ly49	Ly49I ^{129/J}	mice	inhibitory	m157(MCMV)	Immune suppression	9
	Ly49J ^{C57/BL6}	mice	inhibitory	none	Resistant to m157 mediated immune suppression	9
	Ly49H	mice	activating	m157(MCMV)	Increased MCMV clearance	9
	Ly49P	mice	activating	gp38(MCMV) H2 ^K dependent	Increased MCMV clearance	97
Nkrp1	Nkrp1b	rat	inhibitory	RCTL (RCMV)	Immune supression	174
	Nkrp1a	rat	activating	RCTL (RCMV)	Increased resistance to RCMV	174
NKG2	NKG2A	human	inhibitory	UL18 (HCMV)	Immune supression	109
	NKG2E	mice	activating	Unknown pox virus detection mechanism (Qa-1 ^b) dependent	Increased pox virus clearance	64,133
CEACAM	CEACAM1	human	inhibitory	Opa (<i>Neisseria</i> species)	Immune supression	81, 82, 171
				P5 (<i>H.influenzae</i>)		
				UspA1 (<i>M.catarrhalis</i>)		
				Carbohydrates on <i>E.coli</i> and <i>Salmonella</i>	may modulate bacterial colonization of gut (?)	110, 111
	CEACAM1a	mice	inhibitory	Spike protein (MHV)	High affinity interaction, increasing susceptibility to MHV	59, 60
	CEACAM1b	mice	inhibitory	Spike Protein (MHV)	low affinity interaction decreasing susceptibility to MHV	59, 60
	CEACAM3	human	activating	Opa (<i>Neisseria</i> species)	Opsonin independent phagocytosis of these bacteria	145
			P5 (<i>H.influenzae</i>)			
			UspA1 (<i>M.catarrhalis</i>)			
SIGLEC	Siglec-9	human	inhibitory	Sialylated molecules of GBS	Inhibits neutrophilaction	33,34
				Sialylated molecules of <i>P.Aeruginosa</i>	Inhibition of immune response?	96
	Siglec-1	human	Non signalling	Sialylated molecules of <i>N.meningitidis</i>	Increased phagocytic uptake	45
		mice		Sialylated molecules of <i>T.cruzii</i>	Increased phagocytic uptake	45
		pig		Sialylated molecules of PRRSV	Increased viral entry to host cells leading to productive infection	52
	Siglec-7	human	inhibitory	Sialylated molecules of <i>Pseudomonas aeruginosa</i>	Inhibition of immune response?	96
	Siglec-E	mice	inhibitory	Sialylated molecules of <i>T.cruzii</i>	Down-regulates IFN γ production	61
	Siglec-5	human	inhibitory	Beta protein of GBS	Increased attachment but decreased phagocytosis	132
PIR	PIR-B	mice	inhibitory	Unidentified molecule on <i>S.Aureus</i>	Decreased TLR2 mediated immune activation of macrophages	130
	PIR-A	mice	activating	Unidentified molecule on <i>S.Aureus</i>	unidentified	130
ILT	ILT-2	human	Inhibitory	UL18 (HCMV)	Immune suppression	44
				Unidentified molecule on <i>S.Aureus</i>	Immune suppression ?	130
	ILT-4	human	inhibitory	UL18 (HCMV)	Immune suppression	44
	ILT-5	human	activating	Unidentified molecule on <i>S.Aureus</i>	unidentified	130
CD200R	CD200R	human	inhibitory	K14 (HHV-8)	Immune suppression	68, 103, 150
				R15 (RRV)		
		rat		U-85 (HHV-6/7)		
				e157(RCMV)	unidentified	67

Table 1.4 Summary of the known pathogen-paired receptor interactions seen in various species.

Nkrp1 Family

Nkrp1 is a paired receptor family encoded in NK gene complex (NKC) on mouse chromosome 6 together with Ly49 (191). Apart from their chromosomal proximity, these two receptor families share other similarities such as their C-type lectin-like structure, selective expression on NK cells and disulfide linked homo-dimerization properties. However, despite these similarities, the ligand receptor interactions and functional importance of the Nkrp1 family remained unclear until the recent identification of C-type lectin related (Clr) proteins as their ligands (35, 85). Clr proteins share similar structural features with these receptors and are also encoded in NKC region close to the Nkrp1 genes (135). One of these molecules, clrb (the ligand for the inhibitory Nkrp1b), is expressed abundantly on normal leukocytes whereas it is significantly down-regulated in neoplasms of these cells. Therefore, the interaction between clrb and inhibitory Nkrp1's works in a parallel fashion to MHC-I - Ly49 binding in terms of self-detection. The down-regulation of clrb ligands in tumors causes a "missing self" and relieves the inhibition by Nkrp-1 resulting in NK cell activation (35). A rat CMV C-type lectin like molecule (RCTL), previously identified as a clrb-like molecule (175) before discovery of Nkrp1-clr interaction, was shown to interact with Nkrp1b (174). Upon infection with RCMV, this molecule is rapidly expressed on the infected cell, while the same infection causes downregulation in the clrb expression on the cell surface. Therefore, this viral molecule acts as a decoy ligand to occupy the Nkrp1b receptor in order to mask the effects of clrb down-regulation. Although the exact mechanism for the CMV induced clrb down-

regulation remains unknown, it is likely that it is a part of the host defense to alert NK cells upon sensing the “danger” signal. This immunevasin exerts pathogen-mediated evolutionary pressure onto the Nkrp1b receptor which promotes several counter-defense mechanisms in different rat strains. While some rat strains succeed in overcoming this threat by generating a RCTL resistant Nkrp1b receptor, other strains use an activating Nkrp1a receptor to bind (weakly) to RCTL molecule (174). The effect of pathogen-mediated evolutionary pressure on the host Nkrp1 receptor family can be tracked by comparing Nkrp1 genes of various rat and mouse strains. One observation is that, in contrast to the low differentiation rate in the Nkrp1 family, there is a localized high polymorphism rate in the rat Nkrp1b-a and mouse Nkrp1b-c (mNkrp1c is the homologue of rNkrp1a) which cannot be explained with the non-polymorphic nature of the clrb ligand (36, 77). The degree of polymorphism in activating mouse Nkrp1c receptor is considerably higher than its rat homolog Nkrp1a. Although neither a mouse Nkrp1c ligand nor a MCMV RCTL homolog has been discovered, these observations indicate that mouse and rat both went through this pathogenic threat through evolution and while the mouse could overcome the threat with its higher polymorphisms, counter-defense evolution is yet incomplete in the rat (36). Identification of these interactions makes the Nkrp1 paired receptor family a good evolutionary model to understand host pathogen interactions. Moreover, this unique example may lead to the identification of the roles of other C-type lectin like genes such as r153 in RCMV (174) and several others in various pox viruses.

NKG2 Family

The third paired receptor family encoded in NKC region is the NKG2 family. This family consists of NKG2A, an inhibitory receptor involved in self MHC-I detection (see 1.2.2), and activating receptors NKG2C, E, D (190). NKG2 proteins need an invariant molecule (CD94) to pair with, for surface expression, except NKG2D, an activating homo-dimer pairing with DAP10 which is significantly different from other members of the family (107). CD94 is also encoded in the NKC in close proximity (approximately 100kb) to the NKG2 receptor genes. CD94 knock-out mice, despite their lack of NKG2A, C and E expression, showed no developmental or functional abnormalities in NK cells, other than increased susceptibility to mouse pox infections (64, 133). This was due to the NKG2E/CD94 complex detecting mouse-pox infection in a Qa-1^b (non-classical MHC-I) dependent manner. Peptides loaded only on this non-classical MHC-I upon mouse-pox infection can generate a stimulatory signal through the NKG2A/CD94 complex and activate a NK cell response (64). The resistance mediated by NKG2E/CD94 and Qa-1^b works in a synergistic manner with the previously described resistance mediated by NKG2D against the same pathogen (63, 64). Considering that Qa-1^b is normally loaded only with a small piece of signal peptide sequence of the self MHC-I and is this way the natural ligand for inhibitory NKG2A/CD94 complex, the unknown dynamics that regulate shifting from NKG2A to NKG2E are intriguing; and besides, it is not clear whether it is the viral peptide or virally modified self-peptide that is responsible for this activation. Nevertheless, this finding is a leap forward in identifying the roles of activating receptors in pathogen defense.

CEACAM Family

In addition to the NK cell receptors exemplified above, paired receptor families with wider tissue distributions may also play important roles in mediating host-pathogen interactions. CEACAM (a large paired receptor family) is among this group. Members of this family are expressed on a variety of leukocytes as well as endothelium and epithelium in most parts of the body and engage in different roles including cell adhesion, immune regulation, tumor suppression and embryogenesis. CEACAM1, the prototype of the family, is an inhibitory receptor which engages in both homophilic and heterophilic (with CEACAM8) fashion to regulate interactions within leukocyte groups as well as between leukocytes and epithelial cells (88, 180, 181). This widely distributed inhibitory receptor is also targeted by many pathogens as a part of their immune evasion strategies. Pathogenic *Neisseria*, *H. influenzae*, *M. catarrhalis* bind to human CEACAM1 using opa, P5 and UspA1 proteins respectively (81, 82, 171). Among these molecules, opa causes activation arrest in CD4+ T cells through interacting with CEACAM1 (19). The interaction of either opa or UspA1 with CEACAM1 on pulmonary epithelium downregulates the TLR-2 mediated generation of the antibacterial response (152). Although CEACAM1 mediated downregulation of immune responses by these immunevasins clearly provides survival advantage for these bacteria, most infections cause asymptomatic colonization while only a small percentage can lead to severe disease. This observation strongly suggests the existence of some counter defense strategies. One of these strategies could be *in vivo* targeting structurally similar activating pairs of CEACAM1. Recently one of the activating members of CEACAM family (CEACAM3) was shown to bind with all these immunevasins

and stimulate phagocytosis of these bacteria in an opsonin independent manner (145).

Considering that CEACAM3 is not engaging any homo or heterophilic interaction with other members of the family and that no endogenous ligand has been identified for this receptor yet, it is likely that this receptor has evolved as a counter-defense strategy against these pathogens.

Apart from the key role of this paired receptor family in maintaining the immune response against those pathogens which are exclusively seen in human, there are other reports of CEACAMs being targeted by pathogens in various species. For example, several members of CEACAM family have been shown to bind strains of *Escherichia coli* and *Salmonella*, obtained from human specimens, through interaction of carbohydrate residues (110, 111). Although no data on the functional outcome of this interaction have been provided, it is thought that this interaction helps modulation of bacterial colonization in gut. Lastly, CEACAM1 with its two homologs in mice (CEACAM1a and CEACAM1b) acts as the receptor for Murine Hepatitis Virus (MHV) (59, 60). MHV Spike (S) protein can bind to CEACAM1 and this interaction facilitates the membrane fusion events which lead to viral entry.

Among these two isoforms of CEACAM1 in mouse, strains that are homozygous for CEACAM1a are more susceptible compared to strains homozygous for CEACAM1b. This is partly due to the significantly lower affinity of CEACAM1b to MHV, which suggests a pathogen-mediated evolution of host's counter defense strategy. However, studies on engineered mice clearly show the contribution of other unknown host factors to the differential susceptibility as well (83).

The CEACAM family, which has well documented interactions with many different microorganisms, shows clear characteristics of pathogen-mediated evolution of paired receptors. This phenomenon is also supported by a phylogenetic analysis on 20 different mammalian species for the distribution and variation of CEACAM genes which demonstrates the rapid evolution of the family with multiple gene duplication and gene-conversion events resulting in significant differences in numbers, types and sequences of these genes even among very closely related species (90).

SIGLEC Family

Siglecs (sialic acid binding immunoglobulin like lectins) are a family of cell surface receptors expressed on wide range of immune cells. This family can be divided into two groups with regard to their evolutionary conservation and structural similarity. The first group contains distantly related Siglecs such as Siglec-1, myelin associated glycoprotein, CD22 and Siglec-15. They have 25-30% sequence identity but orthologs of this group are seen in all mammals (45). The second group, CD33 related (CD33r) Siglecs, consists of proteins that have higher structural similarity but are more rapidly evolving as well (30, 45). Nine CD33r Siglecs exist in human, eight of which bear ITIM residues in their cytoplasmic tails and are therefore inhibitory while one member (Siglec-14) can pair with DAP12 and act in an activating fashion (45). This paired receptor family is specialized in detecting sialic acid residues found attached to surface proteins of all host cells. They were first observed in cis- interactions binding sialylated proteins on the same cells in which they are expressed (137), but later they were found to be capable of making trans

interactions with sialylated proteins of other cells or surfaces in a competitive fashion (34). As sialylated surface proteins are characteristic of mammalian cells and seen in only a small percentage of microbes, detection of these proteins by inhibitory Siglecs is seen as a part of self- detection and hypo responsiveness to own cells. However, around 20 pathogens are known to benefit from the role of sialylated proteins in self-detection. Pathogens gained access to sialic acid by many different strategies. While only a few of them can synthesize these complex carbohydrates *de-novo* or from host originated substrates, others scavenge them from host cells (170).

The direct interaction of sialylated pathogen surface molecules with Siglecs has recently been shown. These include Group B *Streptococcus* (GBS) (binds with Siglec-9 and inhibits neutrophil action) (33, 34), porcine reproductive and respiratory syndrome virus (binds with Siglec-1 and enters macrophages) (52) , *Pseudomonas aeruginosa* (binds with Siglec-7&9) (96) and *Trypanosoma cruzi* (binds to mouse Siglec-E and down-regulates IFN γ production) (61) are among examples of pathogen-mediated immune evasion strategies through mimicking sialic acid –Siglec interaction. Although pathogens may benefit from sialic acid usage in various other ways such as evading the alternative complement pathway, avoiding contact with host cells by coating themselves with these negatively charged molecules or just utilizing this complex carbohydrate as a nutrient; the only explanation for the identical mimicry of host sialic acids is to interact with Siglec receptors (34, 45, 170). However, it is also noteworthy that the host may use Siglec-1 (non-signaling)

to trap some of the sialylated pathogens and facilitate phagocytosis of these microorganisms probably as a counter defense strategy (89, 126).

In addition to many examples of mimicry of ligand-receptor interaction, Group B streptococci are able to interact with human Siglec-5 in a sialic acid independent manner (32). This interaction is mediated by the beta protein of Group B Streptococci through amino acid residues that are not involved in IgA detection of the same molecule and causes decreased phagocytosis of the bacteria despite the increase in bacterial attachment (32, 132). This finding clearly shows that mimicking ligand-receptor interaction is not the only way to stimulate a receptor and is therefore, crucial in understanding the evolution of paired receptors.

Functional and genomic analysis of the Siglec family puts them in a unique place among all paired receptor families. One of the main reasons for this claim is that Siglec family started to be included as a paired receptor family only after the discovery of Siglec-14 (the only activating receptor in CD33r Siglecs). This receptor bears 99% amino acid sequence identity with the chromosomally nearby located Siglec-5 making them activating and inhibiting pairs of each other. There is clear evidence that each primate line including human has undergone numerous repetitive gene convergence events to achieve this level of sequence identity. It is quite possible that Siglec-14 duplicated from Siglec-5 under pathogen pressure and this pair evolved in a concerted fashion (5). However, there are no data whether Siglec-14 binds pathogens, therefore this puzzle remains unsolved. The rest

of the CD33r like Siglecs are thought to have gone through a different evolutionary process. Although there are several hypotheses to explain the evolutionary history of this polymorphic family, the most widely accepted is that it is mediated by two interdependent primary “Red Queen” effects (45, 167). First, the evolutionary pressure created by invasive pathogens binding host sialic acids to help their attachments which forces host sialome to change. Second, the evolutionary pressure created by pathogens using sialic acids to bind with inhibitory Siglecs which forces host Siglecs to change. These two separate primary Red Queen effects causing continuous evolution of receptor (Siglec) and the ligand (sialome) generate another evolutionary pressure on both of them to adapt to the change of the other which is called as the secondary Red Queen effect.

PIR Family

Paired Ig like Receptors family consists of one inhibitory receptor (PIR-B) and six activating receptors (PIR-A) in mouse (158). They are coded in a gene cluster and there is more than 92% amino acid identity between ecto-domains among members of the family (102, 130). They are expressed on DCs, macrophages and mast cells in a PIR-B dominant fashion and additionally PIR-B is also exclusively expressed on B cells. Both activating and inhibitory PIRs recognize MHC-I types (classical and non-classical) and opposite signals they generate, maintain a critical balance for optimal responsiveness of immune cells (129, 158). PIR-B was shown to play a role in various immune regulatory functions such as DC maturation, maintaining a hypo-immune status of resting immune cells, prevention of

exacerbated Th2 responses etc. while the precise contributions of PIR-A to these functions are not known.

Apart from these immune regulatory functions, recently PIR-B and one of PIR-A receptors (PIR-A1) were shown to bind *Staphylococcus aureus* and these interactions caused a decreased TLR-2 mediated inflammatory response in macrophages (130). Therefore, this is an immune evasion strategy used by that bacteria. It was also shown that PIR has different binding sites for MHC-I and *S. aureus* which suggests that pathogens may target not only the ligand binding site but also different parts of the receptor to achieve the same result. In addition to *S. aureus*, PIRs were bound with *H. pylori* and *E. coli* but not to several other Gram+ and Gram- bacteria. Moreover, some but not all of these bacteria could also bind to human PIR orthologs (ILT-2 and ILT-5). Although the PIR-A1 –bacteria interaction caused an increased response under experimental settings, it may not be enough to counterbalance the pathogen threat on real macrophages. The exact nature of this interaction is not known yet. However, these findings support a pathogen-mediated evolution in this paired receptor family.

CD200R Family

After reviewing all these paired receptor families in the context of host pathogen interactions, clues of pathogen driven evolution could be more easily tracked in CD200R family. Members of this family are also encoded in gene clusters where activating and inhibiting receptors lie in close proximity to each other. The inhibitory receptor is coded

by a single gene while activating genes may exist in multiple copies. For example, the mouse CD200R family in the C57BL/6 strain consists of one inhibitory (mCD200R) and three activating (mCD200RLa-c) genes plus a pseudogene CD200RLd with an incomplete sequence (187). Additionally, a fourth potential activating gene (CD200RLe) was observed in CD1 and NOD-1 mice but not in C57BL/6. This may suggest heterogeneity in numbers and types of the CD200R family genes as seen in many rapidly evolving paired receptor families. Activating members share 39%-91% amino acid identity with the inhibitory CD200R in their extracellular domains which is a sign of common evolutionary origin for all these genes (Table 1.5). However despite the significant similarity, none of the activating members can bind to CD200 (the natural ligand of the inhibitory receptor) at a comparable affinity; and yet no other endogenous ligand has been characterized for these receptors (80, 187). These observations may indicate that the activating members have evolved under pathogen driven evolutionary pressure, as hypothesized for many other paired receptor families. A supporting evidence for this claim is the characterization of viral CD200 homologues from different viruses which try to benefit from the suppression of immune response by mimicking the CD200-CD200R interaction (Figure 4.2). All viral CD200 homologues that have been characterized so far exist in genomes of either Herpes or pox viruses with one exception which is seen in duck adenoviruses (68). Independent emergence of this immune evasion strategy in viruses of different backgrounds shows the importance of CD200-CD200R pathway in downregulation of immune responses.

A B C C'

C'' **D** **E** **F** **G**

Rabbit CD200R or RL9D
Rabbit CD200R
hCD200R
hCD200RLa
Rat CD200R
mCD200R
mCD200RLa
mCD200RLb

T I E L T K I I N C T D E R I T W A S R P D V I P E L Q I H P V A L S H D G Y Y R C Q M A T F Y G I F D H - Y R L Q V S V C P E
G T N E L T K I I N C T D E R I T W A S R P D V I P L Q I H P V A L S H D G Y Y R C Q M A A S N G I F D H G Y R L Q V S V C P E
E T N E L T K E T N C T D E R I T W V S R P D Q I S D L Q I R T V A L T H D G Y Y R C I I V T P D G I F H R G Y H L Q V L V T P E
E T N E L T K E T N C T V E R I T W V S R P D Q I S D L Q I R R V A L T H D G Y Y R G I I V T P D G I F H R G Y H L Q V L V T P E
D T R E L T S I N C S D R S I T W A S R P D L A P D L Q I S A V A L T H G G R Y S G I A V P D G N F Q N I Y D L Q V L V T P E
D T K I T N E L S C L G R N I T W A S T P D L Q I S A V A L T H G E G T Y T C E T V T P E G I F E K N Y D L Q V L V T P E
D K K I T N E L S C L G R N I T W A S T P D S P E L Q I S A V A L T H G E G T Y T C E I V T P E G I F E K N Y D L Q V L V T P E
E L Q I S K K I C T E R G T I R V P A H H Q S S D L P I K S I A L K H D G H Y S C R I E T T D G I F E R H S

A B C C' E F

Rabbit CD200R or RL V T L S P V K N R T A V C K A V A G K P A A Q V S W A P V G D C V T E Q E H W D N G I N G T V T V W S T H W F D - S I V S A V T C S V S H
 Rabbit CD200R V T L S P V K N R T A V C K A V A G K P A A Q V S W A P V G D C V T E Q E H W D N G I N G T V T V W S T C H W F D S I V S A V T C S V S H
 hCD200R V T L F Q I R R T A V C K A V A G K P A A Q A S W I P E I G D C F A T K Q E Y W S N G T V T V K S T C H W F E N V S V T C H V S H
 hCD200R_{La} V T L F Q S R R T A V C K A V T G K P A A Q I S W I P E I G S L A T K Q E Y W S N G T V T V K S T C P W E G H K S T V T C H V S H
 Rat CD200R V T H F P E K N R S T A V C E A I A G K P A A Q I S W T P D G D C V T E S E S H S N G T V T V R S T C H W E Q N V S D V S C L V S H
 mCD200R V T H F P E K N R S T A V C E A I A G K P A A Q I S W T P D G D C V T E S E S H S N G T V T V R S T C H W E Q N V S D V S C L V S H
 mCD200R_{La} V T H F P E K N R T A V C E A I A G K P A A Q I S W T P D G D C V T E S E S H S N G T V T V R S T C H W E Q N V S D V S C L V S H
 mCD200R_{Lb} - I Q V P G E I R T V V C E A I A S K P A M Q L S W T P D E D C V T K S I - K S R I D T M I V R S K C H R E K I N G S V F C F L S H

Rabbit CD200R or RL² C G I K S I S Q R L L P - - - - -
Rabbit CD200R L C - G N I K S L S Q I L L P V T D P K G T S H
hCD200R L C - G N I K S L S Y E L L P V P G A K K S A
hCD200RLa L T - G N I K S L S V K I I S G L R T S G S P A
Rat CD200R L T T G I I Q S L S I E L G R G G D Q L L G - -
mCD200R L T - G I I Q S L S I E L G R G G I I Q S L R - -
mCD200RLa L S T - G I I Q S L S I E L S Q G I T M T P R S -
mCD200RLb L T - G I I W L S I E I Q I R G T T S I L P S -

51

Viral homologues of CD200

The K14 ORF of HHV-8 (KSHV) was the first characterized vCD200. K14 has a relatively low (40%) amino acid sequence identity to human CD200 but still engaged with human CD200R with an indistinguishable affinity to that of the host hCD200 protein (68). The K14-hCD200R interaction led to a decrease in macrophage activity as shown by downregulation of the TNF- α production (68). This is obviously a clear advantage for viral survival. Moreover K14 is transcribed together with a constitutively active G coupled receptor (ORF74) in a bicistronic manner. The ORF74 mediates a series of the events leading to tumor formation (Kaposi Sarcoma) (125). Thus the co-expression of K14 with ORF74 may downregulate macrophage activity at the tumorigenesis site.

The human CD200R family contains one activating member which could not be expressed on cell surface in experimental settings (187). Nevertheless, using a recombinant chimeric protein, this activating receptor cannot bind with K14 (unpublished observation). This rules out the possibility that hCD200RLa can act as another K14 receptor to counterbalance the immune suppression this virus creates. Even so, this receptor most probably evolved in response to pathogen pressure (either K14 or something else). If it evolved against K14, it can be suggested that the virus escaped from this counter-defense strategy by critical changes in K14 and this led to the process that has gradually turned hCD200RLa into a pseudogene which is at the edge of elimination. However hCD200RLa might have evolved against another unknown pathogenic threat or might still have an unidentified function.

The Human herpes virus 6 and 7 (HHV-6, HHV-7) also contain a viral CD200 (U85) in their genome. These molecules are both 24% identical to human CD200 and like K14, they can bind with hCD200R (150). The R15 protein is another vCD200 homolog in Rhesus Rhadinoviruses (a very close relative to HHV-8). The amino acid sequence of R15 has 28% identity to K14 and 30% identity to hCD200. This protein, also binds to hCD200R. The interaction causes a 51% decrease in TNF secretion from the THP-1 cell line (human monocytic leukemia cell line) and a more dramatic 82% decrease in primary rhesus macrophage cells (103).

