THE PATTERN AND FUNCTIONAL CONSEQUENCE OF KILLER IMMUNOGLOBULIN-LIKE RECEPTOR EXPRESSION ON T CELLS

by

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ABSTRACT

Killer immunoglobulin-like receptors (KIRs) are a family of proteins expressed on human natural killer cells and a subset of T cells. Several inhibitory KIRs have been shown to recognise MHC class I molecules (predominantly HLA-C), with their engagement preventing target cell lysis. The ligand(s) and function(s) of activating KIRs, however, are less well characterised. Genetic studies of the association of KIRs with disease have identified an association with viral infections and autoimmune disease and this implicates that these proteins are important in human health.

This thesis was concerned with an investigation of the factors that determine KIR expression on lymphocytes, and how this might influence the cellular functional response. In my initial work I produced soluble recombinant forms of activating and inhibitory KIRs and studied the biophysical interaction of these proteins with HLA-C molecules. I saw some evidence that KIR2DS2 binds to the HLA-C group 1 allele HLA-Cw*0702, supporting the idea that HLA-C alleles are a true ligand for stimulatory KIRs. I then went on to make a detailed 11 colour flow cytometric analysis of the expression of KIR proteins in healthy individuals. I was able to show that total, and individual, KIR protein expression was correlated and defined a pattern of dominance on lymphoid subsets. I then went on to study the distribution of KIR expression on discrete memory T cell subsets and showed that they were found predominantly on late differentiating $CD45RA^+$ T cells. Interestingly there was also considerable expression on central memory $CD8^+$ T cells although the biological basis for this is unclear. I demonstrated that age and CMV infection have a marked effect on KIR expression and I speculate on the reason for this. Finally I studied KIR expression on CMV-specific T cell clones in order to undertake a functional analysis of the consequence of KIR expression. I observed that KIR expression increased when cells were cultured *in vitro* but I could not detect any difference in cytokine production or cytotoxicity between KIR^+ and KIR^- cells. My work has contributed to the literature on KIR biology in relation to lymphoid cells and will have direct relevance to a number of clinical studies.

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DEDICATION

This work is dedicated in loving memory to the original Dr. Chagoury -

Uncle Marius, who planted the seed.....I wish you were here to see me blossom.

"Ignorance more frequently begets confidence than does knowledge: it is those who know little, not those who know much, who so positively assert that this or that problem will never be solved by science."

Charles Darwin

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LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC	Allophycocyanine
APS	Ammonium persulphate
ATP	Adenosine-5'-triphosphate
BCS	B cell serum
bp	Base pair
BSA	Bovine serum albumin
BSP	BirA substrate peptide
CD	Cluster of differentiation
cDNA	Complementary DNA
CEF	Chicken embryo fibroblasts
CMV	Cytomegalovirus
CPE	Cytopathic effect
CSA	Cytokine secretion assay
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DME	Dulbecco’s modified Eagle (medium)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum

FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gB	Glycoprotein B
gH	Glycoprotein H
gM	Glycoprotein M
Gy	Grey
HC	Heavy chain
HCMV	Human cytomegalovirus
HFFF	Human foetal foreskin fibroblasts
HHV	Human herpesvirus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HuS	Human serum
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITAM	Immunoreceptor tyrosine-based activation motif
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
Kb	Kilobase pairs
KIR	Killer immunoglobulin-like receptor
LB	Luria Broth
LDA	Limiting dilution assay
LIR	Leukocyte Ig-like receptor
LCL	Lymphoblastoid cell line
M	Molar
mAb	Monoclonal antibody
mCMV	Murine cytomegalovirus
MES	2-(*N*-morpholino)ethanesulfonic acid
mg	Milligram
MICA/B	MHC class I chain-related gene A and B
MHC	Major histocompatibility complex

ml	Millilitre
mM	Millimolar
MLA	MLA-144
MOI	Multiplicity of infection
mRNA	Messenger RNA
MVA	Modified vaccinia ankara
NCR	Natural cytotoxic receptor
NK	Natural killer
NKR	Natural killer receptor
OD	Optical density
OPD	*o*-Phenylenediamine dihydrochloride
ORF	Open reading frame
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerytherin
PFU	Plaque-forming unit
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
SAP	Shrimp alkaline phosphatase
SEB	Staphylococcal enterotoxin
S.D.	Standard deviation
SDM	Site directed mutagenesis
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TAE	Tris-acetate-EDTA
TCA	Trichloroacetic acid
TCM	Central memory T cell
TCR	T cell receptor
TE	Tris-EDTA
TEM	Effector memory T cell

TEMED	Tetramethylethylenediamine
TEMRA	Effector memory RA T cell
Tfh	T follicular helper cell
Th1	T helper 1 cell
Th2	T helper 2 cell
Th17	T helper 17 cell
TMB	3, 3', 5, 5'-tetramethylbenzidine
Treg	Regulatory T cell
tRNA	Transfer RNA
U	Units
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by volume
β2m	Beta-2-microglobulin
µg	Microgram
µM	Micromolar

Amino Acids

Ala	Alanine	Leu	Leucine
Arg	Arginine	Lys	Lysine
Asn	Asparagine	Met	Methionine
Asp	Aspartic acid	Phe	Phenylalanine
Cys	Cysteine	Pro	Proline
Glu	Glutamic Acid	Ser	Serine
Gln	Glutamine	Thr	Threonine
Gly	Glycine	Trp	Tryptophan
His	Histidine	Tyr	Tyrosine
Ile	Isoleucine	Val	Valine

Chapter 1

Introduction

THE IMMUNE SYSTEM

The immune system is a complex network composed of many interdependent cell types that collectively protect the body from bacterial, parasitic, fungal and viral infections as well as growth of tumour cells. Both innate and adaptive immune responses are vital. A key feature of the innate response is that the mechanisms for immunity are in place even before pathogen exposure, providing a 'set response' which can act immediately upon microbial invasion. The principal components of this include physical and chemical barriers (such as epithelial surfaces and the antimicrobial products produced at these surfaces), serum proteins (such as complement), and phagocytic cells (including neutrophils, macrophages and dendritic cells), natural killer (NK) cells and gamma delta (γδ) T cells (Abbas and Lichtman, 2003).

By contrast, adaptive immunity develops *after* the initial exposure to a microbe and is characterised by exquisite specificity which can be adapted and moulded to any given microbe, and is capable of distinguishing between self and non-self. Furthermore, adaptive immune responses exhibit immunological memory; that is, the ability to respond more

quickly and vigorously following subsequent exposures to the same pathogen. This protection against reinfection can persist for the lifetime of the host. Adaptive immunity is dependent on the function of lymphocytes (T cells and B cells) and their products. The specificity, adaptiveness and ability to provide immunological memory sets adaptive immunity apart from innate immunity. However, the two systems do not work independently; phagocyte activation, part of the innate response, plays a crucial part in activating and shaping adaptive immunity, whilst innate effector mechanisms are directed and utilised during adaptive immune responses.

1.1 INNATE IMMUNITY

The innate immune system is the front-line defense system that provides a non-specific response to pathogens in all plant and animals. Unlike the adaptive immune response, the innate immune system does not generate memory or protective immunity. An essential part of the mammalian innate immune system is the function of NK cells that recognise and destroy cells infected with intracellular pathogens, including viruses, parasites, and bacteria.

1.1.1 Natural killer cell biology

NK cells were first identified on a functional basis in 1975 (Kiessling *et al.* 1975). Murine studies had identified cytolytic cells with specificity for in vitro cultured mouse Moloney leukaemia cells (Kiessling *et al.* 1975). Removal of T and B cells left a population still capable of killing, and it was this population that became known as NK cells. NK cells are large granular lymphocytes of the innate immune system. They are widespread throughout the body, being present in both lymphoid organs and non-lymphoid peripheral tissues (Cooper *et al.* 2004; Ferlazzo *et al.* 2004). NK cells are involved in direct innate immune reactions against viruses, bacteria, parasites, and other triggers of pathology, such as malignant transformation, all of which cause stress in affected cells (Moretta *et al.* 2002; Raulet 2004). Importantly, NK cells also link the innate and adaptive immune responses, contributing to the initiation of adaptive immune responses (Martin-Fontecha *et al.* 2004) and executing adaptive immune responses with the CD16 FcγRIIIA immunoglobulin Fc receptor.

1.1.1.1 Origin

NK cells arise from lymphoid precursors in the bone marrow (Hirose *et al.* 2002). During development within the bone marrow, NK cells undergo maturation and express receptors, some of which are specific for MHC class I molecules. This process of NK cell 'education' or 'licensing' is believed to account for functional NK cell clones that are self tolerant (Fernandez *et al.* 2005; Kim *et al.* 2005; Anfossi *et al.* 2006; Cooley *et al.* 2007). However, a small proportion of NK cell clones that do not express receptors specific for self MHC may leave the bone marrow and enter the circulation. These NK cells are 'hyporesponsive' to activation signals. Although most NK cells become educated, around 10-20% of NK cells do not express any known inhibitory receptors for self MHC class I molecules (Fernandez *et al.* 2005; Anfossi *et al.* 2006; Cooley *et al.* 2007). These hyporesponsive NK cells may be activated by cytokines such as IL-2 and induce cytotoxicity. Immature NK cells can leave the bone marrow without full receptor expression. Further receptor development may also occur in peripheral tissues such as thymus, lymph nodes, liver and spleen (Huntington *et al.* 2007).

1.1.1.2 Function

NK cells were first identified on a functional basis. Called 'null' cells (due to the lack of expression of detectable markers at the time), they were found to be capable of killing tumour cells and transformed lymphoblastoid cell-lines and described morphologically as large granular lymphocytes. Work in the late 1970s and early 1980s confirmed that NK cells played an important role in host immunity (Santoli *et al.* 1979; Marx 1980). Two methods of killing have been demonstrated. Firstly, NK cells constitutively express a lytic machinery and are capable of lysing target cells in a non-MHC restricted manner as compared to cytolytic T-cells, which kill in an MHC-restricted fashion (Phillips 1986). Secondly, NK cells can help

respond to infectious agents via the FcγR (CD16), responding to the immune complexes formed between IgG antibodies and different pathogen components (Tarkkanen *et al.* 1986; Klimpel *et al.* 1988; Cassatella *et al.* 1989; Moretta *et al.* 1989). What was not clear as these processes became understood was by what mechanism host cells escaped destruction by NK cells.

The 'missing-self' hypothesis proposes that NK cells recognise self-antigens on host cells, and that this leads to them being spared (Ljunggren *et al.* 1990). Virally infected cells or tumour cells lack the necessary antigens identifying them as self, and it is this that leads to NK cytolytic activity. MHC molecules are critical to the control of NK function (Karre *et al.* 1986; Storkus *et al.* 1989).