All these four viral CD200 homologs in herpes viruses cause similar outcomes despite their low sequence identities to hCD200 and their different viral origins. Analyses of virus genomes show that U85, seen in beta herpes viruses (HHV-6/7) and K14-R15, seen in gamma herpes viruses (HHV-8, RRV) are coded in different locations in the genome (68). It is also estimated that these two virus subtypes (beta and gamma) diverged approximately 200 million years ago (118). Therefore, these viral CD200 homologues were probably acquired independently by these virus subtypes.

e127 is another CD200 homologue encoded in English strains of RCMV. It codes for a transmembrane protein with 56% amino-acid identity to rat CD200 (176). It can get expressed on infected cells and bind to rat CD200R with the same affinity seen in rCD200-rCD200R interaction (67). However, this study observed no change in macrophage activity with regard to e127 presence in RCMV virus. It is possible that changes in macrophage

activity were too subtle to detect or that a counter-defense mechanism exists and neutralizes the pathogen effect.

Viral CD200 homologues were also identified in the genomes of many members of pox viruses. These homologues have less sequence identity (around 20-23%) to their host's CD200 and there is limited information on their expression or functional importance. The M141R of myxoma viruses with sequence similarity to N terminal domains of CD200 is the most studied one. This gene codes for a cell surface molecule with a single IgSF domain. It has been shown that viruses that contain M141R, have significantly increased virulence in susceptible European rabbits compared to M141R KO virus (27). A follow on study also demonstrated the downregulation of mouse macrophage activity exclusively by M141R+ virus in *in vitro* settings. However, despite the well characterized effect M141R has over immune system, a clear biochemical explanation on how it mediates these functions does not exist. Therefore, this molecule has yet to be shown whether it acts as CD200 homologue or interacts with another receptor. Characterization of that interaction may help identification of the roles of other CD200 like molecules in various pox viruses.

Conclusions on pathogen driven evolution of the CD200R family

CD200R-pathogen interactions and their contributions to the evolution of this family are puzzling in many aspects. The major questions are focused on the mouse CD200R family which contains the highest degree of variability but yet there is no identified driving factor for that. The viral CD200 homologues discussed above bind CD200R in a discriminative fashion and none could interact with any of the CD200R family members in mouse. Other than that, there has not been any vCD200 gene identified among viruses that infect mouse. Perhaps clear identification of the role of M141R (vCD200 of rabbit pox viruses) which has an unexplained functional effect on mouse cells may reveal a wider picture in this field. Additionally, the lack of a viral CD200 engaging with members of mouse CD200R family may suggest that pathogens other than viruses might be engaging with these receptors in a manner different from ligand-receptor interaction. Alternatively, although unlikely, these receptor like molecules might have evolved for different regulatory roles other than counterbalancing the pathogen threats. Moreover, the role of hCD200RLa is another question mark. Is that really an evolutionary relic on its way to elimination or does it have a use at present? All these unknowns emphasize the requirement of in depth research in the evolution of this receptor family. Such investigations may have outcomes that could help our understanding of the evolution of paired receptor families in general.

Aims of this Thesis

Most paired receptor families, as discussed in detail above, diverge under pathogen driven evolutionary pressure. In this co-evolution mechanism, the host either continuously generates activating receptors to counterbalance the pathogen threat over the inhibitory receptor, or generates isoforms of the inhibitory receptor that bind the natural ligand but are resistant to the virulence factors of the pathogen. The CD200R receptor family with various number of activating receptors in different species which do not bind the natural ligand of the inhibitory receptor, show signs of pathogen driven diversification. However, clear evidence is lacking.

Two main foci of the thesis are:

- 1- Analysis of heterogeneity of genes in different mice strains. This may help identification of different diversification rates between activating members and give clues on how they evolve.
- 2- Direct analysis of whether members of CD200R family and other paired receptors bind to pathogen molecules. This screening may help revealing examples of pathogen driven evolution of this paired receptor family.

This thesis also aims to characterize specific monoclonal antibodies that discriminate between highly similar members of the mouse CD200R family. These specific reagents will help identification of the tissue distribution of these molecules. Besides that, these novel antibodies might be used in various future studies to analyse the functions of these receptors.

Chapter 2:

Materials and methods

2.1 Reagents and solutions

2.1.1 General reagents

Water: DNase, RNase, Protease, free 0.1µm filtered sterile water was purchased from Sigma and used sterile for PCR.

Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄ pH 7.3 Tablet form (Oxoid Ltd) was prepared by dissolving 1 tablet in 100ml ddH₂O. Sterile PBS was obtained in 500ml bottles from Gibco. PBS was used in a range of cellular and biochemical experiments.

PBS/BSA: PBS containing 1% (w/v) bovine serum albumin (BSA) (Sigma Aldrich), used in ELISA, flow cytometry (10mM NaN₃ added) and western blot

PBS/FCS/Azide: PBS containing 5% (v/v) Foetal Calf Serum (PAA) plus 10mM NaN₃ used in flow cytometry.

PBS/EDTA: PBS containing 2mM EDTA (Ethylenediaminetetraacetic acid), used sterile in tissue culture.

PBS/Trypsin: PBS containing 10% (v/v) 10x TrypLE Express (Invitrogen), used sterile in tissue culture.

Fixation Buffer: 2% paraformaldehyde in PBS, used to fix pathogenic bacteria.

Tris Buffered Saline (TBS): 10 mM Tris pH 7.5, 150 mM NaCl used in various molecular techniques.

Tris Acetate EDTA (TAE): 40 mM Tris acetate, 2 mM Na₂EDTA.2H₂O, pH 8.5, used as electrophoresis buffer.

Tris Borate EDTA (TBE): 89 mM Tris-borate and 2 mM EDTA, pH 8.3, used as electrophoresis buffer.

6x Agarose Gel Loading Dye: 10 mM Tris-HCl (pH 7.6), 0.15% (w/v) orange G, 0.03% (v/v) xylene cyanol FF, 60% (v/v) glycerol, 60 mM EDTA.

Enzyme Linked ImmunoSorbant Assay (ELISA) substrate buffer: A set of SIGMA FAST p-nitrophenyl phosphate (Sigma Aldrich) tablets were dissolved in 5 ml ddH₂O to obtain a 1.0 mg/ml p-nitrophenyl phosphate, 0.2 M Tris buffer, and 5 mM magnesium chloride solution.

Tris EDTA (TE): 10 mM Tris:HCl pH 8.0, 1 mM EDTA used to store DNA.

2x SDS PAGE sample buffer (non-reducing): 0.5 M Tris:HCl pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue used in protein gel electrophoresis.

20x SDS PAGE running buffer: 50 mM MOPS pH 7.7, 50 mM Tris base, 0.1 % w/v SDS, 1 mM EDTA .

Coomassie Blue Stain: 2gr/L Coomassie blue, 10% (v/v) glacial acetic acid, 50% (v/v) methanol.

Coomassie Blue de-stain: 10% (v/v) glacial acetic acid, 50% (v/v) methanol.

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

Western blot blocking buffer: 1 % BSA (w/v) in TBS.

Western blot wash buffer: 0.05 % v/v Tween 20 and 0.2 % v/v Triton X-100 in TBS.

Hepes Buffered Saline (HBS): 10 mM Hepes pH 7.4, 150 mM NaCl, 0.02% NaN₃.

HBS for BIAcore (HBS-B): HBS, 3.4 mM EDTA, 0.005% surfactant P20.

Substrate buffer for Hexosaminidase Assay: 34mg p-nitrophenyl N-acetyl β -D glucosaminide. (Sigma) and 0.2% (v/v) Triton-X-100 in 20ml 50mM Sodium Citrate, pH 4.8.

Stop buffer for Hexosaminidase Assay: 33mM glycine, 83mM Na₂CO₃, 67mM NaCl.

2.1.2 Bacterial growth media

Competent *E.coli* (Alpha-Select bronze efficiency), purchased from Bioline were used for transformation of various DNA constructs. Among the pathogenic bacteria, used in bacterial binding assays (chapter 5), *N.meningitidis* (Serotypes A and C) were given by Dr. Nigel Saunders and *S.aureus*, *S.Pneumonia*, *H.influenzae*, *E.coli*, *P.aeruginosa* were given by Dr. David Greaves. These pathogens were grown in either blood agar or chocolate agar (VWR international).

Lysogeny broth (LB): 10 g/L NaCl, 1.0% (w/v) bactotryptone and 0.5% (w/v) yeast extract solution in ddH₂O, pH 7.5, was obtained from departmental store and used to grow bacteria. (50-100 µg/ml ampicillin was used for selection of construct containing bacterial clones)

LB Agar: 15g agar was melted in 1 L LB-Broth and 50-100 µg/ml ampicillin was added before the media solidifies.

Super optimal broth with catabolite repression (SOC): 2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 8.56 mM NaCl, 2.5mM KCl ,10mM MgCl₂, 20mM glucose solution in ddH₂O was used in transformation of competent bacteria

2.1.3 Cell culture media

RAW.264.7, 2B4 T-Cell hybridoma, 293 T HEK, Phoenix Eco, NIH3T3 , CHO cell lines were grown in DMEM (PAA) supplemented with 10% heat inactivated FCS, 50 U/ml penicillin (PAA), 50 µg/ml streptomycin (PAA), 0.0004 % (v/v) 2-mercaptoethanol, 2mM L-Glutamine. RBL.2H3 cell line was grown in RPMI (PAA) supplemented with the same ingredients as DMEM. Selection of stable cell lines were made by supplementing the normal growth media with 1 µg/ml puromycin (Sigma) or 500 µg/ml G418 (Sigma) or 300 µg/ml hygromycin-B (Sigma) depending on the transfected vector. Stable cell lines transfected with two different constructs were selected using the relevant combination of these selective agents at the concentrations given above.

CHO-K1 cell line transfected with constructs in PEE-14 vector were selected using CB2 media supplemented with 5% Dialysed FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 25 µM L-methionine sulfoximine (MSX).

Antibody-producing hybridoma clones were grown in serum free Hybridoma-SFM (Gibco) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 0.0004 % (w/v) 2-mercaptoethanol, 2mM L-Glutamine, 0.1 mM nonessential amino acids (Gibco) and 1 mM sodium pyruvate.

Soluble protein secreting transfected 293T cells were grown in serum free X-VIVO 20 (Lonza) supplemented with 50 U/ml penicillin (PAA), 50 µg/ml streptomycin (PAA), 2mM L-Glutamine.

2.2 Nucleic acid methods

2.2.1 PCR

PCRs for confirming the existence of the desired segment within a vector were made using PuReTaq Ready-To-Go™ PCR Beads (GE-Healthcare).

PCRs for generating long (>3kb) fragments out of mice genomic DNA were made using Expand Long Template PCR system (Roche).

PCRs for screening of mCD200RLe in the genomic DNA of various mice strains were made using KOD Hot Start (Novagen) kit, due to its observed superiority in specifically detecting this gene in preliminary experiments.

PCRs for cloning a fragment out of cDNA were made using Titanium Taq Polymerase (Clontech) due to its high specificity.

PCR for sub-cloning a fragment between two vectors were made using Pfu Ultra (Agilent) due to its low error rate.

RT-PCRs were made using SuperScript® III First-Strand Synthesis System (Invitrogen).

Oligonucleotides for PCR reactions were designed in a way that forward and reverse primers have approximately similar melting temperatures. For this purpose $[4(G+C)+2(A+T)=T\text{-melting}(^{\circ}C)]$ formula was used. (A,T,G,C represent the matching adenine, thymine, guanine, cytosine nucleotides respectively.) Designed oligonucleotide sequences were confirmed using Vector-NTI software and synthesized by Sigma.

All PCR reactions, mentioned above were carried out using manufacturer's guidelines.

2.2.2 Nucleic acid isolation

Competent *E.coli* were transformed with a plasmid, the transformed *E.coli* were then used to selectively amplify this plasmid of interest (first by overnight incubation on selective LB-agar at 37°C and then by another overnight incubation of a single colony from the LB-agar

plate in selective LB-broth at 37°C). Harvested bacteria were processed using plasmid isolation kits (Qiagen) according to the guidelines. Quantitation of the isolated plasmids and analysis of their DNA purity were made NanoDrop ND 1000 spectrophotometer.

RNA isolation from eukaryotic cells (both cell lines and primary cells) was carried out using RNeasy kit from Qiagen according to standard protocols.

RNA stocks were stored at -80°C and DNA stocks were stored at -20°C

2.2.3 Agarose gel electrophoresis and gel purification

For size-based separation of DNA fragments, the agarose gel electrophoresis technique was used. For separation of fragments >200 base pairs, 40ml agarose gel at 1% (w/v) concentration was cast using TAE buffer and 0.5-1µg/ml ethidium bromide for visualization of DNA. 60ml running buffer (1xTAE) was poured after gel solidified in the electrophoresis tank. Samples mixed with loading dye in 5:1 ratio were loaded to the wells and run at 100 volts for 1 hour. For separation of fragments <200 base pairs, agarose gel at 2.5% (w/v) concentration was cast using TBE buffer which was also used as running buffer. Samples were run at 100 volts for around 2 hours. When required, gel bands were cut out under UV light and DNA fragments were re-purified using a Qiagen gel purification kit.

2.2.4 Constructs

Restriction digestions and ligations

All cloning and sub-cloning experiments were made using sticky end cloning strategy, in which both fragment to be cloned (insert) and the vector were cut with compatible restriction enzymes (Roche and New England Biolabs). Vectors were subsequently treated with calf intestinal alkaline phosphatase (Invitrogen) to prevent self ligation during ligation step. Both vector and insert were run in agarose gel electrophoresis. Gel bands representing cut vectors and inserts were gel purified. Ligation of insert to vector was made using T4 ligase (Invitrogen). Different vector/insert ratios were tried in the ligation step. Following ligation, reaction mixture was used to transform bacteria. All procedures described above were done following manufacturer's guidelines.

DNA sequencing

Sequences of inserted fragments were confirmed by bi-directional sequencing using vector specific primers. 6µl/sample reaction mix containing 200-500ng of the construct plus 3.2pmol primer (either forward or reverse) were sent to Source-Bioscience (Oxford) for sequencing. Results were analysed using contig and align functions of Vector-NTI (Invitrogen) software.

pEF-BOS Mammalian expression vector based constructs

pEF-BOS is a mammalian expression vector with the size of 5.8kb. It contains a Simian Virus 40 (SV40) origin of replication site and the promoter region of elongation factor 1 α (EF-1 α) gene (124). When transfected into 293T cells, a human embryonic kidney cell line transformed with temperature sensitive large T antigen of SV40 virus, T antigen specifically binds to SV40 origin of replication site on the vector and causes enhanced replication of the plasmid (55). This results in a high copy number of plasmids in the target cell line. EF-1 α gene, whose promoter sequence is encoded in pEF-BOS vector, is one of the most abundantly transcribed genes in all eukaryotic cells and it was shown that the promoter sequence provides enormous contribution to this high transcription level (164). Therefore, this promoter is responsible for the high transcription level of the cloned gene in pEF-BOS vector.

A modified version of pEF-BOS vector which already had rat CD4 leader sequence, rat CD4 domains 3- 4, and biotinylation site sequence for BIR-A enzyme inserted was used in cloning experiments (23) (Figure 2.1 A).

For cloning full length genes of interest, modified pEF-BOS vector was cut using XbaI and BamHI restriction sites to get rid of the already inserted fragment (rCD4 leader, rCD4 d3-4,

biotinylation sequence). Full length mCD200RLa and mCD200RLb, mCD200RLc fragments were amplified from previously made construct in other vectors. mCD200RLc and mouse PIR-A1 were amplified from cDNA generated from peritoneal exudate cells of C57BL/6 mice. All PCR amplifications were made using forward primers with XbaI cutting site plus Kozak sequence and reverse primers with BamHI cutting site added to the 5' ends of the matching primer sequences.

To generate full length proteins with Hemagglutinin tag (HA tag) attached to their N termini, rCD4 leader sequence was amplified and HA tag plus Sall-BamHI restriction sequences were attached to this PCR product by using a specific reverse primer which contained this additional sequences attached to the 5' end of the actual matching sequence. This fragment, cut with XbaI and BamHI, was inserted to empty pEF-BOS vector which was also cut with the same enzymes. This pEF-BOS vector containing rCD4 leader and HA tag sequences was cut with Sall and BamHI enzymes to clone "leader sequence free" full length sequences of the genes of interest (Figure 2.1 B). hCD200RLa and M141R genes were cloned into pEF-BOS vector using this strategy.

To generate full length proteins with flag tags attached to their N-termini, a previously made construct which contained rCD4 leader plus flag tag sequence was used. Leader sequence free full length sequences of the desired genes were inserted into this vector

using a similar strategy as above. Mouse DAP12 was cloned in to pEF-BOS using this methodology (Figure 2.1 C)

Soluble chimeric proteins of members of CD200R and SIRP families containing extracellular regions of the protein attached to rCD4 domains 3-4 plus biotinylation site were also generated, using pEF-BOS vectors. For this purpose pEF-BOS containing rCD4L rCD4 domains 3-4 plus biotinylation sequence were digested with XbaI and Sall enzymes to get rid of the rCD4 leader sequence. Fragments coding for Kozak and leader sequences plus sequences of extracellular domains of the protein were inserted into this cut vector (Figure 2.1 D).

Soluble proteins that contain extracellular domains of desired molecules at the N termini and His tag (6 histidine residues) attached to the C terminus for purification were made using a pEF-BOS vector in which was previously inserted with a His tag between Sall and BamHI sites (designed by Deborah Hatherley). Leader sequences and extracellular portions of the molecules were inserted to this vector using XbaI and Sall digestion sites (Figure 2.1 E). Rabbit CD200 and CD200R molecules were cloned into His tag containing pEF-BOS using this strategy

For all pEF-BOS constructs made using Sal-I restriction site (5'GTCGAC3'), constructs were designed in a way that first **guanine** of the restriction site is the last nucleotide of the last

codon of the molecule that comes 5' to the restriction site. Therefore, TCG of the restriction site that comes after the first guanine codes for a serine amino acid. The last two nucleotides (AC) of the restriction site should code for a threonine and to achieve that, an extra adenosine is inserted to the 5' end of the sequence that comes 3' to the restriction site. This design ensures that, for all chimeric proteins, the transition site between two chimeras is made with serine-threonine amino acids which were predicted to affect the overall structure minimally (Figure 2.1 F).

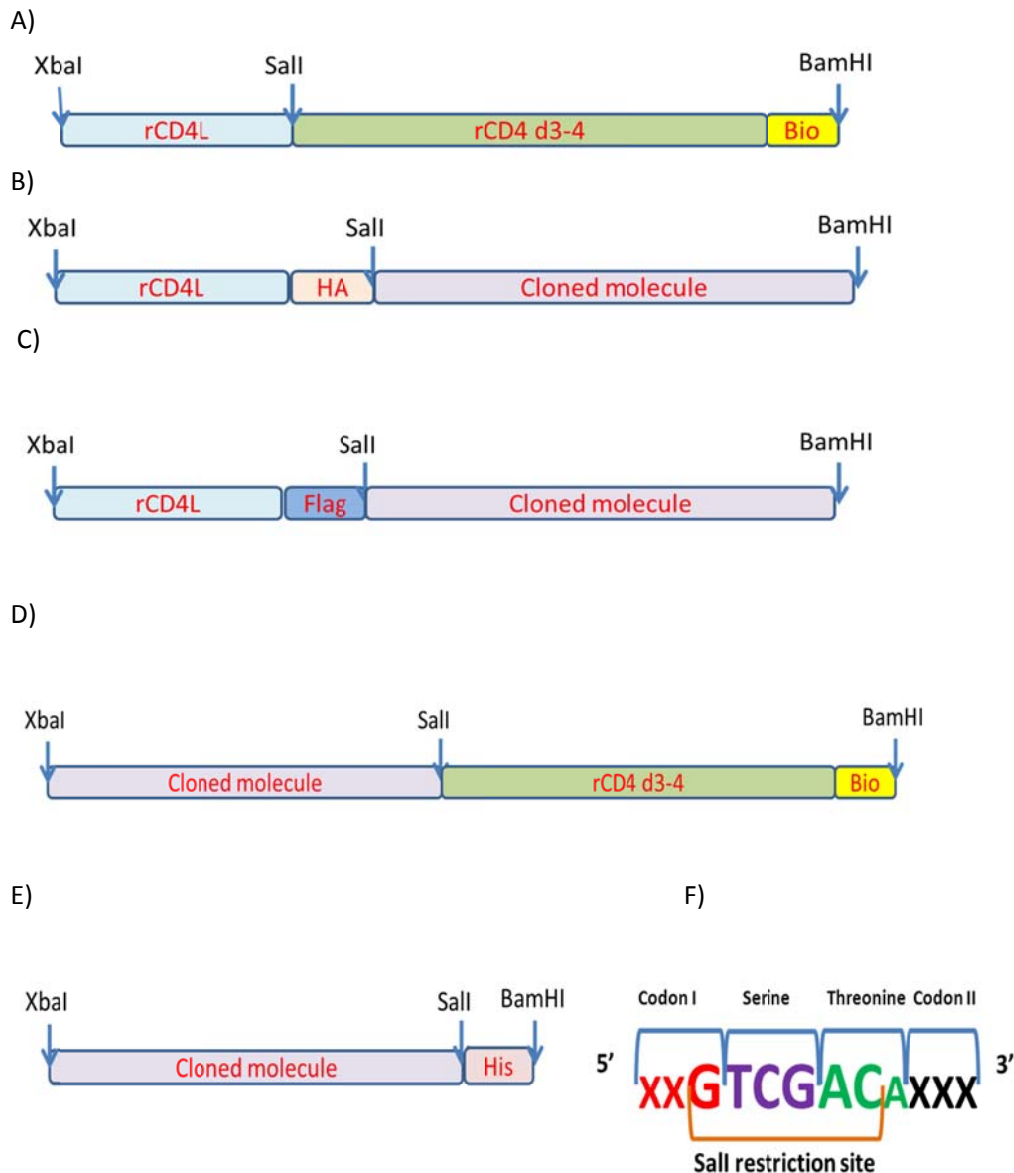


Figure 2.1 Strategies used in cloning different inserts into pEF-BOS vector. The 5' to 3' schematic representations of the cloning sites of different pEF-BOS backbone constructs are shown (A-E). Restriction enzyme digestion sites are shown with blue arrows. Cloned molecule refers to the insert. rCD4L, rCD4 d3-4, HA, Flag, His, Bio stands for rat CD4 leader, rat CD4 domains 3&4, Hemagglutinin tag, Flag tag, His tag and BIR-A enzyme biotinylation site sequences respectively. F) Organization of the transition site around Sall restriction site is shown. Blue braces represent the places of codons (Codon I is the last codon of the first chimeric molecule, codon II is the first codon of the second chimeric molecule) and orange brace represent the place of Sall restriction site. X refers to any nucleotide depending on the construct.

Retroviral vector based constructs

Two different retroviral vector backbones (pBABE and pFB) were used to generate stable cell lines (Figure 2.2). Both vectors were derived from Maloney Murine Leukemia virus (MMLV) and contain the viral package signal, transcription and processing elements as well as the target gene. When transfected into a packaging cell line they generate high titres of viruses that carry the gene of interest but are replication incompetent due to lack of genes required for replication and packaging.

pFB vector (Stratagene) originally contains a Neomycin resistance gene (pFB Neo) which is transferred into the virion and eventually integrates into cell lines genome together with the cloned gene. This allows selection of the viral integrate positive cells with G418 (Geneticin) which is an analog of neomycin sulfate. It also contains an ampicillin resistance gene. This is used to select the transformed bacteria that are positive for the construct. Full length genes of mCD200RL's, cut from pEF-BOS vector backbone constructs using EcoRI and BamHI restriction enzymes, were inserted into pFB Neo cut with same restriction sites. Full length genes of CD200R (mouse, human and rabbit), M141R (both HA tag + and-) and rabbit CD200 were cloned into pFB Neo through BamHI, XhoI restriction sites. A variant form of pFB vector containing hygromycin resistance instead of neomycin, (161) was used to clone mDAP12 (flag tagged), M141R (both HA tag + and-), rabbit CD200, mouse CD200 using BamHI and XhoI restriction sites.

pBABE vector (generated by Bob Weinberg) exist in two forms: One carrying a hygromycin resistance gene (pBABE-Hygro) and the other carrying a puromycin resistance gene (pBABE-Puro). These resistance genes are transferred into the virion which eventually integrates in target cell's genome together with the gene of interest. Therefore, these resistance genes allow selection of positive clones by using the relevant antibiotic. These vectors also carry an ampicillin resistance gene, used during bacterial amplification of the construct. Full length members of the mouse CD200R family cut from pFB Neo backbone constructs with EcoRI and XhoI enzymes were cloned into pBABE Puro (using EcoRI, SalI restriction sites). Flag tag containing mouse DAP12 was inserted into pBABE Hygro using the same restriction sites.

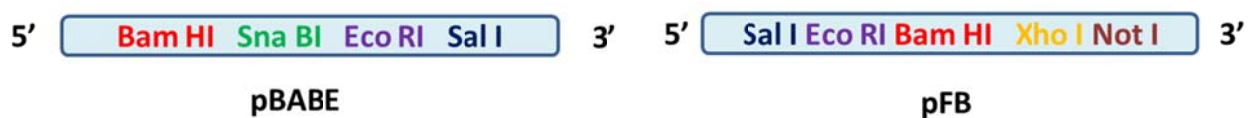


Figure 2.2 Multiple cloning sites of retroviral vectors. Restriction enzyme cutting sites encoded in multiple cutting site regions of pBABE (left) and pFB (right) are shown.

pEE-14 based vectors

pEE-14 (Celltech) vector was used to generate stable CHO cell lines that secrete large quantities of His tagged soluble proteins. It has a CMV promoter to facilitate expression of the inserted gene and a glutamine synthetase gene that allows selection of construct positive cell lines using L-glutamine free growth medium containing L-methionine sulfoximine (MSX). pEF-BOS constructs of the extra cellular domains of the rabbit CD200 and CD200R attached to His tag on their 3' ends were cut using XbaI and BamHI restriction sites. These fragments were inserted into pEE14 vector that was cut with XbaI and BclI.