1.1.1.3 Natural killer cell receptors (NKRs)

NK cells have an abundance of receptors (Table 1-1), although the role of some is not yet clearly understood. As has been demonstrated, NK cells are highly effective at lysing targets that fail to express class I MHC, the implication being that there are receptors on the cell surface recognising ligands other than MHC.

Although human and mice NK cells undergo similar education within the bone marrow they have very different receptor systems. This has occurred through evolutionary pressures that led to the development of greater polymorphism within the human NK cell receptor repertoire. The evidence for this is inferred from the absence of recently discovered NK cell receptor orthologs beyond mammals (Parham 2005). The human NK cell repertoire includes an array of activating and inhibitory receptors working in concert to determine the threshold

for NK cell activation. These receptors are natural cytotoxic receptors (NCR), NKG2 (C-type lectin like receptors), killer immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptors (LIR).

Table 1-1: Table of activating and inhibitory NKRs

Activating	Inhibitory
NKp30	KIR2D (L)
NKp44	KIR3D (L)
NKp46	NKG2A
NKp80	LAIR-1
KIR2D (S)	P75; CEACAM1; MAFA
KIR3D (S)	
NKG2C	
NKG2D	
NKG2E	
CD16	
CD226 (DNAM-1)	
CD244 (2B4)	
CD96; CD160; NTB-A	

Natural cytotoxicity receptors

The NCRs include NKp46, NKp30 and NKp44. Overall, those NK cells that have a high surface density of NCRs have high cytolytic activity (Biassoni *et al.* 2001). This is directly dependent on stimulation via the NCR, as stimulation via CD16 does not induce different degrees of response between NCR^{bright} and NCR^{dull} cells. NCR^{bright} cells have enhanced cytolytic activity against most target cells, the exceptions being T-cell lymphomas, some ovarian and epithelial tumours, which are killed equally as well by both NCR^{bright} and NCR^{dull} cells. Reduced levels of surface expression are seen in infections such as HIV (De Maria *et*

al. 2003), although whether this is cause or effect is unclear. Further interest has been generated in NCRs because of the recurrent finding that their expression is down-regulated in patients with leukaemia (Costello *et al.* 2002) but often returns to normal levels in those patients that achieve a complete remission (Fauriat *et al.* 2007).

Ly49

The Ly49 family represents the receptors responsible for specific class I MHC recognition in the mouse (Correa *et al.* 1995). The natural ligands of these receptors are the polymorphic H-2 class I molecules on target cells, and the subsequent interaction leads to a modification of NK cell function (discussed below). Ly49 is a homodimer type II integral membrane protein, a member of the C-type lectin superfamily. Thus far, eleven functional Ly49 genes have been identified, located within the 'NK gene complex' on mouse chromosome 6 in the C57BL/6 strain. Additional variants have been found in other strains of mice. Diversity appears to be provided by alternative mRNA splicing and allelic polymorphism (Held *et al.* 1995). In addition, the genes differ in their extracellular and cytoplasmic domains. These differences have functional implications, both through the nature of the ligand bound, and potential differences in signal transduction. More than one Ly49 receptor can be expressed by any NK cell, allowing for a diverse repertoire to be assembled.

Ly49A is the first and most thoroughly characterised member of the family. It is expressed on a subset of C57BL/6 NK cells corresponding to approximately 20% of the total NK population. Ly49A$^+$ cells effectively lyse target cells bearing D^b, L^d or K^d, but are unable to kill target cells that express H-2D^d, H-2D^k or H-2D^p. This protective effect is overcome by

'masking' with mAbs against Ly49A or class I molecules (Yu *et al.* 1996), confirmation that the abrogation of the NK cytotoxicity is mediated by receptor-class I interaction. Further evidence has been provided by the demonstration of direct binding of Ly49A to purified D^d or D^k molecules. The site of interaction of Ly49A with the MHC molecule has been localised to the α_2 domain (Tormo *et al.* 1999), which contrasts with the KIRs in the human, where binding specificity is determined by the α_1 domain of the MHC class I molecule.

Lian *et al* demonstrated that Ly49C binds to a broad spectrum of class I MHC molecules (Lian *et al.* 1999), including D^d, D^b, K^b, K^k, H-$2D^s$ and H-$2D^b$. They also managed to localise on the Ly49C molecule residues crucial in the interaction between Ly49 and MHC. A recombinant version of Ly49A (Ly49A-EC) has been created and crystallised with H-$2D^d$, allowing X-ray crystallographic analysis of the binding of the two molecules. Ly49 interacts with two distinct sites on H-$2D^d$, neither of which overlaps with the footprint of the T-cell receptor (TCR) (Tormo *et al.* 1999). Site 1 covers one end of the peptide-binding groove with a high degree of match in the topology of the two surfaces. Site 2 overlaps the CD8 binding site.