2.3 Cellular Methods

2.3.1 Transfection

Circular DNA constructs containing the genes of interest were introduced into various mammalian cell lines by transfection. Transfections in this study were made using two different transfection reagents. FuGENE 6 (Roche) is a lipid based transfection reagent that makes complexes with DNA and transfers it to various animal cells. The other transfection reagent is Polyethylenimine (PEI) which is a cationic polymer that makes complex with the negatively charged DNA. This complex is attracted to anionic cell membrane and internalized by endocytosis. Following this, osmotic swelling causes the burst of endosome and PEI-DNA complex (polyplex) is released (20). This polyplex finally unpacks and allows the DNA to diffuse into the nucleus.

Transfection of 293T cells to analyse surface expression

pEF-BOS constructs carrying the activating members of mouse CD200R family were transfected into 293T cells together with pEF-BOS constructs carrying mouse DAP12. For this purpose, 293T cells were plated on to six well plates the day before transfection (10^6 cells/well in 3ml full growth media). On the day of transfection 2ml media was removed from each plate, leaving attached 293T cells with 1ml growth media. Apart from that $1\mu\text{g}$ of CD200RL and $1\mu\text{g}$ of DAP12 constructs were mixed with $6\mu\text{l}$ FuGENE 6 (DNA/FuGENE 6= 1:3) transfection reaction in $100\mu\text{l}$ DMEM. This mixture was incubated at room temperature for 30 minutes and then applied onto the 293T cells on the plates. One day later cells were supplied with an additional 1ml fresh full growth media and 48 hours after transfection they were harvested to be analysed by flow cytometry.

Transfection of 293T cells to generate chimeric proteins.

Large quantities of soluble chimeric proteins, containing rCD4 d3-4 and a biotinylation site in addition to the extra cellular domains of the molecule of interest, were generated by transfecting 293T cells with relevant pEF BOS constructs. For each chimeric protein, two to three 75cm^2 flasks were seeded with 3×10^6 293T cells/flask the day before transfection. Each flask containing 7 ml of full growth media were incubated for one day. On the day of

transfection, 10µg DNA construct was mixed with 100µl of PEI (1mg/ml) in 300µl DMEM. This mixture was incubated at room temperature for 30 minutes and then applied into a flask of cells. Flasks were incubated at 37°C incubators for 5-6 hours after transfection and then all the media containing transfection mixture was replaced with XVIVO 20 serum free growth media. Cells were then incubated at 37°C incubators for 4-6 days. After this period media were removed and spun at 1250 RPM for 5 minutes to remove cells. Supernatant containing the chimeric protein of interest was collected and filtered through 22µm filter and kept at 4°C.

Transfection of CHO K1 cells for generation of large scale soluble proteins.

CHO K1 is a cell line that is usually grown in L-glutamine containing media. Under this growth condition it consumes the L-glutamine from the media, however in glutamine free growth media this cell line can survive by up regulating its intrinsic glutamine synthetase which generates glutamine by condensing glutamate and ammonia. This enzyme can also bind the inhibitory agent L- methionine sulfoximine (MSX) to its glutamate site which results in phosphorylation of the agent causing non-covalent irreversible inhibition of the enzymatic activity (139). Under growth conditions lacking L-glutamine and containing MSX, CHO K1 cells can survive if they have sufficient copy numbers of transfected PEE 14 vector which bears a glutamine synthetase mini gene. Prolonged incubation of transfected cells under these selective conditions enable selection of cells that have integrated this

vector into their own genome. Stable cell lines secreting the protein of interest could be generated, using this strategy.

1.5×10^6 CHO K1 cells per 75cm^2 flask, were seeded the day before transfection using 5% FCS containing full DMEM media. At least two flasks, one being negative control and other being the experimental were prepared. On the day of transfection $10\mu\text{g}$ DNA was mixed with $30\mu\text{l}$ FuGENE 6 in RPMI media in a total volume of 1ml. After 30 minutes of incubation at room temperature, this mixture was added to the experimental flask while nothing was added to the negative control. The day after transfection cells were trypsinized, counted and re suspended in CB2 (DMEM based and glutamine free) media supplemented with 5% dialysed FCS, 50 U/ml penicillin, $50\mu\text{g/ml}$ streptomycin and $25\mu\text{M}$ L-methionine sulfoximine (MSX) (Sigma). These cell suspensions (at least one experimental and one negative control) were plated onto flat bottom 96 well plates at a density of 10^6 cells per plate. These plates were incubated at 37°C , 10% CO_2 incubators for several weeks until colony growth was observed by colour change in some of the experimental plate wells. Supernatants from these wells were analysed by western blot and highest secreting clones were transferred first to 24 well plates and then to flasks. Upon achieving two to three confluent 75cm^2 flasks of cells, they were transferred into Nunc cell factories (Fisher).

2.3.2 Transduction

Constructs that were made using retroviral vectors (section 2.2.4) were used to generate ecotropic viruses which could deliver the constructs into target rodent cell lines by transduction.

Phoenix Eco is a packaging cell line generated by Garry Nolan. It was made by stable introduction of gag-pol (under hygromycin selection) and env (under diphtheria toxin selection) genes of the retrovirus into 293T cell line. These genes were introduced by using non moloney promoters to minimize recombination potential. Once a retroviral construct is transfected to this cell line, these stable genes manage replication and packaging of the region of construct between Long terminal repeats (LTR). 5' LTR acts as promoter for RNA polymerization and 3'LTR acts as termination signal to this transcription. Therefore the region between LTR's containing the cloned gene and the resistance gene were transcribed and packaged into the virion in Phoenix Eco cell line. Those viruses can infect rodent cell lines and transfer these gene fragments into the genome of those cell lines using reverse transcriptase and integrase enzymes brought within the virus.

Transfection of Phoenix Eco cells was made using the same protocol explained in section 2.3.1. One day after transfection, 1ml full media was added to the cells and 6 well plates

containing transfected cells were moved to 32 °C 8% CO₂ incubator and left there for at least one day. 32 °C is better for the stability of the viruses and therefore incubating at this temperature yields a higher viral harvest. After this duration, supernatants were collected and filtered through 22µm sterile filters to get rid of the cells. Polybrene (hexadimethrine bromide) (Sigma) was added to the filtered supernatant with a final concentration of 5µg/ml. This cationic polymer acts to neutralize charge repulsions between retrovirus and sialic acid of the target cell and increases transduction rate (50). For transduction of plate attaching cells like RBL.2H3, CHO etc. 10⁶ cells/plate had been seeded the day before transduction, for cells that grow in suspension like 2B4 cells 10⁶ cells were arranged on the day of transduction. Growth media of these cells placed on 6 well plates were replaced with the supernatant containing polybrene added filtered virus and this mixture was spun at 2700-3000 RPM for 90 minutes at 32°C. Plates were, then, gently put back to 32°C incubator and left there for around 6 hours. After this incubation period, virus containing media was replaced with normal cellular growth media and incubated at 37°C for one day. Next day selective agents (puromycin 1µg/ml, G418 500µg/ml, hygromycin 300µg/ml) were added to the growth media depending on the resistance gene encoded in initial construct. Selection of the positive clones continued for 1-2 weeks and cells were assayed for the presence of the molecule of interest after the selection is over.

2.3.3 Cell lines

5 different cell lines (293T, Phoenix Eco, RBL-2H3, 2B4 Reay, CHO) were used for different experimental purposes. Growth conditions for these cell lines were summarized in section 2.1.3

293T cell line is a SV40 large T antigen containing variant of 293 human embryonic kidney cell line which was first cultured by Alex Van Der Eb from healthy aborted human foetus. This cell culture was transformed using adenovirus (73). Despite their weakness as a cellular model for normal or cancer cells, they are preferred for their high transfectability. These cells were used to express surface molecules transiently and to generate soluble chimeric proteins containing rCD4 d3-4 and biotinylation site.

Phoenix Eco is a packaging cell line generated out of 293T cell line by Garry Nolan (section 2.3.2). It was used to generate viruses for transduction purposes.

RBL-2H3 is a basophilic leukemia cell line isolated and cloned from Wistar rat basophilic cells in the Laboratory of Immunology at the National Institute of Dental Research. They form monolayer with fibroblast like appearance when grown in tissue culture. These cells were used for their basophil like degranulation potential upon stimulation of activating pathways.

CHO (Chinese Hamster Ovary) cell line was developed by Theodore T. Puck in 1957. This cell line has a rapid proliferation potential and can synthesize abundant amounts of protein. It forms monolayer when grown in tissue culture. CHO-K1 variety contains slightly lower amount of cellular DNA compared to original CHO cell line and was used in generation of stable cell lines for secretion of large scale soluble proteins. CHO-IE^K is the second CHO cell type used in the experiments. This cell line contains a stably inserted mouse MHC Class II (α & β) genes in its genome and thus can act as an antigen presenting cell (183).

2B4 is a mouse T cell hybridoma clone derived from B10 mice. It possesses a T cell receptor (TCR) that is specific for pigeon cytochrome c. TCR triggering by specific peptide loaded MHC Class II was shown to generate IL-2 secretion which could be useful as a read out in cellular assays. Later on, this well characterized cell line, in addition to pigeon cytochrome c, was shown to be responsive to Moth Cytochrome C (MCC) when presented on IE^K (18). Therefore, 2B4 cell line could be used in conjunction with CHO- IE^K cell line in a T cell stimulation model.

Other than the cell lines mentioned above, RAW 264.7, NIH3T3, BaF3, 171, WEHI-3b cell lines were also used in preliminary experiments while searching for suitable cell lines for transductions.

Stable cell lines (Table 2.1) were generated using the transduction strategy explained in section 2.3.2. These cell lines were maintained in selective media and used in various cellular assays. Aliquots of them were frozen in freezing mixture containing 90% FCS and 10% DMSO (Sigma) and kept in -80°C for short term storage and in liquid nitrogen for long term storage.

Cell line	Stable Insert I	Stable Insert II	Uses
RBL.2H3 (Rat Basophilic Leukemia)	mCD200RLa (pFB Neo)	mDAP12-flag (pFB Hygro)	Stimulation assays using antibodies,
	mCD200RLb (pFB Neo)	mDAP12-flag (pFB Hygro)	Flow cytometry (test of antibody specificity)
	mCD200RLc (pFB Neo)	mDAP12-flag (pFB Hygro)	
	mCD200RLe (pFB Neo)	mDAP12-flag (pFB Hygro)	
	mCD200R (type 1) (pFB Neo)	None	
2B4 Reay (Mouse T cell Hybridoma)	mCD200R (Type 1) (pFB Neo)	None	Flow cytometry (test of binding to antibodies and ligand coated beads)
	mCD200R (type 2) (pFB Neo)	None	
	mCD200 (pFB Neo)	None	Flow cytometry
	hCD200R (pFB Neo)	None	
	Rabbit CD200R (pFB Neo)	None	Flow cytometry (test of binding using ligand coated fluorescent beads)
	Rabbit CD200 (pFB Neo)	None	
	M141	None	
	M141-HA	None	
CHO (Chinese Hamster Ovary)	Rabbit CD200 extracellular domains-his (pEE 14)	None	Large scale chimeric soluble protein production
	Rabbit CD200R extracellular domains-his (pEE 14)	None	
	mCD200 (pFB Hygro)	IE ^K (pBJ1-Neo)	Cellular assays to characterize anti CD200R antibodies. To be used for testing functional outcome of possible ligand-receptor interaction
	Rabbit CD200 (pFB Hygro)	IE ^K (pBJ1-Neo)	
	M141-HA (pFB Hygro)	IE ^K (pBJ1-Neo)	
	M141 (pFB Neo)	IE ^K (pBJ1-Neo)	

Table 2.1 List of the cell lines generated for various experimental purposes. Vector names in braces stand for the vector the construct was made into. HA and flag refer to HA tag and flag tag respectively.

2.3.4 T cell stimulation model to test antibodies against mCD200R

Three antibodies OX110 (rat anti mouse CD200R), OX131 (rat anti mouse CD200R) and OX90 (rat anti mouse CD200) were tested for their ability to interfere with the CD200-CD200R interaction. For this purpose CHO-IE^K-mCD200 and 2B4 Reay- mCD200R (type 1) stable cell lines were generated as explained in section 2.3.3. Two days before the assay, flasks containing confluent cells of these types were diluted to 1/10 ratio and their selective media was replaced with normal growth media (DMEM full) (no selection) to propagate their growth. On the day of assay 10^5 cells/50 μ l DMEM full per well from each group were mixed in a well of round bottom 96 well plate. 4mM Mito Cytochrome c (MCC) prepared in 50 μ l DMEM full and 20-40 μ g/ml of relevant antibody in 50 μ l DMEM full were added to the cell mixture. For the wells that do not have one or more of these four components, as a part of the experimental procedure, DMEM full is added at the same amount to keep the volume at 200 μ l/well. The final mixture, containing 10^5 CHO and 10^5 2B4 cells as well as 1mM MCC and 5-10 μ g/ml antibody in a total volume of 200 μ l/well in 96 well plate was incubated at 37°C 10% CO₂ for 16-24 hours. After the incubation, the plates were centrifuged at 1250 RPM for 5 minutes to pellet the cells and supernatants (160 μ l/well) were transferred to a separate round bottom 96 well plate. These supernatants were frozen at -20 °C until they were assayed with IL-2 ELISA procedure (see section 2.3.3).

2.3.5 RBL degranulation model

Stable RBL.2H3 cell lines expressing activating members of mouse CD200R family together with mDAP12 on their surface were tested for their degranulation ability upon stimulation with antibodies against the activating receptors. 2×10^5 RBL.2H3 cells/200 μ l RPMI full per well were plated onto flat bottom 96 well plates the day before experiment. After an overnight incubation at 37°C 5% CO₂ incubator plates were put on ice and left until cool then media were replaced with 50 μ l/well primary antibody at 10 μ g/ml concentration in RPMI full media. This step was carried out on ice and very slowly to prevent spontaneous degranulation of RBL.2H3 cells. This mixture was put back to 37°C, 5% CO₂ incubator and left for overnight incubation. Next day, plates were put on ice, supernatants were collected and transferred into a separate 96 well round bottom plate. After gently washing wells once with 50 μ l RPMI (on ice), secondary antibodies were applied at 5 μ g/ml concentration in 50 μ l/well RPMI full medium (on ice). Plates were taken back to incubator for 45 minutes of incubation at 37°C. After this period, supernatants were collected (on ice) and transferred into a separate round bottom 96 well plate and remaining cells were lysed on shaking incubator using 50 μ l 1% Triton X 100 (in PBS). Cell lysates were collected and transferred to a round bottom 96 well plate. Supernatants and lysates were analysed using Hexosaminidase assay (see section 2.3.4).

2.3.6 Analysis of cell surface interactions using flow cytometry

Staining cell surface receptors with antibodies

Cell surface expression of transfected receptors was tested by staining with primary and secondary antibodies. 10^6 cells/tube were centrifuged in round bottom 5ml polystyrene tubes (BD Falcon) and supernatants were discarded. Tissue culture supernatants containing primary antibodies were applied 50 μ l/tube. In cases when purified primary antibodies were used, antibodies were diluted from original stock to 5 μ g/ml concentration in PBS/FCS/azide (see section 2.1.1) and 50 μ l of the final mixtures were used. Cells, mixed with the primary antibody solutions were incubated on ice for 1 hour. After the incubation, cells were washed with PBS/FCS/azide three times and fluorescent labelled secondary antibodies (AbD Serotec) (5 μ g/ml in 50 μ l/tube PBS/FCS/azide) were applied on ice and incubated for 1 hour. After this secondary incubation, tubes were topped up to 300 μ l volume using PBS/FCS/azide and analysed by flow cytometry.

Staining cell surface receptors with fluorescent beads coated with ligands

The day before the experiment, 12.5 μ l (per sample) avidin coated yellow fluorescent beads (0.1 % w/v) (SPHERO™) were centrifuged in 1.5ml micro centrifuge tubes and the supernatants were discarded. Beads were re-suspended using 30 μ l (per sample) concentrated biotinylated tissue culture supernatant and incubated on rotating wheel at 4°C overnight. Next day tubes were centrifuged and the supernatants were discarded.

After three times washing with PBS/BSA/ azide (section 2.1.1) beads were re-suspended with 100 µl PBS/BSA/azide. Bead solutions were sonicated and applied onto cell pellet containing 10^6 cells/ tube. Cells mixed with beads were incubated on ice for one hour. After incubation tubes were topped up with PBS/BSA/azide to a volume of 300 µl/tube and analysed in flow cytometry.

Flow cytometry

Flow cytometry analyses were carried out in the departmental flow cytometry unit using one of the flow cytometry instruments (CyAn™,BD FACsort™,BD FACSCalibur™). Collected data were analysed using FlowJo and CellQuest softwares.

2.3.7 Production of large quantities of antibodies from hybridomas

Hybridomas secreting antibodies against members of the CD200R family, generated by Marie-Laure Aknin, were grown for large scale antibody production. These hybridomas were grown in Hybridoma SFM supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 0.0004 % (v/v) 2-mercaptoethanol, 2mM L-Glutamine, 0.1 mM nonessential amino acids (Gibco) and 1 mM sodium pyruvate. When four 175cm² flasks reached confluence, cells were pelleted and transferred to Integra CELLLine™ CL1000 flasks and maintained there, according to manufacturer's guidelines. The same growth media were used and antibody containing supernatants from cell compartment were harvested every

5-7 days. These supernatants were filtered and kept at 4°C until the purification of the antibodies was performed.

2.4 Protein methods

2.4.1 Biotinylation of soluble proteins

Filtered tissue culture supernatants harvested from 293T cells, transfected with constructs that encode for rCD4 d3-4 plus biotinylation site containing chimeras (see section 2.3.1.2) were quantified using inhibition ELISA technique (see 2.4.2). Once reasonable amounts of protein were confirmed to exist in the supernatant, samples were concentrated around 25 times using Amicon Ultra-15 Centrifugal Filter Units (10000 MWCO) (Milipore).

Concentrated samples were biotinylated using a biotinylation kit (Avitag). The final biotinylation reaction consisted of one part (10x) Biomix A, one part (10X) Biomix B and eight parts sample-enzyme mix (concentrated sample plus ~1µg BirA enzyme). This mixture was incubated at room temperature overnight for biotinylation. Next day, excess unbound biotin was removed by exchanging the buffer with PBS/azide using dialysis. The samples were frozen at -20°C for long term storage after dialysis.

2.4.2 Inhibiton ELISA for quantification of rCD4 d3-4 containing chimeric soluble proteins.

OX68 is a mouse anti rat CD4 antibody which binds to the rCD4 d3-4 domains in the chimera. This antibody was used to quantify the amount of rCD4d3-4 containing chimeric protein secreted by transfected 293T cells (see section 2.3.1.2) in tissue culture supernatants. Serial dilutions of these tissue culture supernatants (50 µl/well) together with the serial dilutions of 5 µg/ml purified rCD4 (standard) (50 µl/ well) were mixed with 1/300 dilution of tissue culture supernatants containing pre-quantified OX68 (25 µl/ well) in 96 well round bottom plate. These mixtures (75 µl/well) were incubated at 4°C for 1-2 hours to ensure that the rCD4 domains in samples bind with the OX68. In the meantime, a flat bottom 96 well plate was coated with 5 µg/ml rCD4 (50 µl/ well) at 4°C for 1 hour and blocked by PBS/BSA/azide at room temperature for 1 hour. After PBS/BSA/azide was removed, 50 µl/well from sample-antibody mixtures were transferred into a flat bottom 96 well plate pre-coated with rCD4 as described above. This plate was incubated at 4°C for 1-2 hours. During this incubation, the free fraction of OX68 antibodies in the sample-antibody mixture, which had not been inhibited by binding to the rCD4 domains of proteins in the same mixture, bound to the plate coated rCD4. At the end of this duration, supernatants were discarded and the plate was washed three times. The antibodies bound to the immobilized rCD4 were recognized by adding 50 µl/well of a 1:3000 dilution of alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) in PBS at 4°C for 1 hour. The wells were washed three times and 50 µl/well of ELISA substrate buffer (section 2.1.1) was added. Plates were left at room temperature until colour change due to phosphatase

activity is clearly visible. Colour change was assayed spectrophotometrically at 405nm.

The chimeric proteins in each sample were measured by comparing the curves for serial diluted samples with the curve obtained by serial dilution of 5 µg/ml rCD4 (standard).

Microsoft Excel and Graph Pad prism softwares were used in data analysis.

2.4.3 IL-2 ELISA

Quantification of IL-2, secreted by T cells (2B4) as a result of peptide bound MHC stimulation by antigen presenting cells (CHO-IE^K) (section 2.3.4), was made with IL-2 ELISA protocol. For this purpose one day before experiment, a flat bottom 96 well plate was coated with 1/500 dilution (in PBS) of rat anti mouse IL-2 (AbD Serotec) (50µl/well) at 4°C overnight. Next day, the plates were washed and blocked with (PBS/BSA) 200µl/well at room temperature for 1 hour. After the blocking, 50 µl/well of supernatants (from the experiment explained in section 2.3.4) were transferred into this plate and incubated for 1-2 hours at 4°C. Together with the supernatants, the serial dilution of purified mouse IL-2 (standard) (starting concentration was 8ng/µl) was also transferred to the same plate for incubation. This incubation caused the IL-2 from the supernatants and the standards to be captured by the plate bound anti mouse IL-2 antibody. Following the incubation and the three times washing steps, the mixture of 1/1000 diluted biotin anti mouse IL-2 (0.5 mg/ml) (AbD Serotec) and 1/500 dilution of ExtrAvidin Peroxidase (Sigma) was applied on to the plates (50 µl/well). Plates were incubated at 4°C for 1-2 hours. This biotin conjugated antibody binds to the IL-2 immobilized with the plate bound antibody, at a

different epitope. At the same time ExtrAvidin peroxidase binds to the antibody through avidin biotin interaction. After the incubation, plates were washed three times and peroxidase substrate 3, 3', 5, 5'-Tetramethylbenzidine (TMB) liquid substrate system (Sigma) was rapidly applied (50 µl/well). Plates were kept in dark at room temperature while colour change was occurring. After colour gradient was visible for the standards, peroxidase reaction was terminated by adding (50 µl/well) 2N H₂SO₄ solution on to the wells. Colour change was evaluated spectrophotometrically at 405nm. Quantification of IL-2 was made by comparing the values with the standard curve using Microsoft Excel and Graph Pad prism softwares.

2.4.4 β-Hexosaminidase assay

β-Hexosaminidase is among a group of lysosomal enzymes, responsible for cleaving hexosamines from gangliosides or other glycosides. This enzyme is released upon stimulation of RBL.2H3 cell line and thus, could be used as an indication of cellular activation. This assay is based on β-hexosaminidase activity related colour change in the media. 20 µl from each well of collected RBL.2H3 supernatant or lysate (see section 2.3.5) was mixed with 20 µl of substrate buffer (section 2.1.1) on ice and transferred to 37 °C for 1 hour incubation. Reaction was terminated by adding 200 µl stop buffer (section 2.1.1) and assayed spectrophotometrically at 405 nm. Percentage of degranulation is calculated using the formula "*Degranulation % = 100 x (O.D._(Supernatants) / O.D._(Supernatants) + O.D._(Lysate))*"

2.4.5 SDS- PAGE

Size dependent separation of proteins was made by running them in 4-12% gradient Bis-Tris SDS PAGE gels (Invitrogen). Samples were mixed with equal amounts of 2X non-reducing sample buffer and boiled for 5 minutes before loading onto the gel. Gel loaded samples were run in SDS PAGE running buffer (see section 2.1.1) at 200 V for 50 minutes. After running, gels were either visualized using coomassie blue (see section 2.1.1) or transferred for western blot (see section 2.4.6)

2.4.6 Western blot

Western blotting was carried out to quantify SDS-PAGE separated CHO-K1 cell supernatants which contained His tag bound soluble proteins. Transfers of gel separated samples to Hybribond P membranes were done according to manufacturer's guidelines, using Novex blot module (Invitrogen) at 30V for 1 hour. Washing, blocking and staining steps were carried out using SNAP i.d. Protein Detection System (Millipore). After the transfer, membrane blots were washed and then blocked with PBS/BSA 1% for 20 minutes. Mouse anti-His6 mAb (BMG-His-1, Roche) antibody (0.25 µg/ml) was applied for 45 minutes to bind His tags, attached to the proteins. After three times washing, goat anti-mouse IgG conjugated to horse radish peroxidase (Biorad) was applied at 1/3000 dilution of the initial stock and incubated for 45 minutes. This was followed by a final three times washing step. ECL plus peroxidase substrate (GE Healthcare) was then applied according manufacturer's guidelines. Substrate applied membrane was wrapped and carried to the

dark room where it was exposed to LS film (Kodak) and developed using X-OMAT processor (Kodak).