KIRs

The KIRs are membrane-bound receptors found in humans and other primates, with no rodent homologs. Originally described in natural killer cells, they have also been found on the surface of T cells (Vilches *et al.* 2002a). They are glycoproteins of the Immunoglobulin superfamily. The genes encoding KIRs are found on chromosome 19q13.4, near genes encoding related molecules such as the Leucocyte Immunoglobulin-like Receptors (LILRs, previously known as immunoglobulin-like transcripts (ILTs) or leucocyte Ig-like receptors

(LIRs)) and the leukocyte associated inhibitory receptors (LAIRs) (Martin *et al.* 2000). This region of chromosome 19 has been termed the leukocyte receptor cluster (LRC) reflecting the density of receptor genes in this region. The precise number of KIR genes is yet to be elucidated, complicated by the fact that firstly, there are several pseudogenes and secondly, what originally were thought to be separate genes may not be so (KIR2DL2/3). Taking these into account, the expressed gene number is currently fourteen, with at least two pseudogenes (and KIR3DL3 to be confirmed as an expressed gene) (Williams *et al.* 2005).

There are two main types of KIR characterised by their extracellular domains (D). Those with three such domains (3D) specifically recognised certain HLA-A and HLA-B proteins while those with two such domains (2D) KIR bind to HLA-C proteins. For most KIR there is further dichotomy defined by the structure of the cytoplasmic tail which determines whether the outcome of ligation is activation or inhibition. KIR with long tails (KIR-L) contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) that transduce inhibitory intracellular signals whereas short tailed KIR (KIR-S) associate with the DAP12 protein which contains an immunoreceptor tyrosine–based activating motif (ITAM) that transduces an activating signal. The inhibitory KIR therefore include 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2 and 3DL3; and the activating KIR include 2DS1, 2DS2, 2DS3, 2DS4, 2DS5 and 3DS1. Amongst these KIR2DL4 has been shown to be both activating and inhibitory. KIR, their known ligands and corresponding functions are listed in Table 1-2.

Table 1-2: KIR, their known ligands and corresponding functions

Receptor	Ligand	Function
KIR2DL1	HLA-C group 2	Inhibitory
KIR2DL2/KIR2DL3	HLA-C group 1	Inhibitory
KIR2DL4	HLA-G	Activating & inhibitory
KIR2DL5	Unknown	Inhibitory
KIR3DL1	HLA-Bw4	Inhibitory
KIR3DL2	HLA-A3, HLA-A11	Inhibitory
KIR2DS1	HLA-C group 2 (low affinity)	Activating
KIR2DS2	? HLA-C group 1 (low affinity)	Activating
KIR2DS3/KIR2DS4/KIR2DS5/KIR2DS6	Unknown	Activating
KIR3DS1	Unknown	Activating

1.1.2 Leukocyte receptor complex (LRC)

The LRC is approximately 1Mb in length, located at 19q13.4. The precise structure of the complex is not yet elucidated, but recent work by Trowsdale's group has demonstrated that the content of the KIR region can vary dramatically (Wilson *et al.* 2000; Kelley *et al.* 2005). In total, there are at least 24 structurally and functionally related immunoglobulin-like receptors encoded within the LRC.

Sequence analysis of the KIR region in two different haplotypes showed the presence of 'framework' loci that flanked regions of widely variable gene content (Wilson *et al.* 2000). The framework loci are *KIR3DL3* centromerically, *KIR3DP1-KIR2DL4* in the middle of the KIR region and *KIR3DL2* at the telomeric end. Their presence at a frequency of 100% in all populations tested so far is consistent with them being permanent members of all haplotypes. The 'framework' loci provide a fixed skeleton of KIR genes throughout which are distributed a more variable set of genes for other KIRs. The framework genes have adjacent unreiterated

sequence (an unusual finding in the KIR region), which might have helped prevent loss of these genes. All of the genes studied in this paper were in a head-to-tail configuration, suggesting evolution through extensive duplication.

Recently, there have been several significant pieces of work published that have used family studies, and therefore the ability to define haplotype gene content by segregation analysis. Both Hsu (Hsu *et al.* 2002c) and Uhrberg (Uhrberg *et al.* 2002) demonstrated that all of the haplotypes defined by these methods contained the framework genes cited above. Haplotype structure and current thinking on allelic relationships for certain KIRs will be further discussed below.

Population studies indicate that nearly all humans have at least one activating KIR gene (Witt *et al.* 1999; Crum *et al.* 2000; Norman *et al.* 2001; Toneva *et al.* 2001; Rajalingam *et al.* 2002; Cook *et al.* 2003). However, it has been shown that chimpanzees can lack these short-tailed KIRs (Rajalingam *et al.* 2001). Comparing repertoires in the three species, pygmy chimpanzees appear to have a minimal repertoire, chimpanzees a slightly more expanded repertoire and humans a more complex repertoire. Recently, KIR3DL0 has been described. This is an ancestral KIR gene, found outside the known KIR gene cluster in humans and has been highly conserved for approximately 50 million years (Sambrook *et al.* 2006).

These findings give some clues as to the evolution of the KIR region of the LRC. As commented on by Rajalingam, the genomic structure of the KIR region is slightly unusual (Rajalingam *et al.* 2004). The genes are all structurally very similar (including the gene for the Fcα receptor lying telomeric to the region), they are arranged very close to each other and they are separated by short homologous sequences. There are very few unique sequences

over 100bp, with KIR gene sequences, including, intergenic regions, being highly conserved. This arrangement is favourable to several genetic events, in particular unequal crossing over which can either delete, expand or hybridise genes. This would explain the increasing diversity seen in chimpanzee and human repertoires.