2.4.7 Antibody purification using protein G column

Supernatants, collected twice a week from hybridomas that had been grown in integra flasks (see section 2.3.7) were checked for antibody presence using ELISA. In this experiment three dilutions of supernatants (neat, 1/10 and 1/100) were compared with three dilutions of antibody of known quantity (10 µg/ml, 1 µg/ml and 0.1 µg/ml) for their ability to bind plate bound mCD200R and this experiment confirmed the presence of (>10 µg/ml) antibody in each sample. Therefore, samples were combined and applied through HiTrap Protein G HP 5 ml columns (GE Healthcare) which were prewashed with PBS/azide. After 100 ml of supernatants were applied through, columns were washed with 50 ml (10 column volumes) PBS/Azide. Following this step, bound IgG was eluted using 0.1 M glycine solution at pH 2.5. Elutes were collected drop by drop in micro-centrifuge tubes which had already been containing 100 µl/tube Tris HCl solution at pH 8.0. Therefore, each 900 µl of elute in low pH glycine solution mixed with 100 µl pH 8.0 Tris HCl to make 1 ml of roughly neutral pH antibody mixture. Around 20 tubes of elute were collected using this method and IgG concentrations were measured by UV absorbance. Aliquots of antibody mixture that contained ≥ 0.4 mg/ml IgG were pooled and, dialysed in PBS for buffer exchange. Antibody mixture in PBS was re-quantified in nanodrop and filtered sterile. Sterile aliquots of antibody were frozen at -20 °C for long term storage.

2.4.8 Purification of His tagged soluble recombinant proteins

Extracellular domains of rabbit CD200 and CD200R attached to a His tag on their C-terminus were secreted by CHO cells grown in cell factories (see section 2.3.1.3).

Supernatants from cell factories were concentrated 20 times and these concentrates were used for protein purification.

Ni-NTA chromatography

Protein purification was made with affinity chromatography using Ni-NTA agarose (QIAGEN). Ni-NTA agarose was poured into Econo Columns (BIORAD) and washed afterwards with PBS. pH of the concentrated tissue culture supernatant was adjusted to pH 8 prior to applying to the Ni-NTA agarose column. After all the supernatant passed through, the column was washed with 10 column volumes of PBS (pH 8), followed by another two washes with 10 and 20 mM imidazole in PBS (pH 8). These washes with low concentration imidazole (10-20mM) helped get rid of the weakly bound non-specific molecules. Fractions containing His tagged recombinant proteins were eluted with 250 mM imidazole in PBS pH 8 and the OD280nm was measured in nanodrop. Positive fractions were pooled and dialysed into HBS.

Size exclusion chromatography

Fractions purified by Ni-NTA chromatography (see section 2.4.8) contained aggregates of the protein as well as small amount of other contaminant molecules. Monomeric and uncontaminated fractions of the desired protein were obtained by applying the positive fraction pool into size exclusion chromatography. This application was done by using Superdex 75 16/60 columns (GE Life Sciences) and AKTA FPLC at 4°C according to manufacturer's guidelines.

2.4.9 Surface Plasmon Resonance (SPR)

All SPR experiments were performed on a BIAcore™ 3000 using standard protocols. Streptavidin was immobilized onto the sensor CM5 chips, coated with carboxy methyl dextran, according to amine coupling procedure (165). Biotinylated tissue culture supernatants (see 2.4.1) were immobilized on to the flow cells of these chips at approximately equal response units (500-800 RU depending on the experiment).

Characterization of antibodies using SPR

The effect of mCD200R antibodies on the mCD200-mCD200R interaction was assayed by using SPR with settings explained above. Instrumental temperature was set to 25 °C for this experiment. Concentrated tissue culture supernatants containing biotinylated rCD4d3-4 attached to extra cellular domains of mCD200R (type 1 and 2) and biotinylated

rCD4 d3-4 (negative control) were immobilized to the flow cells. Aliquots of purified mCD200 extra cellular domains attached to His tag (given by Deborah Hatherley) in HBS-B were passed over the flow cells at 10 µl/minute speed to demonstrate initial ligand receptor interaction. Concentrated tissue culture supernatants containing relevant antibodies were diluted 1/100 in HBS-B and passed over the flow cells to demonstrate antibody-antigen interaction. Following antibody injections, mCD200 used above, was passed over the cells at the same concentration again to identify any difference in the ligand-receptor interaction. Results were analysed using BIAevaluation software (BIAcore).

Measurement of the affinity between rabbit CD200 and CD200R using SPR

Concentrated tissue culture supernatants containing biotinylated rCD4d3-4 attached to the extra cellular domains of rabbit CD200, rabbit CD200R and rat CD200R alongside with biotinylated rCD4 d3-4 (negative control) were used for immobilization to the streptavidin coated (see section 2.4.9) flow cells. Instrumental temperature was set to 37 °C for the experiment. Purified monomeric fractions (see section 2.4.8) of soluble recombinant rabbit CD200 or CD200R proteins (extracellular domains attached to His tag in HBS-B) were passed over the flow cells in increasing concentrations. Equilibrium binding was calculated by subtracting the control binding values from the ligand-receptor binding values. Langmuir binding isotherm formula $R_{Bound} = ([Analyte] \times R_{max}) / ([Analyte] + K_D)$, where [analyte] refers to the concentration of the analyte and R_{Bound} and R_{max} stand for measured Response units and maximum response units respectively, was used in

generating non-linear curve fitting of the specific ligand responses with increasing analyte concentrations. GraphPad PRISM (GraphPad Software Inc) software was used in curve fittings.

2.5 Methods for screening possible interactions between pathogenic bacteria and various cell surface receptors

2.5.1 Bacteria strains

Pathogenic bacteria strains used in these experiments were gifts from Greaves Lab (Sir William Dunn School) except *Neisseria meningitidis* subtypes a and c which were given by Saunders Lab (Sir William Dunn School). Bacteria were grown on LB agar, Blood Agar or Chocolate agar using standard protocols for each bacteria type. Bacterial swabs were dissolved in LB broth and their concentrations were estimated spectrophotometrically using O.D. 600nm values of the suspensions. Swabs from bacterial culture plates were dissolved in 500 µl sterile FCS in 2ml cryo bank vials (Nunc) and frozen at -80°C for long term storage.

2.5.2 Fluorescent staining of the bacteria

1 µM SYTO 60 red fluorescent nucleic acid stain (Invitrogen) in 1ml LB broth was used to make bacterial suspension with a bacterial swab, taken from overnight grown plates. This

mixture was wrapped with aluminium foil to prevent light entrance and incubated at 37 °C with shaking for 1 hour. After this incubation, bacterial suspension was centrifuged at 6000 RPM for 5 minutes and pellet was washed three times with fresh LB Broth before resuspending them with PBS/azide. Two aliquots, one taken from the stained and the other taken from the unstained bacterial suspension, were fixed using fixation buffer (see section 2.1.1) and analysed in flow cytometry (FL 4 Channel) to confirm bacterial staining.

2.5.3 Analysis of bacterial binding to the magnetic beads coated with the receptors of interest

Streptavidin coated magnetic Dynabeads® (Invitrogen) (10^7 beads/experiment) were incubated with concentrated tissue culture supernatant (40 µl/ experiment) containing biotinylated and rCD4d3-4 attached receptors on rotating wheel at 4°C for 45 minutes. Beads were washed three times and then resuspended with 100 µl PBS/azide containing 10^8 SYTO-60 stained bacteria (see section 2.5.2). The mixture was incubated with rotation at 4 °C for 1 hour. Following the incubation, PBS/azide was removed and beads were gently re-suspended in fixa fixation buffer to kill hazardous bacteria and analysed using flow cytometry. An aliquot from each magnetic bead solution coated with a specific protein was stained with specific antibodies to test protein coating levels for those beads.

Chapter 3:

Screening for the heterogeneity in the mouse CD200R family

3.1 Introduction

Paired receptor families generally show significant levels of heterogeneity so that multiple activating and inhibitory members exist in their gene clusters (36, 120, 130). This heterogeneity is linked with different phenotypic traits between subpopulations of the same species. For example different human subpopulations were shown to have different numbers and types of KIR genes which was thought to be linked to selection by geographically distinct diseases (120, 121). Similarly, significant levels of heterogeneity was observed in Ly49 genes between members of the same species. Among these differences, possession of Ly49H or Ly49P provides resistance against MCMV viruses in some mice whereas their absence is linked to increased susceptibility to the same pathogen (9, 97).

Mouse CD200R paired receptor family consists of one inhibitory (CD200R) and at least four activating (CD200R Like) (187) members. Additionally, the most recently characterized member of the family CD200RLe was shown to be present in mouse strains like NOD1 and CD1 (173) but not in C57BL/6 which may indicate an uneven distribution of these genes among different mice strains. A comprehensive analysis of the distribution of mouse CD200R family members among various wild type and laboratory derived mice

strains can provide information on the extent of heterogeneity in the CD200R family and may reveal additional unknown genes belonging to this family. Additionally, these results may provide insight on the evolution of this receptor family.

For this purpose, genomic DNA from 22 different mice strains was analysed for their CD200R family repertoire using a discriminative PCR approach.

3.2 Sequence analysis of CD200RL genes and the PCR strategy used

Discrimination between closely related genes, such as members of the CD200R family, requires clear identification of the extent of sequence identity in between these genes. Full-Length coding sequences of each member of the family were obtained using the NCBI data base and were aligned using Vector NTI (Invitrogen) software. Both nucleic acid and protein level alignments were used to calculate the percentage of sequence identity (Table 3.1). These sequence alignments were also used to search for a specific DNA region where discriminative primers, specifically binding to one gene but not the others, could be designed. As the DNA sequences were so similar, attempts to discriminate CD200RL genes using this approach on plasmids containing the genes were unsuccessful. As specific primers failed to discriminate each gene, intron lengths between exons were analysed for each gene using ENSEMBL genome browser. Intron 4 had the most variable length in the range of 2-5 kb (Table 3.2) Therefore, instead of using specific primers for each allele,

common primers binding to all CD200RL genes and amplifying the region starting with exon 4 including the intron between and ending with exon 5 were designed and PCR product length was used as discriminative factor.

This approach could discriminate CD200RLa, CD200RLb, CD200RLc. Genomic organization data of CD200RLe was not known therefore an additional PCR strategy (see section 3.5) was used. (The sequence of CD200R in this region allowed a specific primer set to be designed for this allele and therefore that was analysed separately (see section 3.6).

	<i>Protein</i>				
	R	La	Lb	Lc	Le
mCD200R	100	84	41	79	87
mCD200RLa	84	100	41	78	84
mCD200RLb	41	41	100	46	43
mCD200RLc	79	78	46	100	80
mCD200RLe	87	84	43	80	100

	<i>Nucleic acid</i>				
	R	La	Lb	Lc	Le
mCD200R	100	91	68	89	93
mCD200RLa	91	100	69	90	93
mCD200RLb	68	69	100	69	69
mCD200RLc	89	90	69	100	89
mCD200RLe	93	93	69	89	100

Table 3.1 Nucleic acid and protein level sequence identity between members of mouse CD200R family. Alignments were made using VectorNTI (Invitrogen) software-align X tool. Numbers indicate the percentage values. The additional 56 amino acids of the cytoplasmic domain, found only in CD200R, were excluded in this calculation.

	Exon 4	Intron	Exon 5
	(bp)	(bp)	(bp)
mCD200R	252	2502	100
mCD200RLa	249	4413	115
mCD200RLb	219	3390	106
mCD200RLc	249	4833	115
mCD200RLe	?	?	?

Table 3.2 Comparative lengths of exons 4,5 and the intron between them for members of the mouse CD200R family. (bp) stands for base pairs. the genomic organization of CD200RLe is not known therefore values are shown as (?)

3.3 Screen of mice strains for mouse CD200RLa-c genes

The PCR strategy explained above was carried out using mouse genomic DNA (purchased from Jackson Lab USA) from various strains (Table 3.4). To ensure further specificity, experiments were done using two sets of primers each of which bound to the same exon (exon 4 for forward primers and exon 5 for reverse primers) but to independent sequences (Table 3.3). C57BL/6 mouse, whose full genomic sequence was known, was used as positive control. The PCR with an extension time of 8 minutes across intron 4, using C57BL/6 genomic DNA, produced three bands on agarose gel (Figure 3.1). Those bands corresponded to CD200RLc, CD200RLa and CD200RLb (from top to bottom). This matched the sequence data available for this strain. This experiment also showed that CD200RLd which was identified as an incomplete pseudogene (187) did not interfere with the results by producing an additional band.

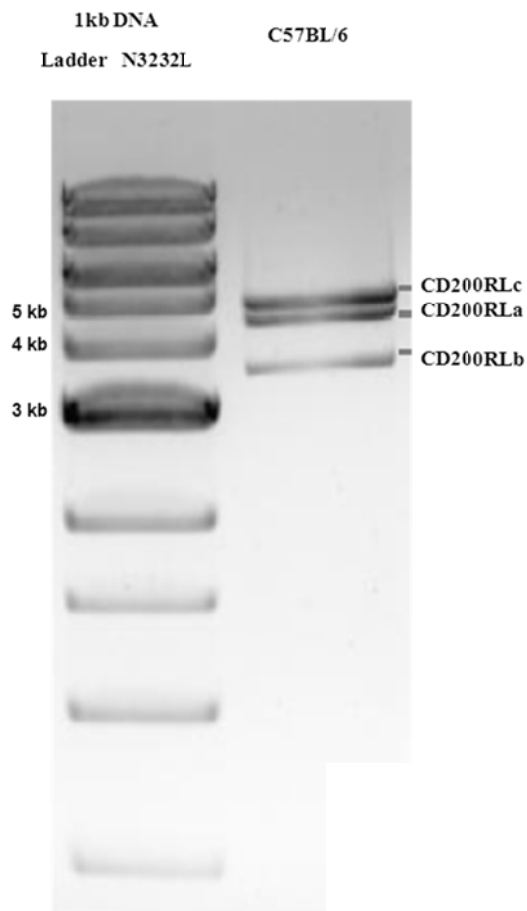
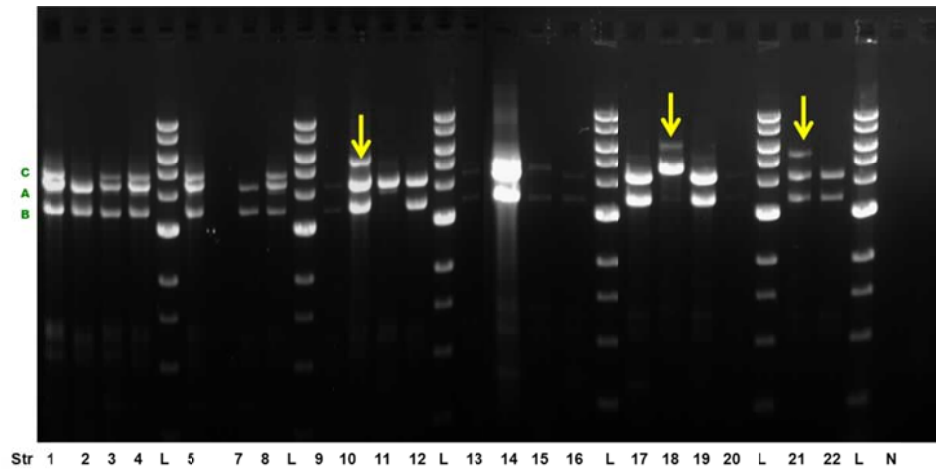


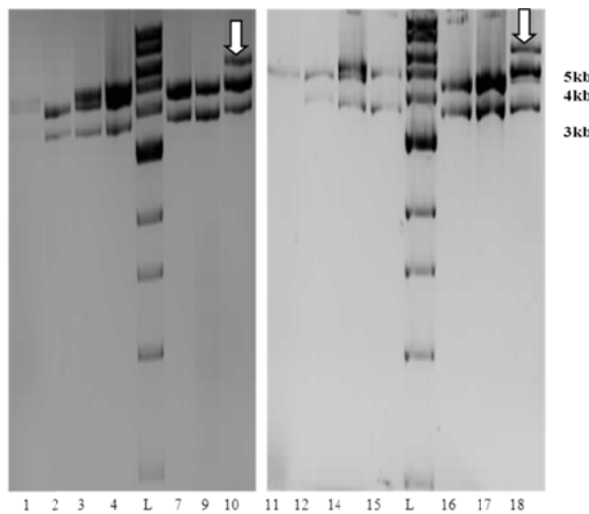
Figure 3.1 Gel image of C57BL/6 PCR. 1kb DNA ladder (N3232L-New England Biolabs) was used for band length estimations. Bands with sizes corresponding to CD200RL a-c were shown (Representative of more than five experiments.)

Following confirmation with C57BL/6, the same PCR strategy was applied for other mouse strains whose genomic sequences were not known. Amongst the 22 mice strains that were studied, twenty of them were positive for CD200RLa and twenty one for CD200RLb while only ten of them were positive for CD200RLc (Figure 3.2)

A)



B)



C)

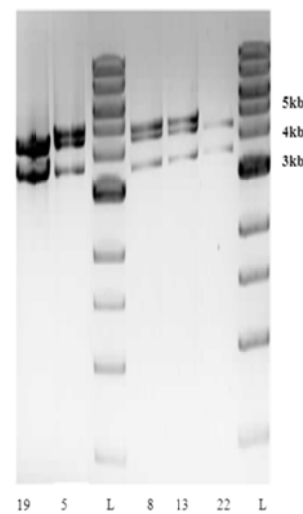


Figure 3.2: Agarose Gel images showing the distribution pattern of CD200RLa-c PCR bands among the 22 mice strains studied. A) Numbers correspond to the strain number in table3.7 and L stands for 1kb DNA ladder and N stands for negative control PCR. A, B, C indicate the bands that correspond to CD200RLa, b and c respectively. Arrow heads point out the position of extra bands whose length do not fit the formerly known CD200R family members. B) Shows the replicate experiments (both upper and lower gel images) using an independent (outer) set of primers as seen in Table 3.3. (Representative of three experiments.)

3.4 Characterization of novel members of the CD200R family

The extra bands shown with arrow-heads in Figure 3.2 which were observed for strains 10, 18 and 21 were significantly longer than the expected sizes of known members of CD200R family. The absence of these bands in NOD (strain 12) and CD-1 (strain 7) mice, which were the two strains CD200RLc was first seen, ruled out the possibility that these bands correspond to CD200RLc. Therefore further exploration was required. These bands were cut out of the agarose gel and were purified. Purified PCR products were sequenced using the outer primer targeting exon 4. However due to heterogeneities and impurities in those PCR products, a clear sequence could not be obtained. Yet those results allowed the design of primers to clone the exon 4 section of the extra bands. That section of the band was amplified using the outer exon 4 primer (Table 3.3) and a reverse primer designed using the unclear sequencing result. Around 300 bp long PCR products (Figure 3.3) were cloned into pEFBOS eukaryotic expression vector through XbaI and BamHI restriction enzyme cutting sites. Sequencing of this construct showed that the predicted amino acid sequences from this region differed slightly from the characterized CD200RL genes (Table 3.3). MOLF/EiJ and WSB/Ei strains have only one amino acid difference in the analysed region of around 70 amino acids. This region is highly conserved between the genes and with only one, two, and three amino acid differences between CD200RLc and the sequences from MOLF/EiJ, WSB/Ei, and SPRET/Ei strains, respectively. Thus, it is not possible to show particular relatedness of the new sequences to an individual CD200RL gene.

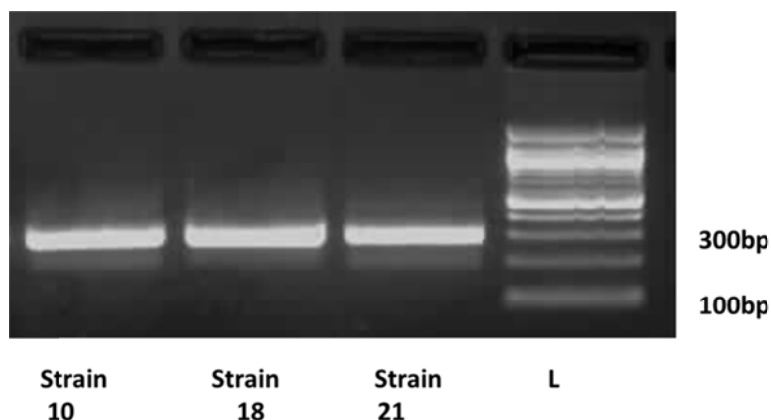


Figure 3.3 PCR bands showing the 300 bp section of the exon 4 region of extra bands before cloning into pEFBOS vector. Strain numbers refer to the mice strains from which those extra PCR bands were generated. L stands for 100 bp ladder.

3.5 Analysis of the distribution of CD200RLe

The genomic organization of CD200RLe, including the places of exon intron boundaries and the lengths of these fragments, has not been identified. Therefore, the genomic screening strategy, used for other CD200RL genes, could not be used for this allele. Instead, discriminative specific primers were designed to PCR amplify the hypothetical exon 5 region which was estimated by aligning the full length coding sequence with the other members of the family. This region was chosen as it contains a unique SnaBI restriction enzyme cutting site (TACGTA) which does not exist in other members of the CD200R family. Therefore digestion of the PCR product with SnaBI enzyme would provide the specificity in this screening (Figure 3.4 A). For NOD 1 and C57BL/6 mice, the 126 bp long region of interest was amplified and the products were cut by SnaBI restriction enzyme after PCR clean up. Cut fragments were visualized on 2.5% agarose gel with ethidium bromide staining together with 50bp DNA ladder (Figure 3.4 B).

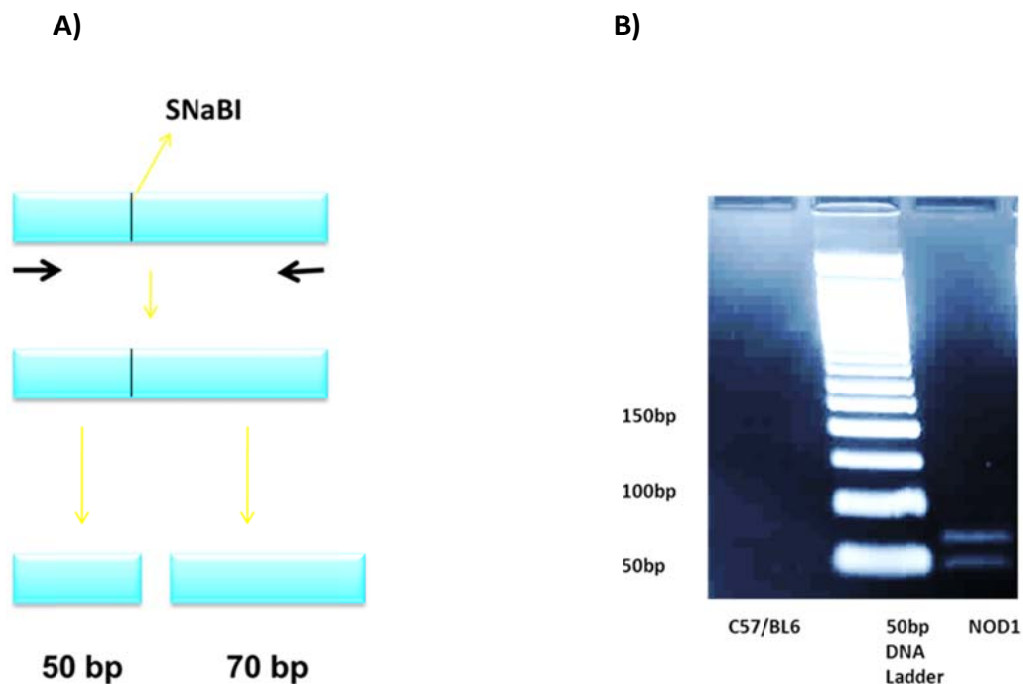


Figure 3.4 Screening for the mouse CD200RLe. A) Illustration showing the experimental design of PCR screening of mouse CD200RLe. B) Agarose gel image showing the SnaBI digested PCR products for NOD1 mouse strain, compared to the negative control C57BL/6 strain. (Representative of three experiments.)

The NOD1 mouse produced a 120 bp long PCR band (not shown) which could be separated into barely visible 50bp and 70bp fragments after SnaBI digestion (Figure 3.4 B). C57BL/6 did not provide any PCR product which was an indication of CD200RLe specificity of the primers that were used.

Following experimental confirmation of the discriminative PCR strategy, all 22 mice strains were tested. However, the shortness of the fragments caused a visualization problem which prevented clear identification of positive and negative results. Thus a further

modification in the experimental design was required. For better visualization of discriminative cut fragments on agarose gel, the fragment size and quantity of DNA in these fragments were increased by adding non pairing poly A sequences to 5' ends of forward and reverse primers (48 and 27 nucleotides respectively). These elongated primers yielded a PCR product of around 200 bp instead of 120 and the restriction digestion of the elongated PCR product with SNaBI enzyme produced two fragments, each being 100 bp in length, which would overlap in agarose gel electrophoresis and produce one band with double amount of sample (Figure 3.5 A).

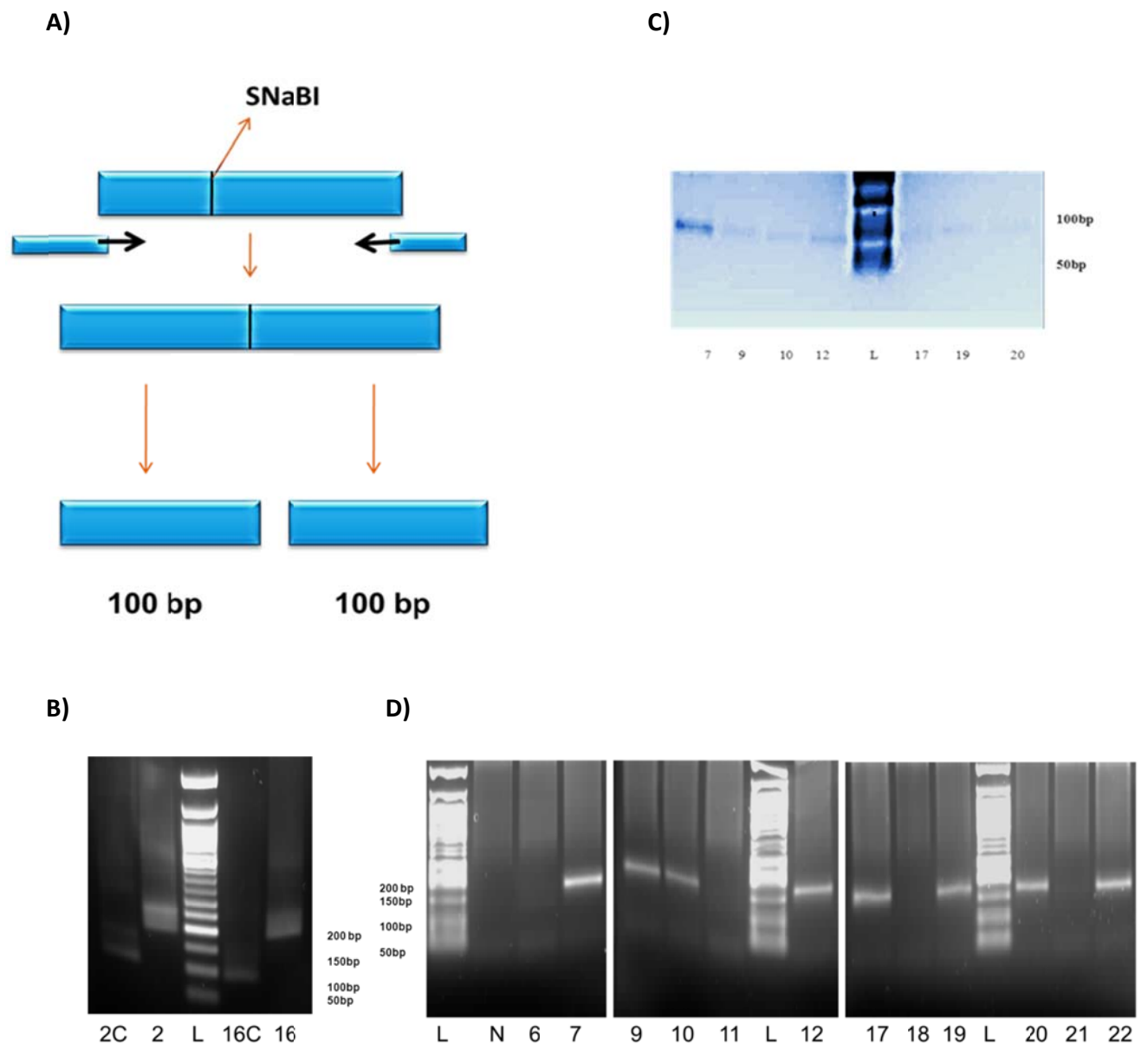


Figure 3.5. PCR screening for distribution of CD200RLe among mouse strains. A) Schematic representation of the experimental design based on elongated primers. **B)** Gel image showing PCR product from two CD200RLe positive strains. 2 and 6 stand for uncut PCR bands from relevant strains while 2C and 6C indicate the SnaBI digested form of the same PCR products. **C)** Gel image showing SnaBI digested PCR products from strains that are positive for CD200RLe. **D)** Gel image showing uncut CD200RLe specific PCR products in CD200RLe positive and negative strains. Numbers correspond to the number of strain (Table 3.4), L stands for 50 bp DNA Ladder and N stands for the negative control PCR.

22 mouse strains were screened using the strategy explained above and ten were positive for CD200RLe (positive strains are shown in Figure 3.5 B-D) and all the CD200RLc containing strains were negative for CD200RLc. This suggests that CD200RLe is a widespread member of the mouse CD200R family and possibly an alternative for CD200RLc.

3.6 CD200R is present in all mouse strains in two slightly different allelic variants.

The inhibitory receptor has a homeostatic role and therefore is expected to be well conserved among different mice strains while the activating members evolve rapidly to neutralize the various changing pathogenic treats towards the inhibitory receptor (14, 36, 169). To confirm the existence of CD200R in all mice strains that were tested, a similar PCR screening strategy, using CD200R specific primers targeting exon 4 and 5 was carried out. Two independent sets of primers were used to ensure further specificity. Around 2800bp PCR product (Table 3.2) specific for CD200R was observed in most of the tested strains, whereas the remaining produced a band of shorter size instead (Figure 3.6 A). This result was consistent in replicate experiments using independent sets of specific primers. This suggested an alternative allele of CD200R in those strains and full length CD200R was generated using cDNA from CD1 strain which was among the strains that generated a shorter PCR band. Sequencing of this allele showed a seven amino acid variation from the C57BL/6 type CD200R (Accession No: BC052682) which made it identical to a previously reported variant sequence of CD200R found in NOD mice (Accession No: BAE32516). That seven amino acid difference is spread across exon 2 and exon 3 while the 4th and 5th exons

remained identical. Therefore the difference observed in the length of 4th intron suggests only an intronic length polymorphism. Further analysis of the distribution of the NOD-like variation in CD200R was made using a discriminative PCR screening. For this purpose, forward and reverse primers were designed in a way that they specifically target variant nucleotides in exon 2 and 3 respectively. All of the twenty two mice strains were screened using this strategy and eight of them produced PCR bands indicating the presence of this variant allele (Figure 3.6 B).

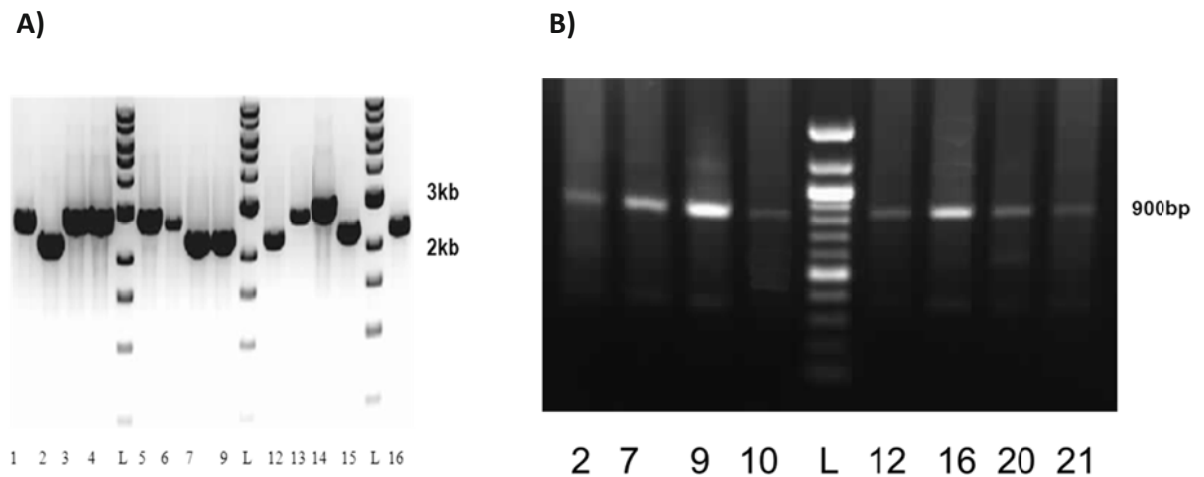


Figure 3.6 Analysis of the distribution of CD200R among mouse strains. A) Agarose gel image showing CD200R screening across 4th intron (a selection of the strains shown numbered. Numbers refer to strain codes in Table 3.4) L : 1kb DNA ladder. B) Agarose gel analysis shows strains that contain the 900bp specific band for the CD200R type found in NOD1 mice. L: 100 bp DNA ladder.

These sets of experiments confirmed the presence of CD200R in all tested strains in two slightly different sequences. The polymorphism lies in the V domain of the protein which

is responsible for ligand binding (79). The biochemical and functional consequences of this variation is characterized in detail in chapter 6.

In summary these results are in concordance with the expectation that the inhibitory member, which is CD200R in this case, is conserved during evolution while a variation in the activating members is more significant.

3.7 Discussion

The methodology used in this study was successful in covering all of the known members of the family. Using two independent sets of primers and extended polymerization time to allow much longer products to be synthesized by PCR, have optimised gene detection. However, it is to be noted that in case a highly divergent member of the family or another member with an intron 4 length above detection limits of the used PCR strategy (>11 kb) exist, this experiments may miss that gene(s).

These comprehensive screenings of CD200R family members in mice from different backgrounds have revealed that there is significant variation in CD200RL genes. This variation is more significant in wild derived mouse strains whereas laboratory mice of different strains showed lesser variation. However no link between the geographical origin of that wild derived mouse and the repertoire of genes they possess could be seen. Mice from different geographical origin have been transported to remote areas of the world in

man made vehicles since the age of discovery and thus, this finding may partly be due to lack of isolation of native mice populations in different parts of the world.

Although there is no exact pattern on how these genes distribute and what drives this distribution, most mice strains showed one inhibitory and 3 activating genes CD200RLa and Lb being positive in the vast majority of strains (Table 3.4). This may suggest less evolutionary pressure on these two genes. The observation of CD200RLe and CD200RLc being always mutually exclusive may refer to these genes being alternatives of each other, despite the structural differences they possess. (amino acid sequence identity between CD200RLe and CD200RLc is 81%, compared to 84% between Le & La and 88% between Le and CD200R). However, this could only be made clear with chromosomal mapping studies which is currently lacking for CD200RLe. The extra alleles that were discovered and partially sequenced during the course of this study have high similarity to CD200RLc. Although with this little sequencing data it is hard to estimate how these extra genes are in reality, this gives us an idea on higher evolutionary pressure towards these members of the family, especially with the finding of CD200RLe possibly being an alternative to CD200RLc.

Mouse strains	Strain Number	CD200RL genes	Number of genes	CD200R	Origin of the strains
A/J	1	A,B,C	3	(+)	Laboratory derived
B10.129P2	3	A,B,C	3	(+)	Laboratory derived
Balb/cWt	4	A,B,C	3	(+)	Laboratory derived
C3H/HeJ	5	A,B,C	3	(+)	Laboratory derived
C57BL/6	6	A,B,C	3	(+)	Laboratory derived
DBA/1J	8	A,B,C	3	(+)	Laboratory derived
NZB/B1NJ	13	A,B,C	3	(+)	Laboratory derived
NZW/LacJ	14	A,B,C	3	(+)	Laboratory derived
AKR/J	2	A,B,E	3	(+)Type 2	Laboratory derived
CD1-TgN	7	A,B,E	3	(+)Type 2	Laboratory derived
NOD/Lt	12	A,B,E	3	(+)Type 2	Laboratory derived
WMP/Pas	20	A,B,E	3	(+)Type 2	Laboratory derived
IS/CamRk	9	A,B,E	3	(+)Type 2	Wild derived from ISRAEL
PERA/EiJ	16	A,B,E	3	(+)Type 2	Wild derived from PERU
SKIVE/Ei	17	A,B,E	3	(+)	Wild derived from DENMARK
TIRANO/Ei	19	A,B,E	3	(+)	Wild derived from ITALY
ZALENDE/Ei	22	A,B,E	3	(+)	Wild derived from SWITZERLAND
Mus Pahari Ei	11	A	1	(+)	Wild derived from THAILAND
PANCEVO/Ei	15	B,C	2	(+)	Wild derived from SERBIA
SPRET/EiJ	18	B,C,X	3	(+)	Wild derived from SPAIN
WSB/Ei	21	A,B,X	3	(+)Type 2	Wild derived from USA
MOLF/EiJ	10	A,B,X,E	4	(+)Type 2	Wild derived from JAPAN

Table 3.4 Summary of the distribution of members of CD200R family. A,B,C,E refer to CD200RLa,Lb,Lc,Le respectively. X refers to extra alleles found in three strains. Type 2 stands for NOD variant of CD200R. The strains are numbered and these numbers are used in other figures.

The degree of diversity observed in CD200R family is obvious but still much less compared to other paired receptor families like ones found in NK cells. For example Ly49 screens in BALB/c, C57BL/6, and NOD mice have seven, ten, and thirteen functional genes, and one,

five, and eight pseudogenes respectively (36). Similar screens on SIRP receptors which are also a myeloid cell receptors like the CD200R family also showed limited diversity (189). These findings may correlate with the more regulatory function of myeloid cells against pathogen attacks compared to more first line defensive role of NK cells which should put them under higher evolutionary pressure.

Another intriguing point is the presence of two isoforms of the inhibitory receptor. The differences are limited to seven amino acids which do not affect the ligand binding ability (see chapter 6). However, those seven amino acids are conserved in eight of the screened strains (Table 3.4) which might indicate a role for these residues. Although it is not possible to clearly identify that role without further investigation, those isoforms might have evolved under pathogen pressure against the inhibitory receptor. A pathogen targeting the inhibitory receptor through a non-ligand binding area of the extra cellular domain might have caused an evolutionary pressure which could have favoured alteration in those residues to ones that either don't bind with the pathogen or bind weakly with them. However, no such pathogenic threat have been identified against mCD200R to back such a claim yet.

Polymorphisms in leukocyte surface proteins are common and were instrumental in the early days of their study in their identification as alloantigens such as Ly-1, Ly-5 and Thy-1 (10). Whereas there is only one amino acid difference between the alleles of Thy-1 there are 9 residues that differ between the alleles of CD45 (157).

Finally, the screenings explained in this chapter provide how the different members of the mouse CD200R family are distributed in different mice strains. That information, on its own, does not identify the rationale behind the heterogeneity but may provide insight on the phenomenon which should further be evaluated using future observations on the role and structure of the activating receptors and the interactions of pathogens with this family.

Chapter 4:

Characterization of the rabbit CD200R family and the test of the possible interaction between the rabbit CD200R and M141R (a candidate viral CD200 homologue)

4.1 Introduction

The CD200R receptor family is one of the various paired receptor families of the immune system whose ligand receptor interactions have been shown to be mimicked by several viruses (67, 68, 103) . In these studies, some members of the herpes virus family were shown to possess viral homologues of CD200, through which they can down regulate immune response directed towards them (see section 1.2.4). These viral homologues of CD200 are thought to be captured from the host genome during the life cycle of virus and maintained through evolution to increase viral virulence. Recently, in addition to those in Herpes viruses, some viral proteins in various members of Pox virus families were also suggested as viral homologues of CD200 (Figure 4.1) (27). The studies mainly focus on a rabbit myxoma virus protein called M141, *in vivo* (27) and *in vitro* (194) findings indicate that this protein with considerable similarity to host CD200 is an important virulence factor for that virus (Table 4.1). However, despite these functional data, biochemical evidence on the viral molecule host CD200R interaction has been lacking. An important reason for this missing biochemical evidence was that rabbit CD200 and CD200R proteins and their interactions had not been characterized.

Therefore, two questions remain: Is M141 really a viral CD200 homologue binding rabbit CD200R and, if not, what mechanism is responsible for its role in down regulation of the immune response?

In this chapter, rabbit CD200 and CD200R molecules and their specific interactions were characterized and the interaction of viral M141 molecule with rabbit CD200R was tested in various settings to identify the underlying molecular mechanisms in M141-host relation.

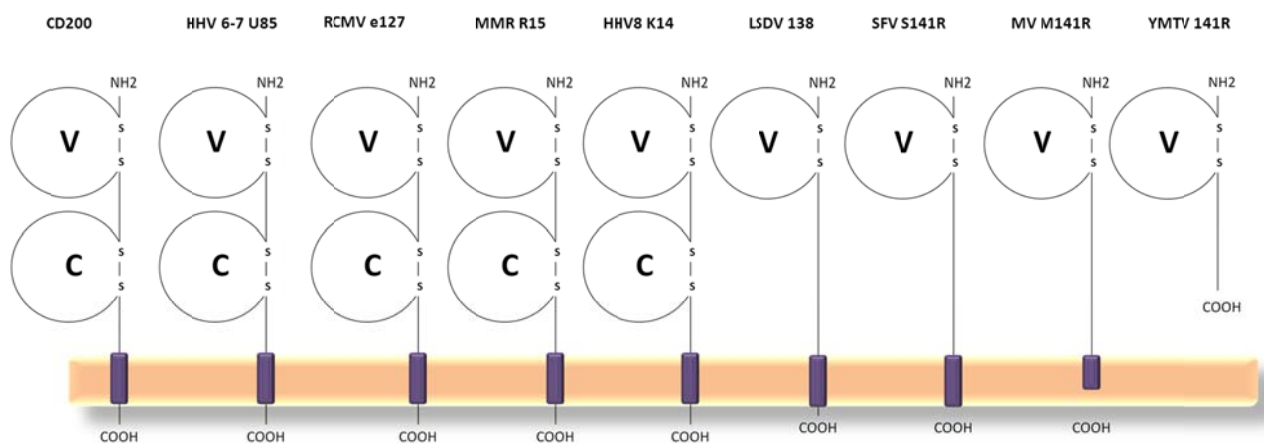


Figure 4.1 Schematic representation of various viral homologues of CD200 compared to CD200. e127, U85, R15 and K14 belong to herpes virus families. The rest are from different members of Pox virus families. Orange layer represents the cell membrane and V& C represent the two extra cellular IgSF domains.

	Amino acid sequence identity (%)	Amino acid sequence similarity (%)
M141 and rabbit CD200	23	36
M141 and mouse CD200	22	34
M141 and human CD200	23	34

Table 4.1 Amino acid sequence comparisons between M141 protein and CD200 from various species. Sequences of the first Ig-like domains of CD200 proteins are compared with the Ig-like domain of M141 protein. Amino acid similarity indicates the sum of identical and similar amino acids.

4.2 Characterization of the rabbit CD200R family

Rabbit CD200R was characterized using genomic sequences available for rabbit in Ensembl genome browser. Knowing that Myxoma virus causes fulminant myxomatosis in European rabbit (*Oryctolagus cuniculus*) whereas it only causes a benign cutaneous fibroma in American counterparts belonging to *Sylvilagus* genus (95), only the European rabbit sequence was analysed. Using alignments with CD200R sequences from other known species, rabbit CD200R was identified in chromosome 14. Amongst two similar sequences identified in that chromosomal region, one was a complete inhibitory receptor sequence with all extracellular, transmembrane and intracellular regions; the other was a sequence without the intracellular region most probably being either a pseudogene or another receptor-like gene (Table 1.5). The complete coding sequence of rabbit CD200R was codon-optimized, generated and cloned into a plasmid by Genart Gene Synthesis,

Invitrogen. A similar investigation was performed for the identification of rabbit CD200 and its full length sequence was also cloned by the same method above.

The sequence of rabbit CD200R was similar to CD200R in other species (e.g. 65% identical amino acid residues with the human CD200R). This is in accordance with the evolutionary conserved nature of the CD200R.

4.3 Characterization of the CD200 CD200R interaction in rabbits

Clear identification of the receptor-ligand interaction for rabbit CD200R was essential to confirm the sequence data explained in (chapter 4.2). To characterize this interaction two recombinant soluble proteins containing the extracellular domains for both receptor and ligand attached to a His tag were generated (see section 2.3.1.3) and these proteins were purified to achieve pure monomeric fractions (see section 2.4.8). Those monomeric proteins were used to detect the ligand receptor interaction using surface plasmon resonance. (Figure 4.2)

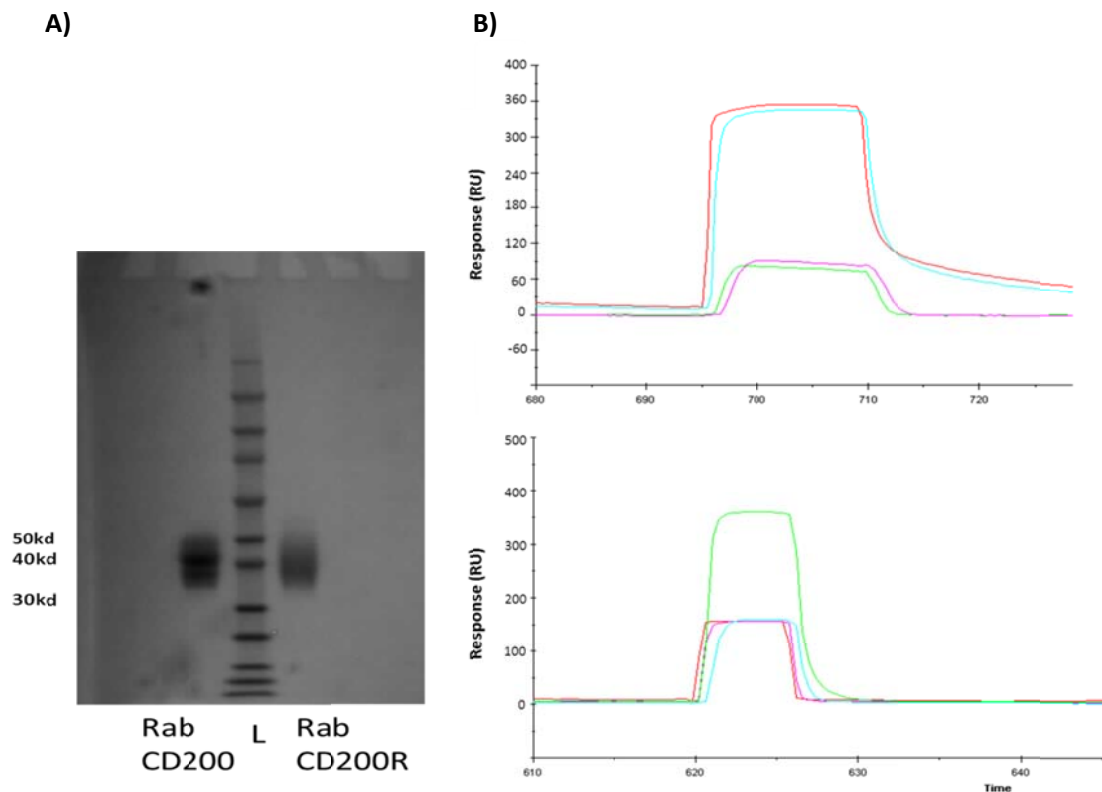


Figure 4.2 Rabbit CD200 binds rabbit CD200R. A) Coomassie blue stained Protein gel image showing purified monomeric rabbit CD200 and CD200R, used for the analysis of the binding kinetics. L stands for Protein Ladder. **B)** Surface Plasmon resonance binding graph for the CD200-CD200R interaction. Soluble purified rabbit CD200 (top graph) or rabbit CD200R (bottom graph) (20µM) was passed over immobilized recombinant proteins (extracellular domains of the related protein attached to ratCD4 d3-4-biotin). Immobilized rabbit CD200R (red), rat CD200R (blue), rabbit CD200 (green) and rat CD4 alone (pink) are shown. Immobilizations on the chip were done through the biotin-streptavidin interaction. For each experiment around 600 RU of recombinant proteins were immobilized. (Representative of two independent experiments)

Recombinant purified rabbit CD200 and CD200R proteins were sequentially passed over four flow cells to which rabbit CD200-rCD4 d3-4, rat CD200R-rCD4 d3-4, rabbit CD200R-rCD4d3-4 and only rCD4 d3-4 had been immobilized. Clear binding was observed between rabbit CD200 and rabbit CD200R and also between rabbit CD200 and rat CD200R (Figure4.3.1). A range of concentrations of the analytes were passed over the chips to

determine the affinity of the rabbit CD200-CD200R interaction. Similar values of $K_D = 3.3$ - $3.6 \mu\text{M}$ were obtained from this two way determinations (Figure 4.3). In these experiments, the cross species interaction of rabbit CD200 with rat CD200R was also measured and that interaction was shown to have a slightly lower affinity ($K_D: 5.1 \mu\text{M}$). Interpretation of the collected data were also made using Scatchard plots (Figure 4.3). This results match the previously identified CD200-CD200R interaction kinetic values measured for rat, human and mouse ($K_D: 1.0 \mu\text{M}$; $0.5 \mu\text{M}$ and $4.0 \mu\text{M}$) respectively (80, 187).