1.1.2.1 KIR structure

The genomic organisation of a KIR gene was first described in 1997 (Wilson *et al.* 1997) and there has been little published subsequently to significantly change our current understanding. The particular gene studied was NKAT2 (now known as 2DL3). Structurally, there are several similarities with the gene for Fcα receptors (de Wit *et al.* 1995), the gene(s) for which are located immediately telomerically to KIRs. The leader sequence is encoded by two exons (exons 1 and 2). The immunoglobulin domains are each encoded by a single exon (exons 3-5), and the stalk region also has a separate exon (exon 6 in KIRs). One difference highlighted is that the transmembrane and cytoplasmic domains are each encoded by separate exons in FcαR. Inhibitory KIRs have two exons encoding the cytoplasmic domain (exons 8 and 9), the second exon encoding the ITIM motif.

The extracellular regions of KIR molecules consist of either two (KIR2D) or three (KIR3D) Ig-like domains. It is likely that the 'template' KIR was a KIR3D with a long tail. The three domains of KIR3D are named D0 (membrane – distal), D1 (middle) and D2 (membrane – proximal). The 2DKIRs are derived by two different mechanisms (Vilches *et al.* 2000a). 2DL1, 2DL2 and 2DL3 are structurally similar. They have the 2 immunoglobulin domains homologous to D1, D2 type seen in 3DL KIRs. The third (D0) domain is not translated. This can be due to nonsense mutations and/or altered splicing sites. Some pseudoexons have no

major structural abnormalities i.e. the correct reading frame is maintained suggesting alternative mechanisms for the failure of transcription. The pseudoexon 3 has ~80% sequence identity with the exon 3 encoding the D0 domain in KIR3DL1 and KIR3DL2. KIRs 2DL4 and 2DL5 (Vilches *et al*. 2000b) arrive at two immunoglobulin-like domains by a different route. They have two domains homologous to D0 and D2 of the 3D KIRs. A deletion of approximately 2kb in size, encompassing exon 4 which would encode the D1 domain, results in the truncated receptor.

The cytoplasmic tails of KIR are classified into long and short but the sequences encoding them are similar in length. The difference arises from variation in the position of the stop codon, usually due to single nucleotide substitutions or short indels in exon 9. Most of the long cytoplasmic tails carry two ITIMs. The short cytoplasmic tailed KIRs are truncated before the first ITIM. This has a significant bearing on function (see Figure 1-1).

The nomenclature of KIRs is based on the above discriminating features, namely number of domains, and the length of the cytoplasmic tail e.g. 2DL1 has two Ig-like domains and a long cytoplasmic tail.

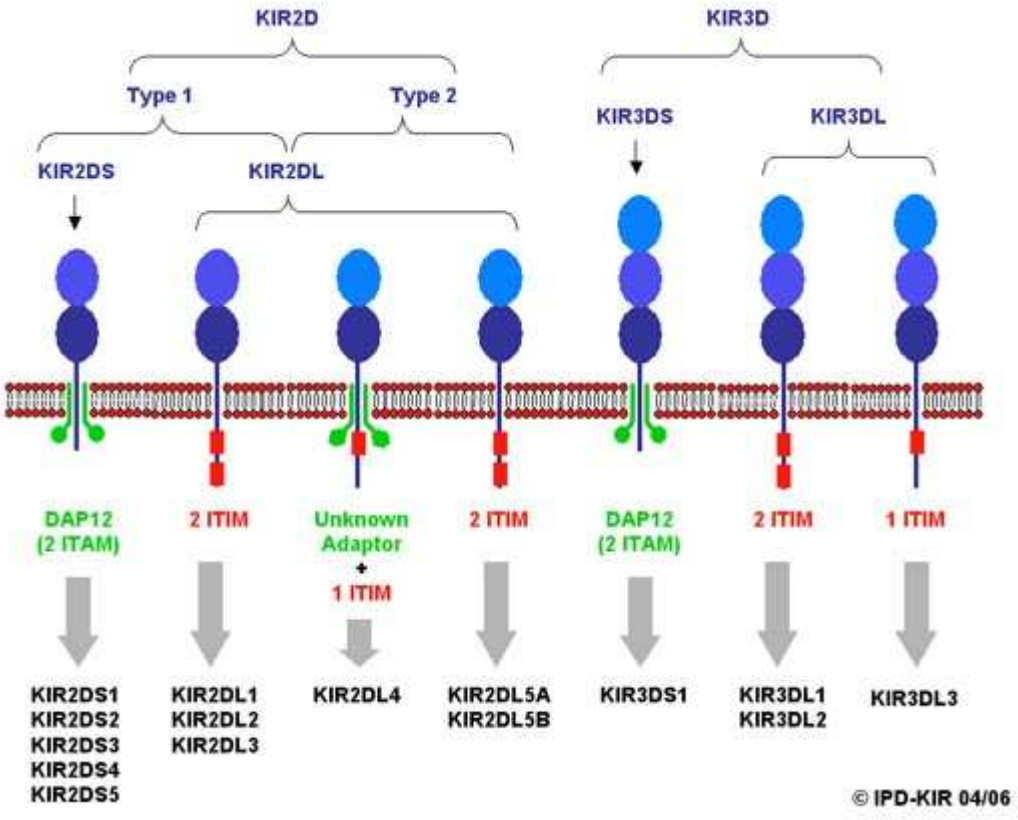


Figure 1-1. The configuration of 2 domain and 3 domain activating and inhibitory KIRs. (IPD-KIR database http://www.ebi.ac.uk/ipd/kir/).