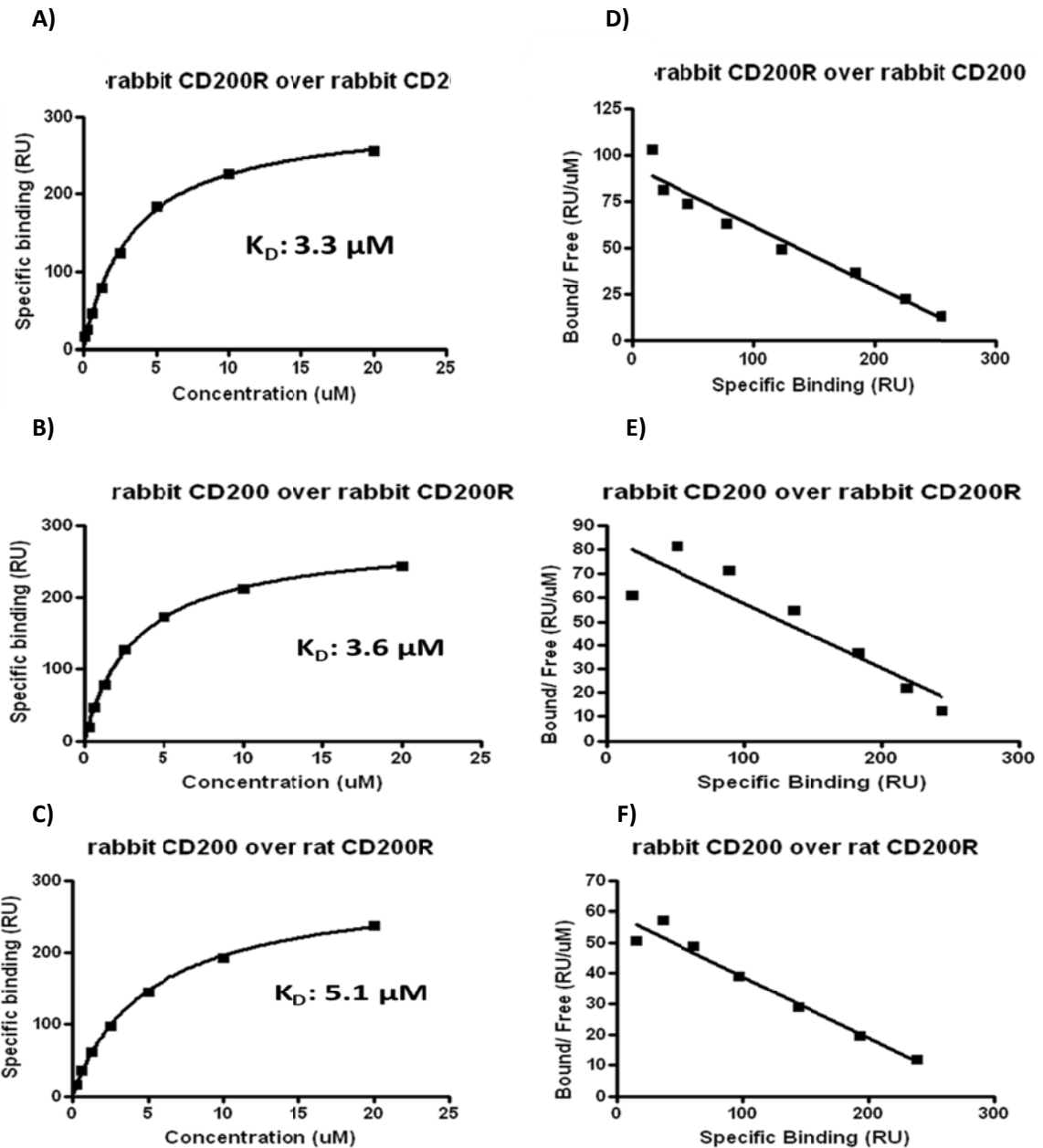


Figure 4.3 Analysis of the equilibrium binding of the CD200 - CD200R interaction. The rabbit CD200- rabbit CD200R interaction was tested using immobilized rabbit CD200 with soluble purified rabbit CD200R (A); and immobilized rabbit CD200R with soluble purified rabbit CD200 (B). Cross species interaction was tested using immobilized rat CD200R with soluble purified rabbit CD200 (C). Scatchard analysis of the binding data showing good linearity are given for rabbit CD200-rabbit CD200R interaction (D,E); and for rabbit CD200-rat CD200R (F). For each experiment around 600 RU of recombinant proteins were immobilized. Specific binding represents the values after subtraction of the negative control binding.

4.4 Investigation of the interaction between myxoma M141 protein and rabbit CD200R

Codon optimized full length myxoma virus M141R gene was a gift from Grant McFadden's group (University of Florida). Ig-like domain of that construct was sub-cloned into a chimera containing rCD4 domains 3 and 4 and a biotinylation site at the C-terminus (see section 2.2.4). This chimeric protein was designed for testing the interaction of this viral protein with rabbit CD200R using flow cytometry and surface plasmon resonance techniques. However, this soluble chimeric protein was expressed at low levels (<200ng/ml). The chimeric protein was re-designed by including different portions of the extracellular region to achieve better expression but these attempts failed to promote expression and there was no way of verification for the proper folding of that low expressed recombinant protein. When concentrated tissue culture supernatant was biotinylated and coated on fluorescent beads to test binding to cells, nonspecific binding to different cells were observed which was attributed to improper folding of that protein (Figure 4.4).

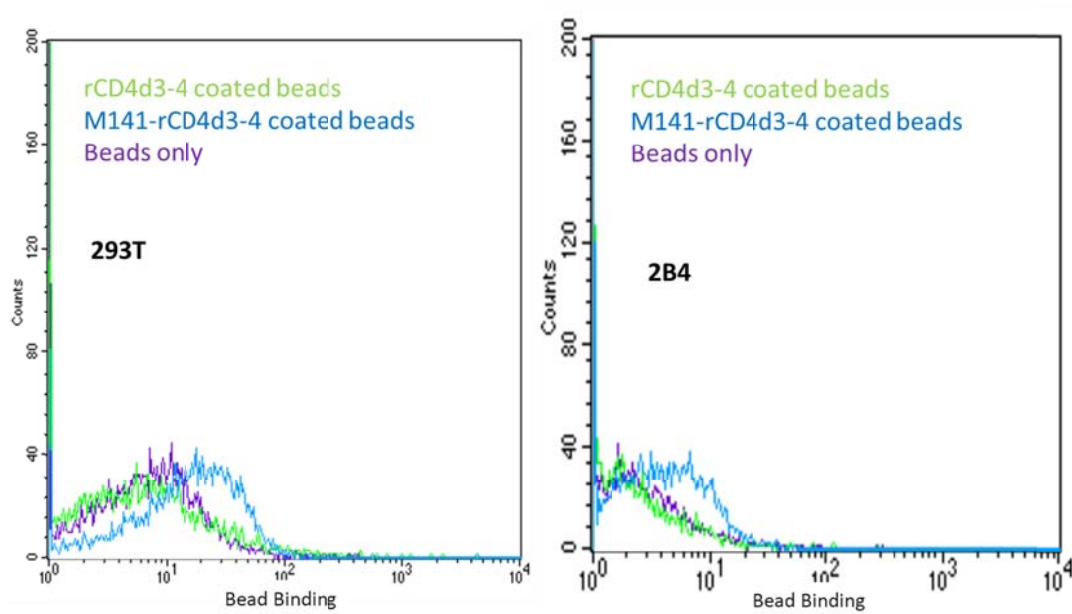
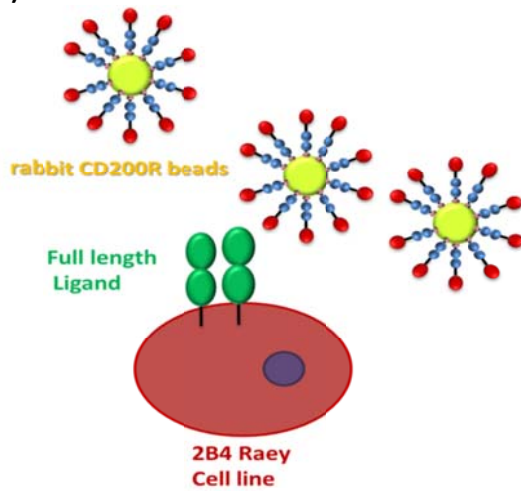


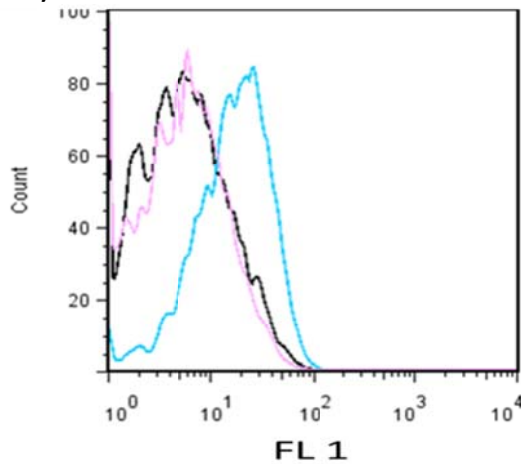
Figure 4.4 M141 coated beads bind non-specifically to different cell types. Flow cytometry plots showing binding of M141 coated biotinylated chimeras (light blue) to 293T HEK (left panel) and 2B4 Reay (right panel)

Therefore, instead of using recombinant M141 proteins, cell lines stably expressing either full length M141 or full length rabbit CD200 were generated. To detect M141 expression on the cell surface a Hemagglutinin tag was added to the N-terminus of the protein. Cells were incubated with fluorescent beads coated with rabbit CD200R- rCD4 chimeric protein and analysed by flow cytometry but no interaction between M141 and rabbit CD200R could be identified (Figure 4.5)

A)



B)



C)

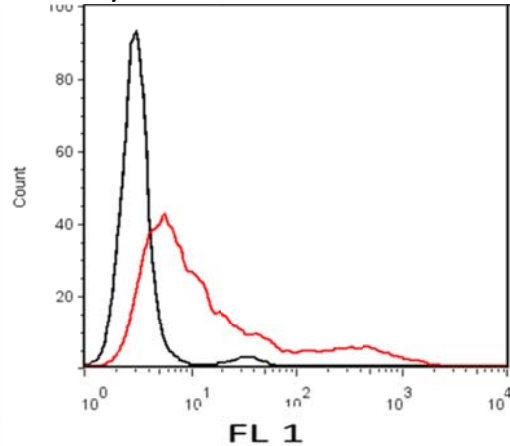


Figure 4.5 Analysis of the interactions of M141 protein using flow cytometry. A) Schematic representation of the experimental design. 2B4 Raey cells, transfected with CD200 or M141 ligand were incubated with green fluorescent Sphero beads coated with rabbit CD200R-rCD4 chimeric protein. B) Histogram showing the interaction between the rabbit CD200 expressing cells and rCD4 coated beads (negative control) (black line) or rabbit CD200R coated beads (positive control) (blue line). The interaction between M141 expressing cells with rabbit CD200R coated beads were shown as pink overlay (pink line). The interaction with the rCD4 coated (negative control) beads was the same for both M141 and CD200 expressing cell lines. C) Confirmation of M141 expression on cells. Histogram showing the fluorescent anti HA antibody staining of the HA tag attached M141 expressing 2B4 cells red line compared to the same antibody staining of HA tag negative protein expressing 2B4 cell line (black line) (representative of three independent experiments).

4.5 Discussion

In this chapter, cloning, characterization and interactions of the rabbit CD200R and CD200 were described. The interaction between rabbit CD200 and CD200R was shown by both cellular and biochemical experiments. In biochemical assays, the CD200-CD200R interaction observed for rabbit had similar affinity compared to the CD200-CD200R interactions observed for other species (187).

These proteins were used to test the possible interaction between rabbit CD200R and M141. However, the cellular assays performed in this study failed to detect the interaction between these two proteins. This either suggests that M141 is not a viral CD200 homologue or that this molecule needs other unknown viral molecules to become effective. Another possibility is that it has evolved to target a different receptor.

Although M141 had 23% amino acid sequence identity with rabbit CD200 (Table 4.1) and this level of identity is close to the sequence identities observed for other vCD200 homologues (see section 1.2.4), it differs from the viral CD200 homologues seen in herpes viruses by having only one extracellular domain and an incomplete transmembrane domain (Figure 4.1). This may suggest a more significant differentiation from the original CD200 molecule initially captured by the virus from the host genome. Another striking difference between the M141 molecule and the host CD200 molecule is the difference in the N-linked glycosylation levels of the first Ig domain. While the original CD200 molecule contains three glycosylation sites, the M141 molecule has six (27) the myxoma virus and

seven sites in the Shope Fibroma Virus, another pox virus which causes cutaneous and ophthalmic diseases in rabbits (94) (185). This level of glycosylation might be responsible for the loss of CD200R interaction ability and puts carbohydrate binding inhibitory receptors as candidate binding partners of these molecules. In conclusion, the M141 protein may have evolved from a vCD200 homologue in the beginning but lost its ability to interact with CD200R while gaining an additional virulence advantage. This hypothesis is supported by the experimental observations discussed in this chapter.

Additionally, a previous study (194), showing M141 mediated down regulation in macrophage activity using RAW 264.7 cell line, a mouse macrophage cell line with no detectable surface mCD200R expression (data not shown), also indicates a CD200R independent mechanism for this protein.

The key difficulty in searching for a receptor for M141 molecule is the difficulty in producing recombinant soluble protein. The rat CD4 and biotinylation site containing chimeric proteins of M141 were generated in three different lengths and with different signal peptide sequences to facilitate expression. However transfected 293T cells failed to produce good amounts of soluble proteins and the low level of secreted protein, after concentration, biotinylation and immobilization on beads, resulted in non-specific binding with different cell lines (Figure 4.4). Together with the low expression, non-specific interactions were interpreted as mis-folding of the chimeric protein. This is a major difficulty in expressing viral proteins in eukaryotic cells. Other than that, this molecule

contains a free cysteine residue on the Ig domain which might also be facilitating the non-specific interactions. This possibility can be tested by using reducing conditions. In addition to this, recombinant molecules with his tag instead of rat CD4 tag and cell lines other than 293T could be tested.

The possibility that M141 uses a different receptor from CD200R can be tested by binding WT and KO viruses to CD200R. Another approach would be to screen other inhibitory immune regulatory receptors, expressed on RAW 264.7 cell line for interaction with M141. Once the puzzle is solved for M141 molecule, these findings may give insight for the other candidate viral CD200 homologues observed in various pox viruses (Figure 4.1).

Chapter 5:

Screening for possible bacteria-receptor interactions among members of the CD200R family

5.1 Introduction

Different examples of how pathogens target inhibitory immune regulatory receptors were discussed in detail in Chapter 1. One way of doing this is to mimic the host ligand-receptor interaction by using a ligand captured by the pathogen from host, examples of which are mainly seen in herpes and pox viruses as well as sialylated bacteria (targeting Siglec family of receptors) (45, 67, 68). Another more recently identified way is targeting the inhibitory receptor without mimicking the ligand-receptor interaction. This is exemplified by bacterial binding to PIR receptors where the non-ligand binding extracellular domains of PIR-B react with an unknown bacterial molecule (130). This second approach does not require a ligand like pathogen molecule to mediate the interaction between pathogen and the receptor and therefore, any pathogen can be a candidate for such an immune downregulation.

The counterbalance theory suggests that activating paired receptors with very high amino acid sequence identity to their inhibitory partner might have evolved to counterbalance the pathogen threat that is targeting anywhere on the inhibitory receptor (14). This may

also explain the reason behind the existence of activating members of the CD200R family which bind neither the ligand (CD200) nor any other identified host molecule despite their very high sequence identity with their inhibitory partner (CD200R) (80, 187). Therefore, various bacteria were screened for binding to CD200R family proteins and also some other paired receptors.

5.2 Development of a screening strategy

Screening different bacteria for possible interactions with different members of CD200R paired receptor family required a quick and robust assay. Different approaches including using plate bound receptors, receptors expressed on cell surfaces, or oligomerization of soluble receptors using enzyme linked secondary antibodies were tested for bacterial binding during the optimization of the assay. Finally, incubating receptors coated on magnetic beads with fluorescently stained bacteria was shown to be better in terms of receptor expression levels and signals generated by stained bacteria binding to receptors, and this was used as the experimental screening strategy (Figure 5.1). Bacteria were visualized by incubating with a fluorescent DNA binding stain SYTO-60 and analysed by flow cytometry (Figure 5.1 A,B). Extracellular domains of all receptors of interest were generated as chimeric soluble proteins which also contained rat CD4 domains 3-4 and a biotinylation sequence attached at the C terminus of the receptor sequences. These protein containing tissue culture supernatants were quantified using inhibition ELISA strategy (see section 2.4.2). Upon confirmation of adequate protein yield, Supernatants

were concentrated and biotinylated. Biotinylation of correct sized chimeric proteins were checked by western blot using streptavidin conjugated horse radish peroxidase labeling (Figure 5.1 F). coated on streptavidin coated magnetic beads and expression levels were detected using specific antibodies (Figure 5.1 C).

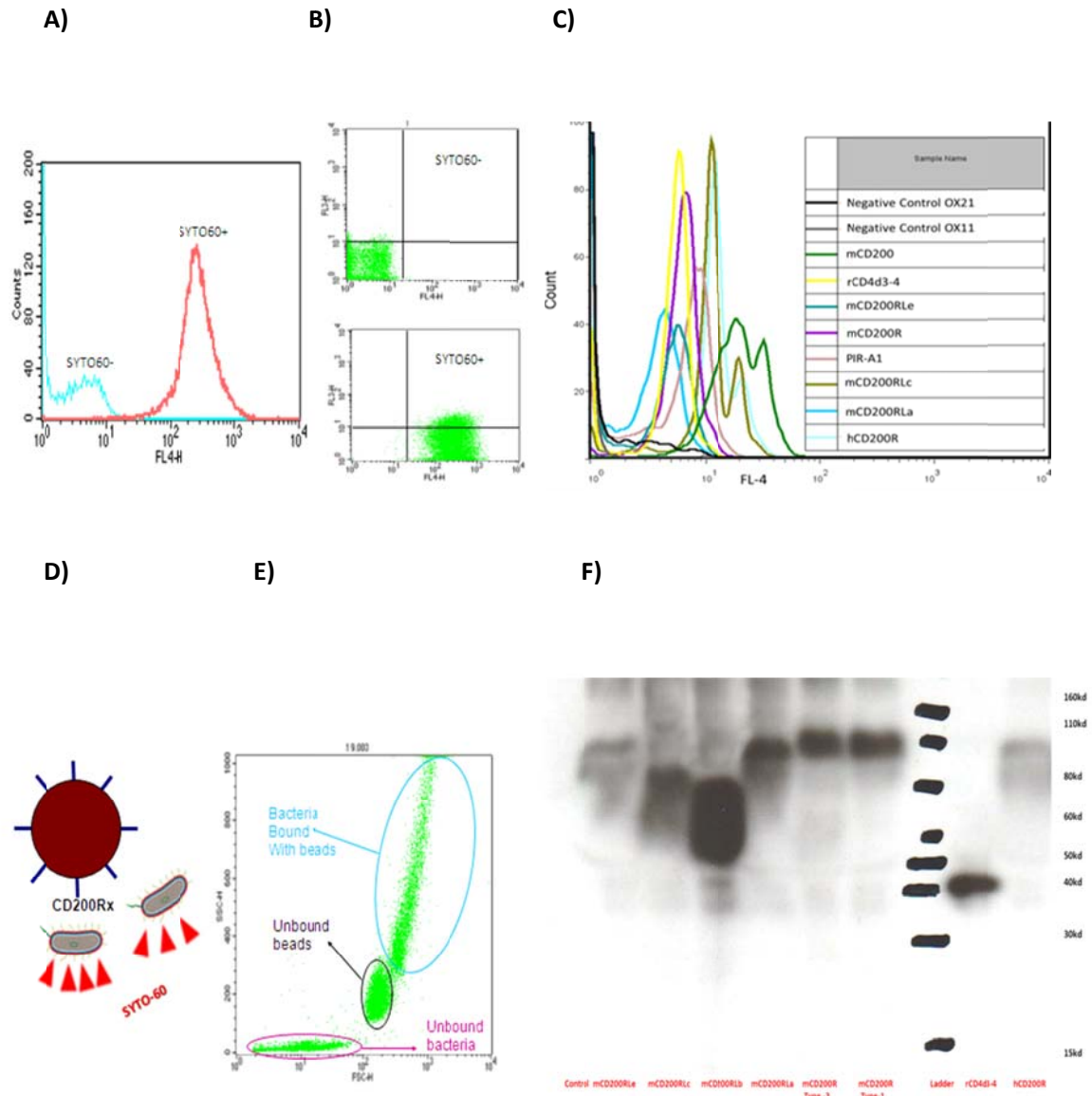


Figure 5.1 The experimental design for bacterial binding A) Histogram showing the difference in FL4 channel between SYTO-60 stained (red) and unstained (light blue) *E. coli*. B) Dot plot showing unstained (upper) and SYTO-60 stained (lower) *E. coli*. C) Levels of proteins (as detected by their specific antibodies) coated on beads are shown for a selection of samples. D) Illustration of the experimental design. Magnetic beads (dark red big dots) coated with the receptor of interest (blue spikes) were co-incubated with fluorescent SYTO 60 stained (light red triangles) bacteria. E) Representative dot plot showing that unbound beads, unbound bacteria and beads bound with bacteria can be discriminated as sub-populations according to relative particle sizes F) Western Blot image showing the biotinylated CD200R family members used in the bead coating experiments. Control stands for mCD200R-rCD4 d3-4 containing tissue culture supernatant which was not biotinylated.

All receptors were shown to bind, although at different levels, on magnetic beads with more than a log difference compared to negative control and this was accepted to be sufficient thus, no further optimizations were made. Beads have a greater size compared to bacteria which allows gating out the unbound bacteria in forward-side scatter analysis. Therefore, any fluorescence detected would only represent bacteria bound to the specific receptor coated on the beads (Figure 5.1 D-E).

5.3 *S. aureus* was shown to interact with PIR-A1 but no other receptors tested

Receptor coated beads were co-incubated with SYTO-60 stained bacteria in a ratio of 10 bacteria per bead (10^8 bacteria vs 10^7 beads). After 90 minutes of incubation at 4°C, 2% formaldehyde was added to kill the bacteria and the samples were analysed by flow cytometry. PIR-A1 (first two domains only)-rCD4 domains 3-4 biotinylated chimeric protein was used as a positive control due to its known binding ability with *Staphylococcus aureus* (130) and rat CD4 domains 3-4 biotinylated protein was used as a negative control. Members of CD200R family were tested together with members of the SIRP family for their binding to the fluorescently labelled *S. aureus* and only PIR-A1 (positive control) was shown to bind to the bacteria (Figure 5.2). The results indicated that none of the tested receptors are able to bind to *S. aureus*. The same experimental settings were used to test interactions of other bacteria with these receptors (Table 5.1) but no interaction has been detected (Figure 5.3).

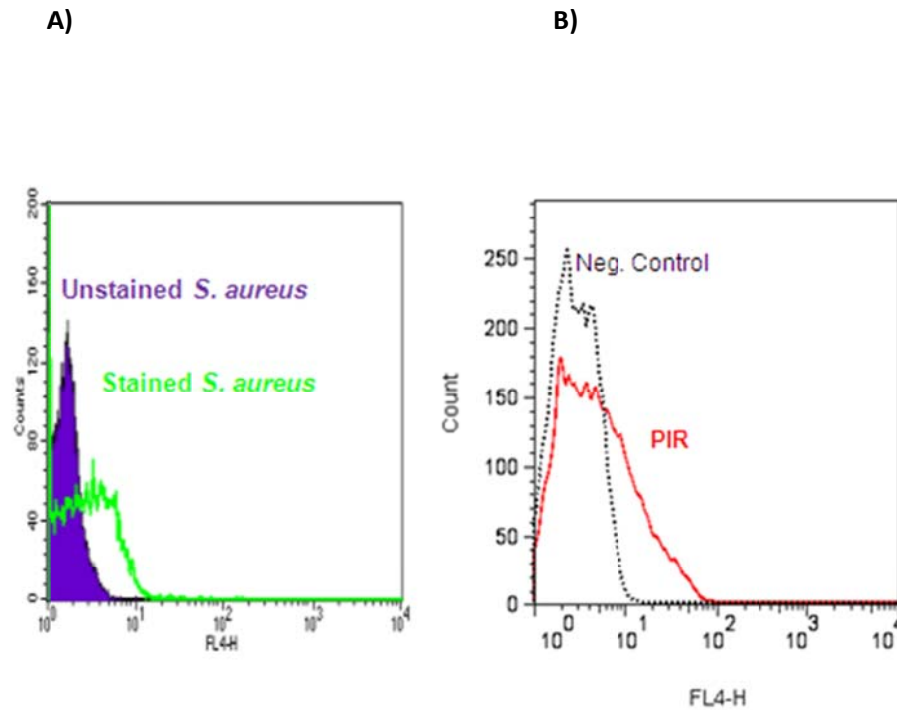


Figure 5.2: PIR-A1 binding to fluorescently labelled *S. aureus* can be detected. A) Histogram showing the level of SYTO-60 staining for *S. aureus* (green) compared to unstained bacteria (purple). B) Histogram showing fluorescence emitted from bacteria bound to PIR-A1 coated magnetic beads (shown as red line) compared to rCD4 d3-4 coated beads (shown as “doted” black line). PIR refers to PIR-A1 and Neg. Control refers to rCD4 d3-4. (Representative of three independent experiments. Bead integrity was not checked after the experiment.)