1.1.2.2 Two domain KIRs

KIR2DL1

KIR2DL1 is a type 1 inhibitory KIR with the immunoglobulin-like domains in a D1-D2 configuration. It is recognised by the antibodies EB6 or HP-3E4 (which also recognise the activating KIR2DS1). To date, there are 15 alleles whose sequences are publicly available. KIR2DL1 is a component of the 'A' haplotype – the commonest haplotype in nearly all populations (Witt *et al.* 1999).

The natural ligands of KIR2DL1 are the group 2 HLA-C alleles characterised by Lysine (Lys) at position 80 (Winter *et al.* 1998). The crystal structure of KIR2DL1 in complex with HLA-Cw4 has been elucidated (Fan *et al.* 2001). There has been some debate over the effect of peptide on KIR-HLA binding. Recently, Betser-Cohen and colleagues demonstrated that not only is peptide crucial in the interaction but that the phosphorylation of individual residues within the peptide can itself impact on the binding of KIR2DL1 with MHC (Betser-Cohen *et al.* 2006). Both findings should come as no surprise – the presence of peptide stabilises the MHC complex thus setting a context for improved interaction and post-translation modifications have been shown to impact significantly on many protein-protein interactions.

KIR2DL2/3

KIR2DL2 and KIR2DL3 were originally treated as separate genes. However, a combination of functional and population genetic data have resulted in them being treated as behaving in an essentially allelic fashion as they do not appear on the same haplotype. Both KIR2DL2 and KIR2DL3 are recognised by the same antibodies (DX27 and GL183) and both interact with group 1 HLA-C. They both have 2 domains in a D1-D2 configuration. KIR2DL2 has been crystallised by itself and in complex with HLA-Cw3 (Boyington *et al.* 2000).

KIR2DL4

KIR2DL4 is another 2 domain KIR. The two domains are in a D0-D2 configuration, a 2Kb deletion accounting for the 'missing' D1 domain. 2DL4 is the only KIR with homologues in all primate species (Grendell *et al.* 2001; Guethlein *et al.* 2002).

There is now in vitro evidence that despite being a long-tailed receptor, the structure of 2DL4 is such that it has an activating rather than inhibitory function (Kikuchi-Maki *et al.* 2003). The cytoplasmic tail of 2DL4 contains one, rather than the usual two, inhibitory motif. This motif has been shown to retain inhibitory potential, but its true role may be inhibition of other intracellular processes. In addition, 2DL4 has a charged residue in the transmembrane region, a feature of the activating receptors. Target cell lysis by 2DL4-induced activation does not require an intact cytoplasmic ITIM, but does require the transmembrane region (with its charged arginine residue). There is evidence that FcRI-γ associates with KIR2DL4 to promote cell surface expression and signal transduction function (Kikuchi-Maki *et al.* 2005a). Finally, the state of the cell bearing the receptor has to be taken into account. Resting NK cells activated via 2DL4 secrete IFNγ, whereas NK cells already activated by IL-2 increase both IFNγ production and cytotoxicity (Kikuchi-Maki *et al.* 2005b). There is evidence that the natural ligand of KIR2DL4 is HLA-G (Rajagopalan *et al.* 1999), although this still remains to be convincingly demonstrated.

KIR2DL5

KIR2DL5 is the most recently described of the KIRs (Vilches *et al.* 2000b). Like 2DL4 it has a D0-D2 configuration, also because of a 2Kb deletion through exon 4, and there is approximately an 80% identity in amino acid sequences (reviewed in (Vilches *et al.* 2002a)). At present, the ligand of 2DL5 is unknown. Because of the similarity in configuration to 2DL4 and the conservation of both in primates, it has been suggested that the ligand may also be a non-classical HLA molecule. 2DL5 is a common constituent of the 'B' haplotype along with KIR2DL2 and KIR2DS2 (Uhrberg *et al.* 2002).

KIR2DS1 and KIR2DS2

There remains some doubt over the nature of the true ligands of the activating receptors. KIR2DS1 and KIR2DS2 are grouped together here because of their similarities in their extracellular structures with their inhibitory counterparts (2DL1 with 2DS1 and 2DL2 with 2DS2). Based on the sequence similarity, it would be expected that the activating KIRs bind HLA-C with the same affinity as their inhibitory counterparts. However, despite these similarities, the evidence for binding of the same ligands is actually quite weak.

KIR2DS3 and KIR2DS5

There has been no identification of any ligands for either of these receptors. Both bear some similarity to inhibitory receptors at residues that interact with HLA-C. These two KIRs are more commonly found on the 'B' haplotype. The 'B' haplotype is more prevalent in certain populations and this raises the possibility that the activating KIRs have arisen in these populations in response to certain environmental factors – particularly infective agents such as CMV. Investigations seeking to determine the ligands will have to take elements such as viral peptides/class I homologues into account.

KIR2DS4

There have been several reports of the interactions of KIR2DS4. It has been reported to interact with HLA-Cw3 and Cw4 (Campbell *et al.* 1998). These do not have a consistent amino acid at position 80. In addition, most of the interactions described have been weaker than the interaction seen between inhibitory receptors and HLA-C. More recently, weak affinity binding to Cw4 was described. Further exploration also demonstrated binding to

ligands expressed on melanoma cell-lines, the ligands not being class I MHC but unfortunately not identified within the paper (Katz *et al.* 2004).

There has been much interest in the genetics of KIR2DS4 recently. A variant has been described (initially termed KIR1D – now known as KIR2DS4*003 (Hsu *et al.* 2002c; Maxwell *et al.* 2002) that has a homologue in the Rhesus monkey (also called KIR1D). This variant has a 22 nucleotide deletion in the coding sequence that leads to a truncated protein due to a premature termination codon following the first amino acid of the putative transmembrane domain.

1.1.2.3 Three domain KIRs

KIR3DL1/3DS1

First identified in 1995, 3DL1 has 3 domains in a D0-D1-D2 configuration. Most of our current understanding of this KIR comes from a complex study looking at antibody binding and genotype for the KIRs 3DL1/3DS1 and 3DL2 (Gardiner *et al.* 2001). There are at least eight KIR3DL1 allotypes and they can be divided into three groups on the basis of cell phenotype as detected by the antibody DX9 (Gardiner *et al.* 2001). Binding can be either low, bimodal or high (as well as no binding for those people who are negative for 3DL1 or have an allele that does not bind- 3DL1*004). What Gardiner et al demonstrated was that these binding patterns are predictably determined by the alleles of KIR3DL1 an individual possesses. Those individuals heterozygous for two alleles have these alleles expressed in a differential fashion giving rise to four populations of NK cells – those positive for either allele alone, those positive for both alleles and those negative for both alleles. Different alleles have different DX9 binding properties, reflecting sequence variations in four positions of the KIR

amino acid sequence. It was also demonstrated that differing binding was detectable between individuals of the same genotype. This would suggest the influence of variations in the promoter regions.

The natural ligands of KIR3DL1 are those HLA-B allotypes containing the Bw4 motif and the HLA-A allotypes that also carry the Bw4 motif (HLA-A23, A24, A25 and A32). This interaction is particularly dependent on amino acids at positions 80, 82 and 83. Again, the binding is dependent on the antigenic peptide bound by the HLA molecule. It is likely that the D1 and D2 domains interact with HLA-B in a manner similar to the KIR2DL-HLA-C interaction. This appears to be stabilised by D0. The precise nature of this remains unclear and requires crystallographic studies. Carr demonstrated that in addition to the variable cell surface expression described by Gardiner, different alleles bind Bw4 (and thus inhibit) differentially, with 3DL1*002 being a much stronger inhibitory receptor than 3DL1*007 (Carr *et al.* 2005).

KIR3DS1 was originally defined separately from KIR3DL1 but is now considered an allele of 3DL1, albeit without a long cytoplasmic tail and the associated ITIMs. There is a 6-12 amino acid difference in the structures of 3DL1 and 3DS1. Interestingly, these differences are found in the Ig-like domains and are predicted to affect binding – one possible explanation for why there have been no reports of 3DS1 binding Bw4 allotypes. Occasionally, 3DL1 and 3DS1 can be found on the same haplotype (Williams *et al.* 2003).

KIR3DL2

KIR3DL2 is structurally similar to KIR3DL1. The gene for *KIR3DL2* is one of the 'framework' genes present on all KIR haplotypes. At present, the true ligand is not known. It has been mooted that HLA-A is the natural ligand (discussed above) but further work is required to clarify this. In particular, needing explanation is why a ubiquitous KIR should have HLA-A3/A11 as a ligand. Hansasuta et al have demonstrated that HLA-A3 and HLA-A11 tetramers will bind KIR3DL2 and that this interaction is peptide-specific (Hansasuta *et al.* 2004).

KIR3DL3

The gene for *KIR3DL3* is the most centromeric of KIR genes identified (Hsu *et al.* 2002b). It is a framework gene being present in all described haplotypes since it was discovered. Originally thought to be a pseudogene, there is certainly evidence that mRNA is detectable in $CD56^{bright}$ cells (Trundley *et al.* 2006). Demethylation results in cell surface expression (Trompeter *et al.* 2005). It appears that in 'normal' cells therefore, that cell surface expression does not occur or does so only at a very low level. It remains to be demonstrated whether this alters in pathological states.

1.1.2.4 Interaction with class I MHC molecules

HLA-C

The classical KIR-HLA interaction is between the inhibitory KIRs 2DL1 and 2DL2/3 and HLA-C, first identified as the likely site of alloantigen recognition in 1992 following careful

genetic mapping of the NK-defined specificities NK-1 and NK-2 (Colonna *et al.* 1992). This interaction inhibits the killing activity of the cell via ITIMs. Once the ligand is bound, the tyrosine residue contained within the ITIM is phosphorylated and phosphatases are recruited through their SH2-domains. SHP1, SHP2 and SHIP have all been identified as partners, depending on the receptor analysed (Long *et al.* 2001). Subsequent phosphatase activity is near the membrane, dampening or preventing NK effector functions such as cytotoxicity and cytokine production. In the absence of an inhibitory KIR interaction with ligand, such functions continue (Figure 1-2).

On the basis of the interaction with KIR, HLA-C can be assigned two groups due to a dimorphism at position 80 (Mandelboim *et al.* 1996). Group 2 HLA-C alleles are the natural ligand of KIR2DL1(Winter *et al.* 1998) and are defined by the presence of lysine at position 80 on the α_1-domain. Group 1 has an asparagine (Asn) at this position and is recognised in only a weak to moderate fashion by KIR2DL1. The amino acid at position 77 is also dimorphic (Ser-Asn) and is in strong linkage disequilibrium with the amino acid at position 80 (Ser77 with Asn80 and Asn77 with Lys80). However, there is no evidence that the amino acid at position 77 affects binding; indeed the evidence is stronger that the amino acid at positions 73 (Ala-Thr) and 90 (Ala-Asp) may be more influential.