Bacteria used in this experiment	Receptors used in this experiment
<i>S. aureus</i> <i>N. meningitidis</i> (serotypes A and C) <i>P. aeruginosa</i> <i>S. pneumonia</i> <i>H. influenzae</i> <i>E. coli</i>	hCD200R hCD200RLa mCD200R (type 1 and 2) mCD200RLa mCD200RLb mCD200RLc mCD200RLe hSIRP α hSIRP β 2 mSIRP α mSIRP β mPIR-A1 rCD4 domains3-4

Table 5.1 Summary of bacterial strains and receptor types used in the screening studies

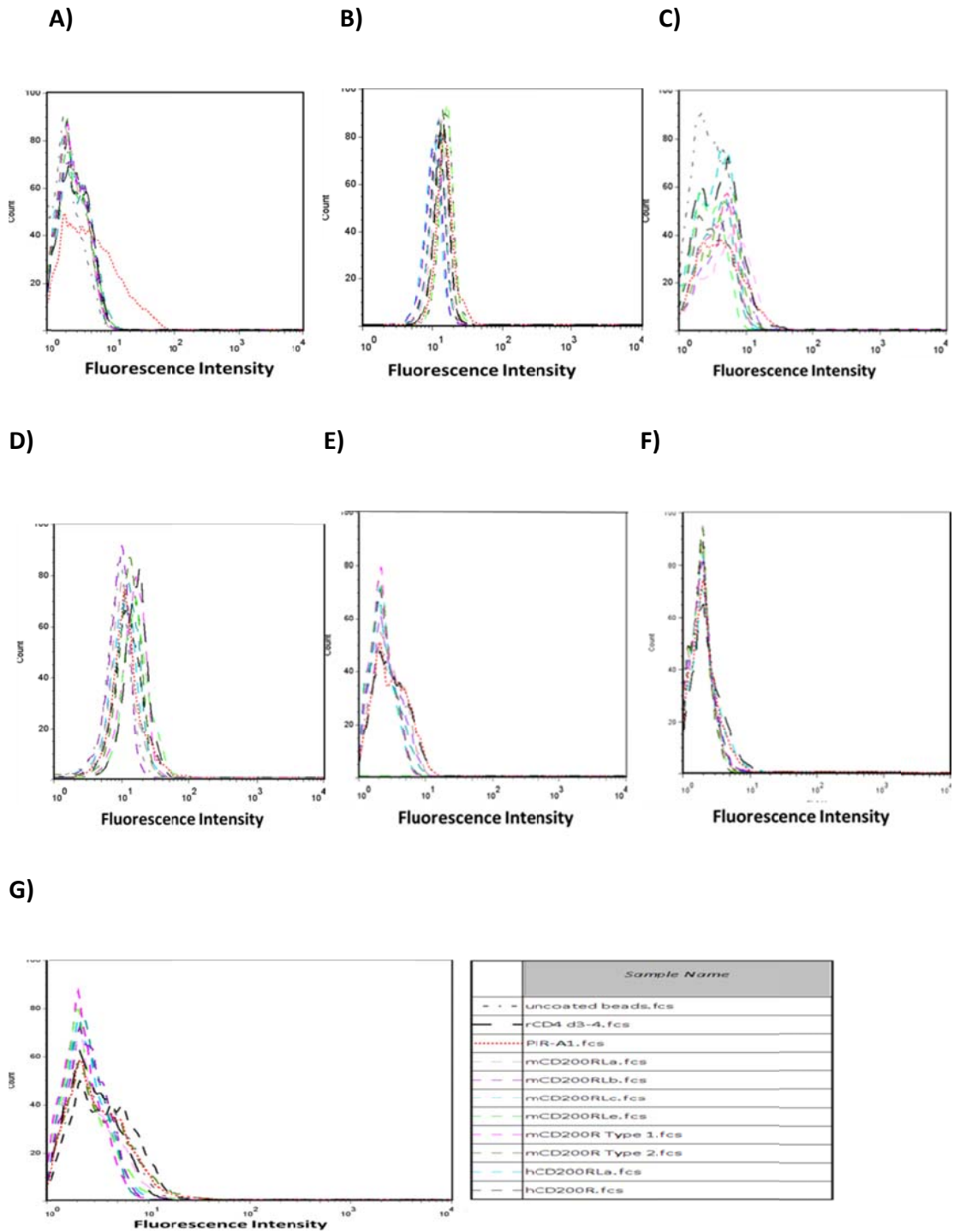


Figure 5.3 Flow cytometry plots showing binding of various fluorescent labeled bacteria to magnetic beads coated with members of CD200R family. A-G represent binding of coated beads to *S. aureus*, *E. coli* (K12), *N. meningitidis* (serotype C), *N. meningitidis* (serotype A), *H. influenzae*, *P. aeruginosa*, *S. pneumoniae* respectively. H) Schematic representation of the proteins used in this experiment. (Representative of at least two experiments.)

5.4 Discussion

The experimental strategy has been shown to be capable of detecting possible bacteria-receptor interactions as seen in the example of *S. aureus*- PIR-A1. This bacterium was extensively studied in previous research on binding to both PIR-A1 and PIR-B and that result was replicated using the novel experimental strategy explained above (130).

Therefore, it is clear that there is no binding of any other tested receptors to *S. aureus*. Additionally, in the same study the interactions of PIR-B with other bacteria such as *E. coli* was also mentioned but no information was given for interactions of PIR-A1. With this novel strategy, no interaction between PIR-A1 and *E. coli* was detected.

Although no interaction has been detected for members of CD200R family (Figure 5.3) and SIRP, further experimental investigations using a wider range of bacteria or different media conditions should be made before concluding that the receptors don't bind pathogens.

The extended range of bacteria to be tested should particularly include members of commensal flora especially those in gastrointestinal system which have been shown to have interactions with the components of mucosal immunity (100). Other than the members of commensal flora, pathogens of the gastrointestinal should be included in the list of pathogens to be screened. The large number of bacteria types in this area may

cause difficulties to the current screening system which can screen for one interaction at a time. An alternative approach could be incubation of receptor coated beads with the contents of the healthy or inflamed gut taken from laboratory animals and introducing a bacteria specific PCR strategy to identify the bacteria that bound with the beads.

However, it should also be noted that the PIR family of receptors which form the basis of this hypothesis are very different in terms of structure and function compared with the CD200R family that has been the focus of this thesis. Therefore, despite other examples in different paired receptor families such as PIR binding to *S. aureus* and Siglecs binding to various bacteria, this does not necessarily mean that CD200R family members should bind with pathogens and other possibilities for the ligands of the activating members should be considered as well.

Finally, pathogens other than bacteria and viruses can be candidate binding partners of these receptors. It has been noted that some *Leishmania* species are capable of inducing host CD200 as a virulence factor to downregulate immune response against them (43). Another parasite *N. brasiliensis*, when infested, was shown to upregulate CD200R and CD200RLb expression on various cell types (173). These findings may suggest interaction of protozoans with members of the family which might be screened using a similar strategy as described in this chapter.

Chapter 6:

Characterization of novel antibodies against members of the mouse CD200R family

6.1 Introduction

The mouse CD200R family, when it was first characterized in C57BL/6 mice, consisted of one inhibitory (mCD200R) and three activating genes (mCD200RLa-c) plus an incomplete pseudogene sequence (mCD200RLd) (187). A few years later, another activating member was identified in NOD and CD1 mice and named as CD200RLe (173). Finally, a polymorphic form of inhibitory receptor was reported (Accession No: BAE32516) for NOD mice and it was shown to be present in eight out of twenty two different mice strains screened (Chapter 3). Although the scientific knowledge has increased dramatically on identification and characterization of novel members of mouse CD200R family, there has not been a comprehensive study on the tissue distributions of most members of the family. So far only mCD200R, mCD200RLa and mCD200RLb were studied for how they are expressed on different cells and no information is available for CD200RLc and CD200RLe. This is partly because of the unavailability of discriminative antibodies against these proteins. OX110, which can detect CD200RLc, cross-reacts with CD200R; and dx109, which detects CD200RLe, also cross-reacts with CD200R. Therefore, novel antibodies are required to study the distribution of these molecules which might provide insights on their functions.

6.2 Generation of stable cell lines expressing members of CD200R family

To generate novel antibodies and analyse the functional characteristics of the receptors, stable rat cell lines (RBL.2H3) expressing the receptors of interest were made using full length constructs of the CD200R family together with DAP12 (transmembrane adapter for CD200RL's) for immunization of rats (see section 2.3). Expression levels were detected by flow cytometry after staining with specific antibodies (Figure 6.1). Except for mCD200RLa whose expression level was barely detectable using dx87 antibody, there was clear expression for all CD200RL's and CD200R (type 1). In particular, good levels of expression were obtained for CD200RLc and CD200R (used in antibody production).

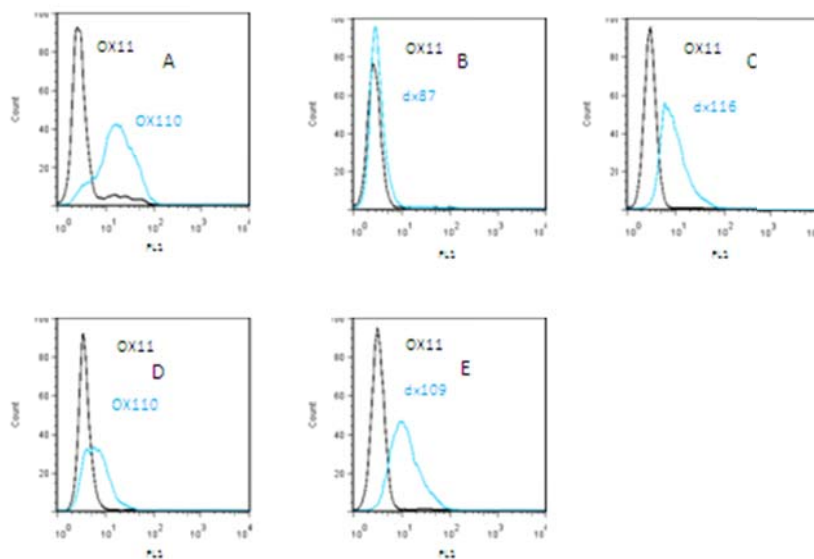


Figure 6.1 Members of the mouse CD200R family were expressed on RBL.2H3 cell lines.

Expression of CD200R, CD200RLa, CD200RLb, CD200RLc, CD200RLe are detected using specific antibodies (blue) in flow cytometry plots A-E respectively. Negative control staining (OX11) is shown in black in each plot.

6.3 Functional analyses on CD200RL-DAP12 molecules expressed on RBL.2H3 cell

Line

The functional outcome of antibody stimulation was tested using specific antibodies against CD200RLa-c. CD200RLe is omitted due to a lack of the specific dx109 antibody in adequate amount. Each cell line was stimulated first with primary antibody and then cross linked with mouse anti rat IgG (see section 2.3.5). The supernatants were analysed for released hexosaminidase (see section 2.4.4). mCD200RLb and mCD200RLc could be stimulated with primary antibody alone whereas mCD200RLa stimulation required a secondary cross linker (anti rat IgG antibody) (Figure 6.2). This degranulation assay confirms the existence of properly functioning receptor-adaptor complex on the surface for all three receptors. Considering that there was very low staining for CD200RLa (Figure 6.1), this assay is more sensitive than flow cytometry for demonstrating the receptor expression. However, the requirement for a secondary cross-linker only for this receptor is in accordance with the low staining observed in flow cytometric analyses which might indicate either a low affinity interaction between dx87 and mCD200RLa or a low level expression of that receptor.

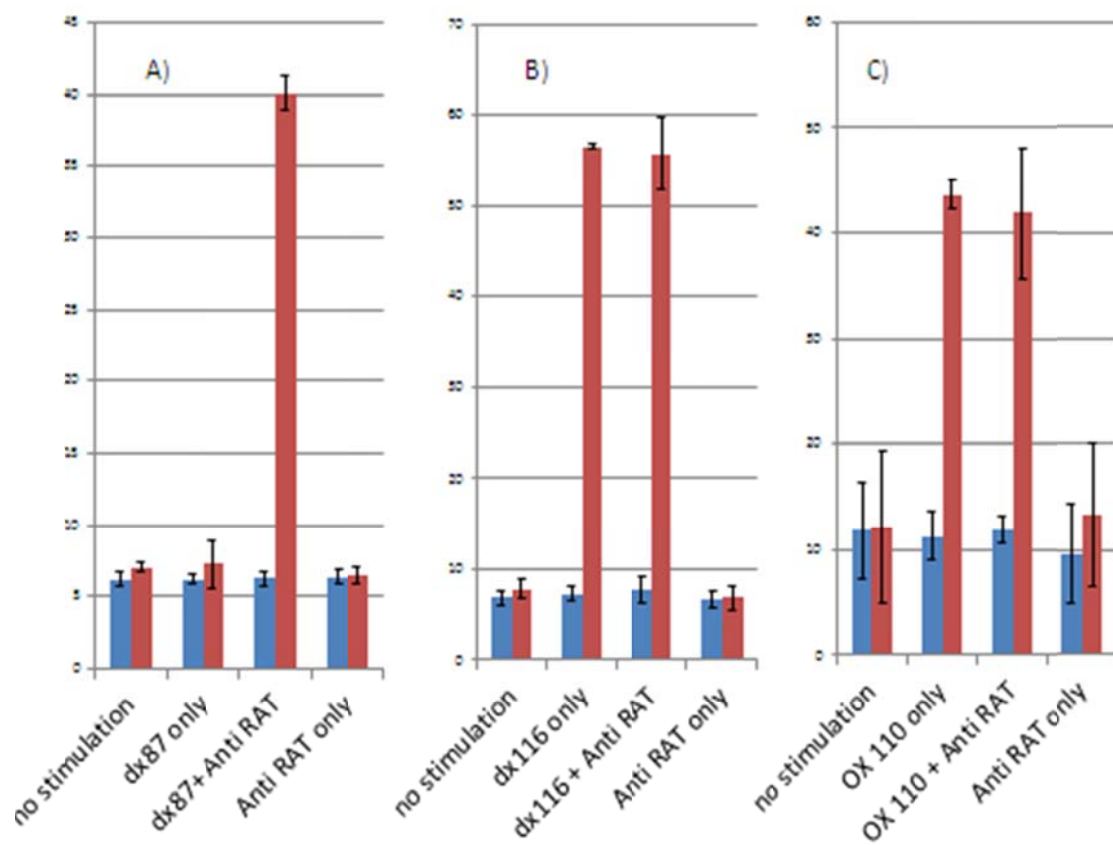


Figure 6.2 Stable RBL.2H3 cell lines expressing CD200RL's can generate cellular activation upon stimulation with specific antibodies. A-C) Percentage degranulation achieved by stimulating each stable cell line was shown by red bars for mCD200RLa, mCD200RLb and mCD200RLc respectively. Degranulation in mock transfected cell line was shown in blue bars. dx87, dx116 and OX110 refer to specific antibodies (Rat anti mouse CD200RL) against these receptors. Anti RAT stands for the mouse anti rat IgG antibody.

The transfected RBL.2H3 cell lines also provided an assay for screening for possible ligands. No effect was found with CD200 coated beads, when they were used instead of antibodies to stimulate degranulation as expected from the very weak binding of CD200 to the receptors (80). This assay could not be used to screen for bacterial binding due to nonspecific degranulation of the RBL.2H3 cell lines when incubated with the bacteria.

6.4 Characterization of Antibody Binding Properties for Novel Antibodies

These cell lines together with soluble chimeric proteins were used by Marie-Laure Aknin (research assistant) to immunize rats for the generation of specific antibodies and two monoclonal antibodies OX131 and OX132 were generated. These monoclonals were tested for cross reactivity between members of CD200R family using biotinylated rCD4 domains 3-4 containing chimeras of extracellular domains of mouse CD200R family members (Figure 6.3).

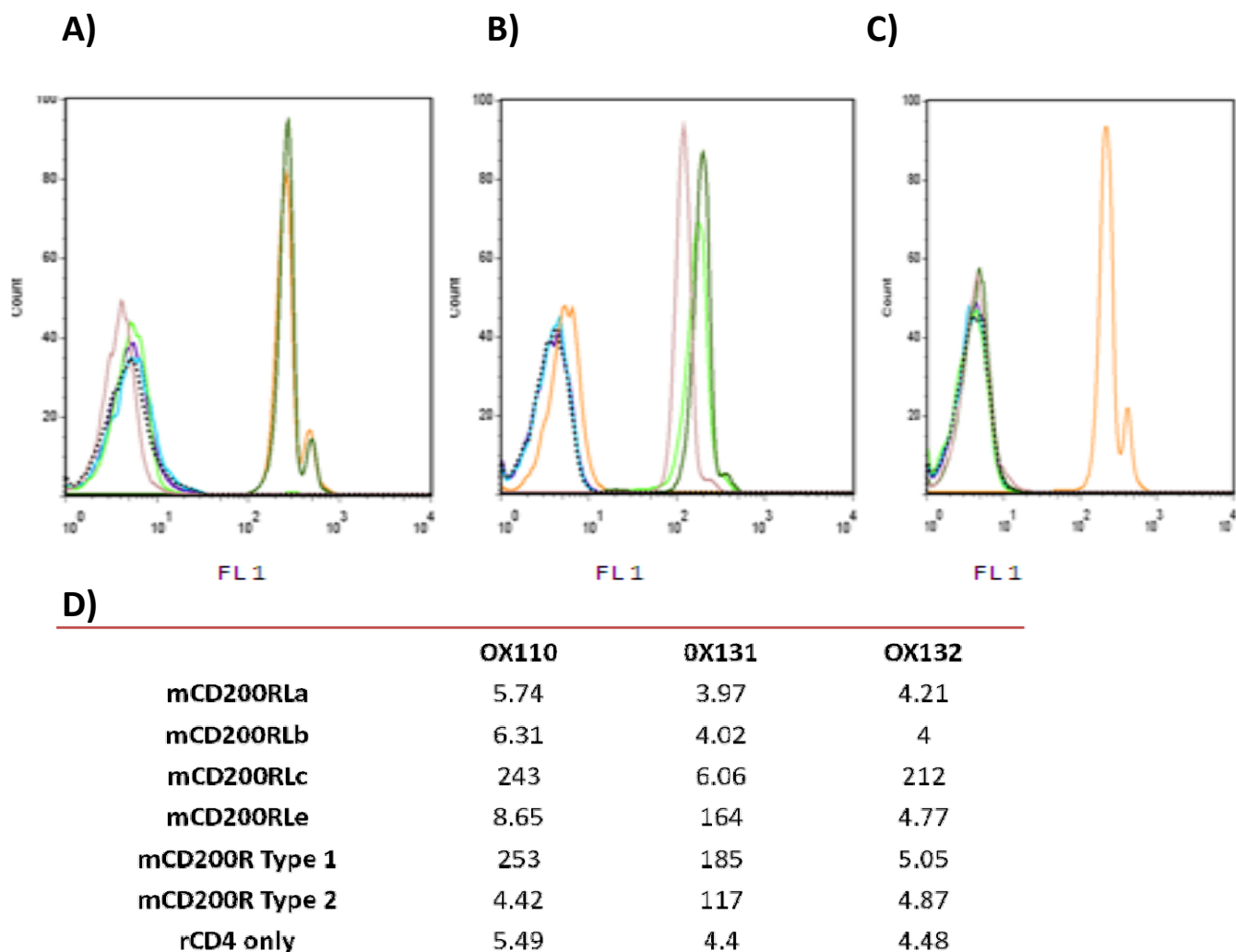


Figure 6.3 Analyses of the specificities of novel antibodies. OX110 (A), OX131 (B) and OX132 (C) were applied to magnetic beads coated with all members of mouse CD200R family. Colour codes for lines are as follows: mCD200R (type 1), mCD200R (type 2), mCD200RLa, mCD200RLb, mCD200RLc, mCD200RLe, rCD4 d3-4 only (dotted). Adequate bead coating levels (at least one log difference compared to negative control) for each molecule were identified using specific antibodies (not shown). Thus OX110 binds to mCD200R (type1) and mCD200RLc, OX131 binds to mCD200R (both types) and mCD200RLc, OX132 binds to mCD200RLc. D) Table showing the mean fluorescence intensities for each antibody-receptor interaction represented in the histograms (A-C).

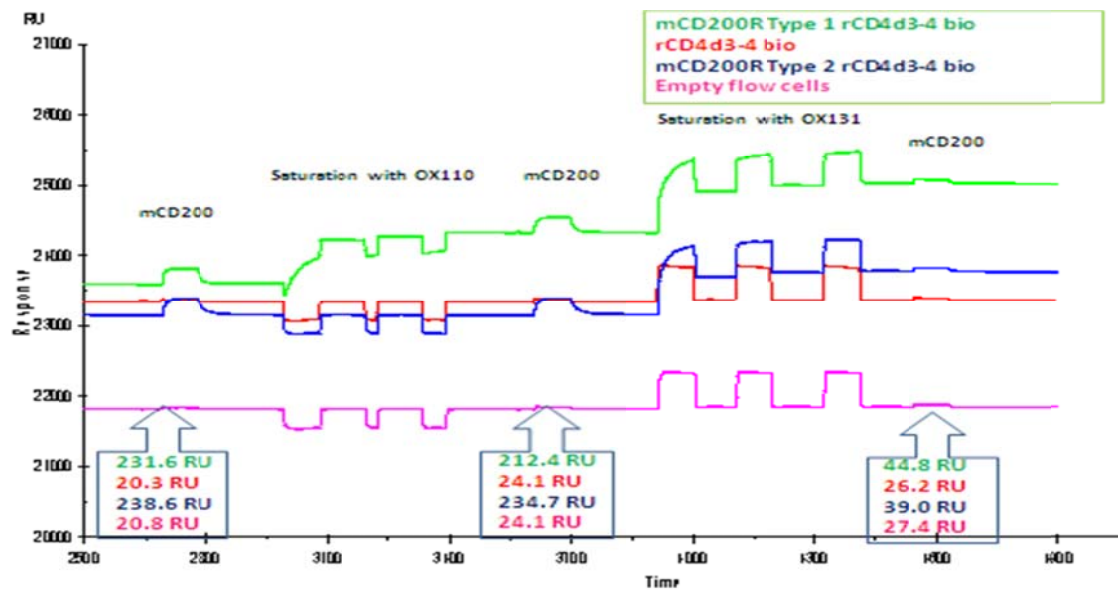
OX131 was shown to detect both CD200R types (type 1 and 2) whereas OX110 could only detect CD200R (type 1). OX131 had no binding with CD200RLc but bound with CD200RLe. Although this antibody is not entirely specific due to this cross binding, it can still be used to study the distribution of CD200R in C57BL/6 mice as this strain is devoid of CD200RLe. On the other hand the second novel antibody OX132 was shown to be completely specific against CD200RLc which may be used to reveal the tissue distribution of this molecule and for functional studies.

6.5 OX131 but not OX110 can block CD200-CD200R interaction

The effects of mCD200R antibodies (OX110 and OX131) on the mCD200-mCD200R interaction were studied using surface plasmon resonance. Recombinant proteins containing the extracellular domains of both mCD200R types (type 1 and 2) + rCD4 (d3-4) biotin chimera were immobilized onto a streptavidin coated CM5 chip together with the negative control which only has rCD4 (d3-4) biotinylated protein (Figure 6.4 A). Recombinant soluble mCD200 was passed over both receptor types and gave indistinguishable binding. Multiple doses of OX110 antibody were applied to both receptors which caused saturation of mCD200R type 1 but had no binding to mCD200R type 2. After that, mCD200 was re passed and bound with the same levels as before which indicated OX110 had no effect on mCD200-mCD200R interaction. This was repeated for OX131. OX131 caused the same level of saturation for both receptor types which indicated that the previous OX110 saturation on mCD200R type 1 did not affect sequential

OX131 binding. However, when mCD200 was re passed, there was no binding. This indicated that OX131 blocked the mCD200-mCD200R interaction for both receptor types and OX110 recognized a different epitope on CD200R type 1 and did not block CD200R binding. mCD200 binding to both receptor types was also confirmed by showing that fluorescent beads coated with recombinant mCD200 binds indistinguishable to 2B4 Reay cell lines expressing either mCD200R type 1 or type 2 (Figure 6.4 B).

A)



B)

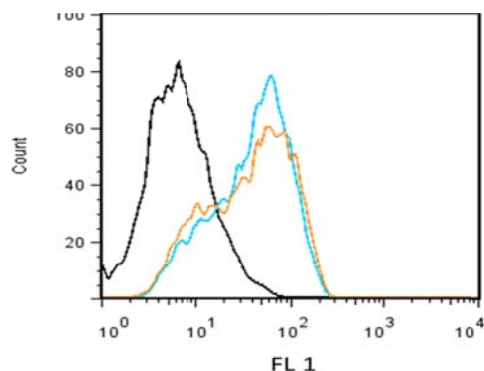


Figure 6.4 OX131 but not OX110 blocks the mCD200-mCD200R interaction. A) Surface plasmon resonance analysis on the CD200-CD200R interaction and the effects of antibodies: Y axis shows the response units, X axis refers to change in time. Flow Cells 1-3 were loaded with approximately equal response units of mCD200R type 1-rCD4 d3-4 biotinylated (green), mCD200R type 2-rCD4 d3-4 biotinylated (blue) or rCD4d3-4 biotinylated only (red). Flow cell 4 was left empty (pink). Sequentially applied soluble proteins are noted on the figure. Numbers in the boxes indicate the level of CD200 binding in response units (RU) at indicated points (shown with arrows) B) Flow cytometry plot showing that mCD200 binds equally to both types of mCD200R. 2B4 Reay cells transfected with mCD200R type 1 (blue line), mCD200R type 2 (orange line) and mock transfected (black line) were incubated with fluorescent beads coated with mCD200 extracellular domains-rCD4 d3-4 biotinylated. Equal levels of CD200R are expressed on each cell line (Figure 6.5 C&D) and no natural CD200R is expressed on mock transfected 2B4 Reay cell line (not shown).

The OX110 and OX131 monoclonal antibodies were tested in cellular assays to assess their capabilities to block the CD200-CD200R interaction. A Stable CHO (Chinese Hamster Ovary) cell line expressing transfected IE^K (mouse MHC Class II molecule) was transduced with full length CD200 using the pFB Hygro vector. These cells were co-incubated with the 2B4-Reay (mouse T cell hybridoma line)- mCD200R cell line which expresses TCR and also the transduced mCD200R. In this model IE^K loaded with MCC (Moth Cytochrome C) generates 2B4 cell stimulation through TCR (assayed as increased IL-2 secretion) that can be inhibited by the CD200-CD200R interaction (see section 2.3.4). Antibodies were tested to see whether they cause any change in the inhibition of the TCR response generated by the CD200-CD200R interaction (Figure 6.5). In addition to the antibodies against mCD200R (OX110 and OX131), another antibody which binds mCD200 (OX90) was also tested in this assay.

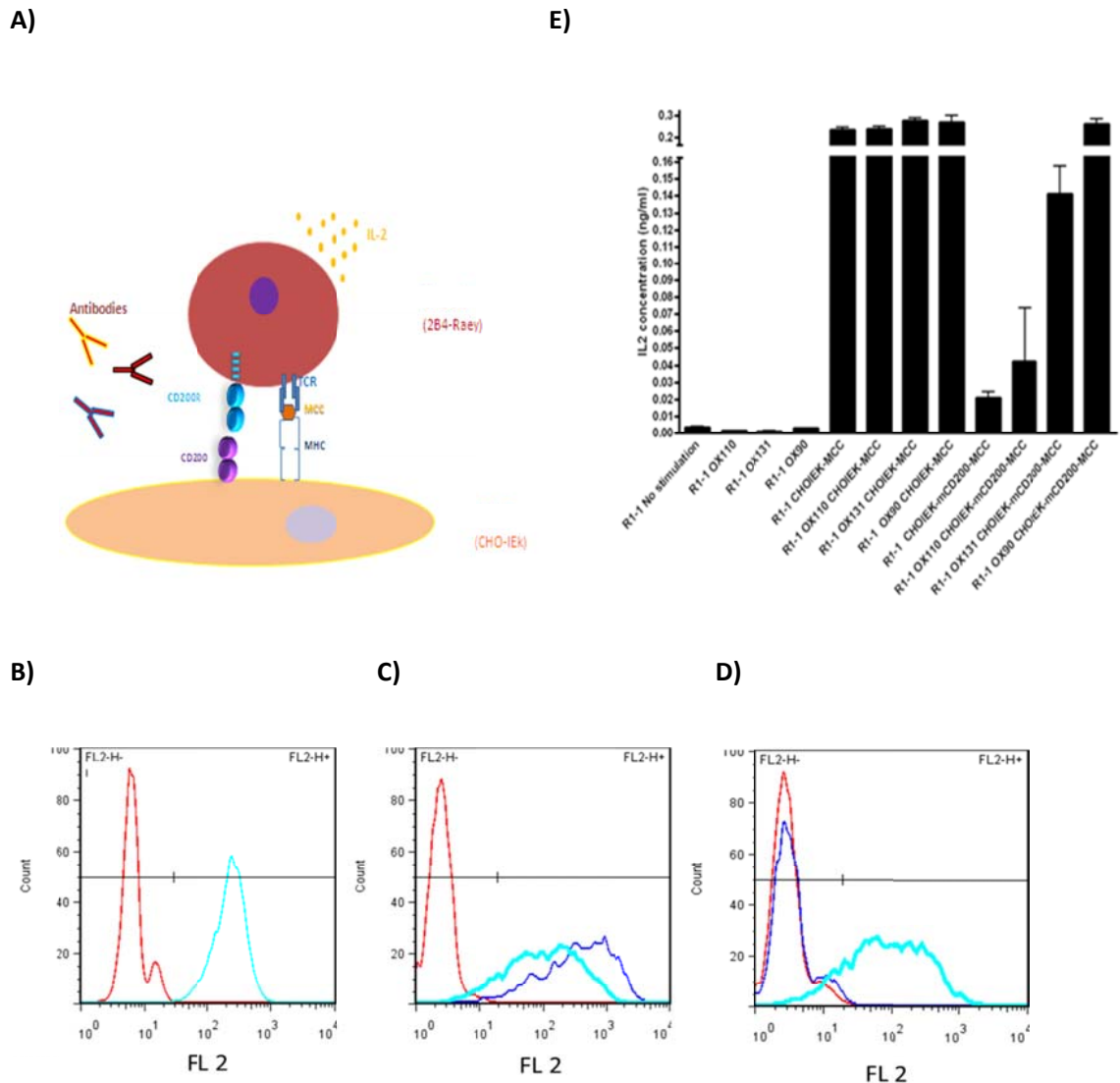


Figure 6.5 OX131 and OX90 can block the inhibition of IL-2 secretion mediated by the mCD200-mCD200R interaction. **A)** Schematic representation of experimental design (APC stands for Antigen Presenting Cell and MHC stands for IE^K). **B)** Flow cytometry plot showing mCD200 expression on full length mCD200 transduced CHO.IE^K cell line. Blue line is OX90 (anti mCD200) staining, red line is OX11 negative control staining. Untransfected CHO. IE^K does not get stained with OX90 (not shown) **C-D)** Facs plots showing mCD200R type 1 and type 2 (respectively) expression on mCD200R transduced 2B4 Reay cell line. Dark blue line is OX110 staining, light blue line is OX131 staining, red line is negative control OX11 staining. 2B4 Reay cell line does not express mCD200 or mCD200R naturally (not shown). **E)** IL-2 secretion levels under different conditions are shown. R1-1 refers to mCD200R type 1 expressing 2B4 Reay cell line. MCC refers to Moth Cytochrome C Protein added in growth media (Representative of four triplicate experiments. Error bars indicate the standard deviation in triplicate results)

This assay has proven to be robust in detecting antibody mediated changes in the inhibition of CD200R mediated IL-2 secretion. OX110, despite binding successfully to cells (Figure 6.5 C), did not cause any significant alteration to the inhibition as predicted, since it does not block the CD200-CD200R interaction. However, OX131 caused a significant but incomplete level of blockage in CD200-CD200R effect. This partial blockage might be because of either the usage of tissue culture supernatant which does not contain high concentration antibody and therefore may not have saturated the receptor, or a possible inhibitory signal being generated when antibody oligomerizes the receptor. Purification of OX131 antibody using Protein G column and low pH elution led to precipitation of the antibody therefore alternative purification strategies were required. Nevertheless, these results correlate with the biochemical characterization findings shown in Figure 6.4. Similar results were observed using mCD200R type 2 expressing 2B4 Reay cell line (not shown). Finally, the CD200-CD200R effect could be blocked totally by using OX90 antibody (formerly developed in this laboratory) which targets mCD200 in contrary to the other antibodies developed against CD200R.

6.6 Discussion

The results of the biochemical characterizations of OX110, OX131 and OX132, the three anti CD200R family monoclonal antibodies, are summarized in Table 6.1. Among these antibodies OX110 (formerly developed in this laboratory) bound to mCD200RLc and mCD200R. The interaction of OX110 with mCD200R was shown not to affect binding of

CD200 or OX131 (novel antibody against CD200R). The OX110 antibody also failed to detect CD200R type 2. Therefore, this antibody probably binds to a region involving the residues that are different between CD200R type 1 and type 2. The CD200R type 1 and type 2 differ by seven amino acids in the N terminal domain (see section 3.6) Both type 1 and type 2 receptors bind to CD200 (see section 6.5) but the failure of OX110 to bind the type 2 receptor indicates that some of these residues are involved and hence are distal to the ligand binding site. However, this seven amino acid residue difference is conserved in eight out of twenty two strains screened (Chapter 3) which may indicate a role for these residues.

Although there is no evidence, this difference might have evolved under a pathogen pressure targeting non-ligand binding regions (such as this one) and in this case it might be involved in differential susceptibility against that threat.

	CD200 R Type 1	CD200 R Type 2	CD200 RLa	CD200 RLb	CD200 RLc	CD200 RLe
OX110	+ (non-blocking)	-	-	-	+	-
OX131	+ (blocking)	+ (blocking)	-	-	-	+
OX132	-	-	-	-	+	-

Table 6.1 Binding specificities of 3 antibodies (OX110, OX131 and OX132) to the members of mouse CD200R family are summarized. +,- indicate presence or absence of binding respectively. Blocking refers that the antibody inhibits the ligand –receptor interaction.

In contrast to OX110, OX131 could detect both types of mCD200R and also was shown to block the CD200-CD200R interaction in both biochemical and functional assays. This indicates a binding site close to ligand binding region for that antibody. Together with OX90, another blocking antibody (but against CD200), this antibody might be useful in various functional studies where blocking the CD200-CD200R interaction is desired.

The blocking of the CD200-CD200R interaction using OX131 (anti receptor antibody) or OX90 (anti ligand antibody) differed slightly in terms of IL-2 release (Section 6.5). This difference may be due to using OX90 from a purified antibody stock and OX131 from tissue culture supernatant whose concentration could not be measured. Another contributing factor for less blocking of IL-2 release inhibition by OX131 could be that

OX131, by targeting the receptor, might be causing a receptor dimerization which generates an inhibition signal that agonizes the ligand binding. To test this hypothesis, effect of antibodies could be screened in settings where cells are stimulated externally such as by anti CD3 antibodies and CD200 is not present.

Finally, OX132 was shown to be specific for mCD200RLc. Therefore, this antibody can be used for characterization of mCD200RLc distribution in various tissues. Although OX131 was similarly designed to re-analyse distribution of CD200R, it has significant cross binding to CD200RLc. Nevertheless, provided that mCD200RLc does not exist in C57BL/6 mice, this antibody is still promising to analyse distribution of mCD200R in this strain.

OX110 has been widely used as anti CD200R antibody. In one study it was used to show CD200R as an activated basophil marker (163). On the other hand, another study showed CD200RLc expression in basophils using microarrays (173). Provided that OX110 is an antibody cross reacting between CD200R and CD200RLc, the observation of CD200R as an activating basophil marker should be reassessed since those cells are also expressing CD200RLc. In another example where OX110 was used as an agonist for triggering CD200R to ameliorate inflammation upon influenza infection, it was shown to decrease inflammation when given intravenously but not when given intraperitoneally (154). However CD200 Fc recombinant protein was effective in both settings. This difference may also be due to the differential expressions of CD200R and CD200RLc, which are both triggered by OX110 administration, in target tissues. These findings illustrate the problems of reagent specificity for rapidly evolving receptors. This type of cross-reactivity

also has implications for the development of therapeutic antibodies against paired receptors as discussed for the SIRP family (195).

CHAPTER 7:

General discussion

7.1 Concluding Remarks on the evolution of paired receptors

Pathogen driven evolution of paired receptor families is a very common phenomenon which follows the general principles of Red Queen effect (166). Interacting species (both paired receptors of the host and the virulence factors of the pathogen) must continuously evolve to maintain relative fitness to each other. The basic principles shared in many examples discussed include the high evolutionary rate and the increased gene duplications, conversions and recombinations that contribute to the level of polymorphisms. Most paired receptors evolve in a fashion that can be explained by the “birth and death process” (131). According to this process, new alleles are born from existing ones under evolutionary pressure and they are maintained with regard to the advantages they provide as long as the pressure continues. However, when the pressure is overcome, most of them rapidly perish. This explains the dynamics behind the evolution of gene families in general, but more specifically there is another important point that is shared between various paired receptor families which is the very high level of sequence identity between members, and this suggests other mechanisms. A likely explanation for these conserved sequences is the role of concerted evolution which is especially dominant over genes that are localized next to each other (112). A good example is the evolution of Siglec-5 and Siglec-14 which are 99% identical (5). In this case numerous gene conversions cause all contributing genes to be highly similar to each other and therefore genes in that

species evolve all together which makes them very homogenous in the species but considerably different among species. However, although homogenization is desired, so that activating receptors can bind with the pathogen that is targeting the inhibitory one, it needs to be kept at optimum levels to let differentiation proceed. Therefore, some paired receptors use strategies to limit concerted evolution such as spacing genes apart by putting genes that are not members of the family in between them (seen in Nkrp1, LILR and CEACAM families) or inversely positioning members (inverted clusters) to limit conversions (seen in Nkrp1, Siglec, LILR and CEACAM families) (15).

Another contributing factor for the high homogeneity between genes of the same species is that activating members are formed over and over independently for every species. They are short lived and mostly needed until pathogen pressure is gone which enhances their elimination. Therefore unlike inhibitory receptors, activating ones do not follow an evolutionary path from an ancestral activating gene that diverges into homolog activating genes in different species (1). Instead they are generated independently, from the single inhibitory receptor of that species and this makes them highly similar within species.

Although dynamics behind pathogen driven evolution of paired receptors have been resolved for many individual cases, there are still many receptors without an identified ligand or function in all paired receptor families. One difficulty in search for novel ligand-receptor interactions is that working with strains of lab animals and selected types of pathogens only gives a limited selection of the repertoire of receptor genes and virulence

factors that exist in the genomic pool of those species. Therefore most probably virulence factors like m144 in MCMV or M141R in myxoma virus bind to some inhibitory and activating receptors in a sub-population of the wild type animals which we do not know of yet. Similarly, there are probably pathogenic ligands in wild forms of that microbe for at least some of the activating receptors which, for the moment, remain without any known functionality.

Another limiting factor is merely searching for pathogen molecules that have mimicry to host's ligands. Although examples of host ligand mimicking virulence factors are abundant (m157-Ly49, vCD200-CD200R, sialated pathogen proteins-Siglecs), there are exceptions such as GBS beta protein- Siglec 5 interaction or *S. aureus*-PIR interaction. In these cases pathogens do not use immunevasins which resemble natural ligand and bind to ligand binding region but use a completely independent molecule that engages somewhere else on receptor to generate the same outcome. "The Counterbalance theory on evolution of paired receptor families" addresses this phenomenon and sees that as the main driving force behind high sequence identity between extracellular domains of activating and inhibiting receptors of the same family (14). Because, that is the easiest way for the activating receptor to counterbalance the pathogen threat, which may be directed to anywhere on the inhibitory receptor. With this new approach, it can be assumed that any pathogen with any molecule regardless of its resemblance to the natural ligand can be a candidate ligand for a paired receptor family which makes a search for ligand extremely difficult.

Apart from that, some activating receptors might have been formed against a pathogenic threat that does not exist anymore. In this case the pathogen easily gets rid of the unsuccessful ligand that has been counterbalanced by host but it takes a much longer time for the host to delete the activating receptors that are not required anymore (see discussion on RCTL-Nkrp1 interaction). In such conditions, finding a ligand is impossible.

Finally although the term “paired receptor” defines a structural similarity in organization of these receptors, it does not give us general clues on how they function. Since forming an activating pair is a simple strategy for the inhibitory receptor to overcome an evolutionary pressure, it has been adopted by many independent receptors with wide range of functions. Therefore an interaction that is identified for a receptor family may or may not help solution of the puzzle for the other one. Because of this, it is also possible that some receptor families might have adopted this strategy against requirements other than pathogens and in this case endogenous ligands or an unrelated exogenous ligand may possibly exist.

7.2 Achievements of this study

The CD200R family presents the general characteristics of paired receptors. This includes well known homeostatic roles for the inhibitory receptor (187, 188), presence of activating members which have high sequence identity with their inhibiting partner (CD200R) but no interaction with CD200 (80), identified pathogen driven evolutionary

pressure on the inhibiting member of the family (67, 68, 103, 150) and considerable level of polymorphism among members of the family (Chapter 3) (4). However, despite the increasing scientific knowledge on the evolution of other paired receptors, studies on the evolutionary factors shaping the CD200R family have been limited. Instead, ongoing scientific researches in this area have mostly focused on the clinical aspects of CD200-CD200R interaction, how it is modulated in certain diseases and the possibility of therapeutic interventions in those settings (141, 159, 179). This study has focused on the CD200R family as a whole and in different species to resolve the questions mainly on the roles of the activating receptors and the evolution of the family.

7.2.1 Observations on the heterogeneity of the mouse CD200R family

The mouse CD200R family, containing the highest number of activating genes among species studied, has been the central target of this study. This family was shown to have significant level of heterogeneity in terms of distribution of activating members among different mice strains (Chapter 3). This heterogeneity mainly existed on mCD200RLc and mCD200RLe. Each was found to be present in ten of the twenty two studied mice strains in a mutually exclusive fashion. This observation suggests that mCD200RLe and mCD200RLc are allelic genes. Additionally, three strains contained formerly unknown CD200RL genes which had higher sequence identity to mCD200RLc than other activating members. These findings may indicate a more rapid evolution on the CD200RLc, CD200RLe members of the family compared to CD200RLa and b each of which were found to be present in twenty one of the twenty two studied strains. However, although there is

significant heterogeneity in the distribution of members of the mouse CD200R family, this is still lower than the heterogeneity observed in Ly49 and KIR paired receptor families.

Although the reason for this difference is not clear, two claims can be made on this observation: Firstly, Ly49 and KIR family of receptors are found on NK cells which are the frontier defense line against viral infections. Therefore, pathogen driven evolutionary pressure might be more significant on shaping these receptors. Secondly MHC-I molecules which are natural ligands for the inhibiting Ly49 and KIR receptors evolve rapidly whereas CD200 is rather stable. The rapid evolution of MHC-I probably exerts a second independent evolutionary pressure on the inhibiting Ly49 and KIR types and the higher polymorphism in the inhibitory receptors eventually results in higher levels of polymorphisms in the activating ones which evolutionary originate from the inhibitory receptor.

In this study, the inhibitory member of the mouse CD200R family (CD200R) was observed to exist in two widely distributed isoforms (Chapter 3). The seven amino acid difference between two isoforms, despite being on extracellular regions of the protein, did not alter the interaction with the ligand CD200 but affected binding to one of the rat anti mouse CD200R antibodies (OX110) (Chapter 6). Then why do these two isoforms exist in such a well conserved fashion? The reason behind this polymorphism is not clear. However, that diversification may have formed under evolutionary pressure caused by a pathogen which targets the non-ligand binding site of the inhibitory receptor. Under such a threat, those

regions could evolve so that the pathogen cannot bind anymore but the essential ligand receptor interaction is conserved.

7.2.2 Novel reagents to study the mouse CD200R family

Clear identification of the roles of activating CD200RL proteins depends on specific antibodies that can discriminate between members of the family. Those antibodies may help revealing the tissue distribution of these proteins and this may give some clues on how these receptors function. Apart from that, specific antibodies could be used in cellular assays to dissect out the functions of those receptors.

As discussed in section 7.2.1, CD200RLc appears to be the most rapidly diverging member of the family which may be linked to a pathogen driven evolution. However, the expression pattern of this molecule is not known. This is because of the previously used OX110 antibody which binds to both CD200R and CD200RLc, preventing specific distribution analysis for these proteins. In this study that problem was addressed and two specific novel antibodies were characterized (Chapter 6). One of these antibodies, OX131, specifically binds mCD200R and the other, OX132, to mCD200RLc. Although OX131 cross-reacted with CD200RLc, this is not a problem when used in C57BL/6 mice which does not contain that allele. Another observation on OX131 is that it can effectively block the CD200-CD200R interaction. This requires an antibody binding site on or very close to the ligand binding region of CD200R. The interesting point in this observation is that OX131 antibody binds within close proximity to the ligand binding region and the residues it

detects have to be conserved in mCD200RLe (as the antibody binds this two proteins in undistinguishable level (Figure 6.2)) which does not bind CD200 (80) . This shows the importance of very few amino acid differences between mCD200RLe and mCD200R in binding to CD200.

7.2.3 Screenings on pathogen binding.

The investigations on the evolutionary dynamics of the CD200R family have been one of the key elements of this study. Most probable candidates for driving the evolution of these proteins are pathogens. Several viral proteins have already been shown to interact with CD200R to generate immunosuppression which is beneficial for the microbial survival (67, 68, 150). However, despite having the most populous CD200R family known, no viral pathogens have been identified against the mouse CD200R family. The lack of viral CD200 homologues targeting mouse CD200R family suggests that a different mechanism is responsible for the evolution of this family. As explained by the counterbalance theory, it is not only the natural ligand homologues that can be used by pathogens to bind with the desired receptor but also other pathogen molecules can be effectively used to hit the inhibitory receptor through non ligand binding, extra cellular regions (14). Examples of this type of interaction were noted for PIR family which interacted with unidentified bacterial molecules (130). Therefore, in this study, possibility of bacterial interaction was tested for all CD200R family members. Although no interaction could be identified, the screening method was confirmed to be efficient by showing the interaction between PIR-A1 and S.

aureus. Thus a wider range screening, using different bacteria, may reveal such an interaction.

Another receptor-pathogen interaction, addressed by this study was the previously suggested interaction between myxoma virus M141 molecule and the rabbit CD200R (27). M141 protein has significant sequence identity with the membrane distal domain of rabbit CD200R (Table 4.1) and the presence of this molecule generates a massive immunosuppression in myxomatosis. However, despite the clear advantage this protein provides for viral survival, biochemical characterization of CD200R-M141R interaction was missing. That point was addressed in this study and rabbit CD200 and CD200R proteins were generated for the first time (Chapter 4). However, no interaction between M141 and rabbit CD200R could be observed. This suggests that either M141 is effective through a CD200R independent mechanism, or M141 used in our studies need the presence of some other viral molecules to gain ability to bind to the CD200R.

7.3 Unanswered questions and future work

Two key questions are how this family evolves and whether it is driven by pathogens. Despite the various pathogen-receptor interactions identified for human and rat CD200R and another pathogen-receptor interaction suggested for rabbit CD200R, still there has been no sign of pathogen-mediated evolution of host defense strategies. In this concept, human and rat CD200R families should be re-evaluated and the interaction of identified viral proteins with activating members of these families should be tested.

There is one activating receptor in human CD200R family which was observed not to be binding with K14 (vCD200 in HHV-8) in preliminary studies. However, a comprehensive study testing all viral CD200 homologues binding with hCD200R, against hCD200RLa is missing. One reason for this is the lack of clarity on the expression of hCD200RLa. First observations claimed this allele is a pseudo gene and does not get expressed on the surface due to lack of essential cysteine residues, but later in this study, cell surface expression was identified after transient transfection of 293T cells (not shown). That expression was not paired with DAP12 and no other adapters could be identified in preliminary immune precipitation experiments. Therefore, it is still not clear whether hCD200RLa could get expressed on primary cells. Despite the lack of a specific antibody against this protein to screen for its surface expression, cDNA s generated from various leukocytes can at least give a hint on how active that locus is. In any case, a comprehensive study including any observed polymorphisms of hCD200RLa and hCD200R and all the viral molecules that bound with hCD200R, should be carried out.

In the rat CD200R family there is one activating receptor but that has not been cloned yet. Cloning that receptor is essential to test whether it binds to the e127 molecule of RCMV virus which targets the rat CD200R. Although the interaction of rCD200R and e127 was characterized, the expected immunosuppression as the functional outcome of that interaction could not be observed (67). This might be due to the counterbalancing effect

mediated by the activating receptor. In that case biochemical and cellular assays testing the interaction of e127 and activating member of the rat CD200R family could be helpful.

Mouse CD200R family, which has a variety of activating members that do not bind CD200, should also be tested for their ability to bind pathogens. Bacteria, different from those used in that study, can be used to test such an interaction. Other than that the sensitivity of the method, used for that screening can be increased by further optimizations and that may help detection of low affinity binding between bacteria and the receptors tested.

Additionally, the novel antibody, designed against mCD200RLc should be used to screen for the tissue distribution of this protein. Does it have a distinct expression pattern or does it get co-expressed with the inhibitory receptor? Knowing the answer of this question would surely generate insight on the possible role of that receptor in immune regulation.

Finally, the function of M141 protein in myxoma viruses is not clear. Although it has significant similarity to CD200, the interaction of that protein with CD200R could not be shown by this study. That point should be addressed again and rabbit CD200R should be tested for binding to wild type virus and the M141 KO virus. Fluorescent viruses designed by Grant McFadden will be imported for this assay to resolve the confusion. If it does not bind to CD200R, then how does it down modulate the immune response? Hunting for a

possible receptor for this molecule is critical as such a discovery would be unique in showing a viral CD200 homologue that has evolved further to gain an independent role.

7.4 Conclusions

This thesis has increased the scientific knowledge on the CD200R family in different species. Novel methodological approaches, generated for various screening studies, have been used to test many possibilities that might have played roles in the evolution of these proteins. Similar methods can be used to answer various other questions for both the CD200R family and other paired receptor families. Besides those methodological contributions, the novel antibodies characterized in this study may be used in various *in vitro and in vivo* assays.

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