Binding experiments have shown that (in solution) binding of KIR to HLA occurs in a 1:1 ratio (Fan *et al.* 1996). The same study showed that carbohydrates were not required for binding or function in the experimental conditions used. There is however, evidence that the peptide bound to HLA-C may affect the interaction between KIR and HLA (Rajagopalan *et al.* 1997), thereby affecting the protection from lysis conferred by the presence of the KIR.

Firstly, it was demonstrated that a peptide bound by HLA-Cw*0304 is required for protection. Secondly, the protection conferred varied widely between peptides (Zappacosta *et al.* 1997). Of interest was the finding that these differences were detectable between different endogenous peptides, not just between endogenous and synthetic peptides. The effect of peptide was found to be different between some NK cell clones, possibly reflecting the contributions of different receptors on the same clone.

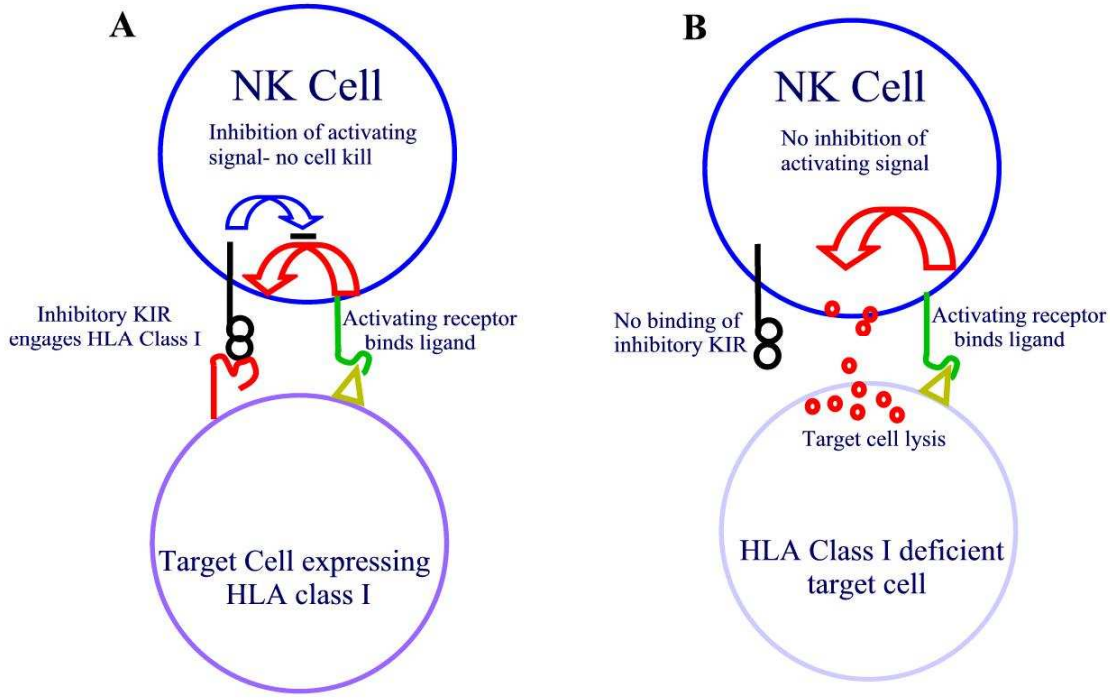


Figure 1-2: NK cell interaction with target cells is modulated by KIRs. (a) When inhibitory KIR receptors are bound by ligand (class I MHC) activating processes are inhibited (b) When the ligand is absent, activation continues resulting in target cell kill.

HLA-B

Akin to HLA-C, HLA-B can be collapsed into a dimorphic system on the basis of the Bw4 and Bw6 motifs. This system is slightly more complex than that of HLA-C as it is based on the amino acids at positions 77-83 of the α-helix of the α_1-domain. This is a highly polymorphic area, with three sequences described that define Bw4 (as defined initially by serological interaction). KIR3DL1 has been demonstrated to recognise the Bw4 motif but not Bw6. Recent work has highlighted the role of the 'third' domain (D0) (Khakoo *et al.* 2002). Deletion of residues 50 and 51 enhanced binding with Bw4. 15 different point mutations were induced in DO, but none of these affected binding. This contrasts with point mutations that were carried out in areas of the D1 and D2 domains predicted to affect binding to Bw4 on the basis of KIR2D-HLA-C interactions. These mutations were capable of disrupting binding. These results indicate that the D0 domain enhances the interaction following initial contact between D1 and D2 and HLA-B. This is of interest, not only in further clarifying KIR-HLA interactions but also in explaining how the loss of a domain during the evolution of KIRs has not prevented continued interaction with HLA. Interestingly, deletion mutant experiments where D0, D1 or D2 were deleted demonstrated that the D0 domain is required for KIR3DL1 to bind to HLA-B.

HLA-A

Many reviews of KIR function mention that KIR3DL2 recognises some HLA-A molecules- in particular HLA-A3 and HLA-A11. Published results are not always consistent, but it does appear that HLA-A3 does have an inhibitory effect via KIR3DL2 in several experimental models (Pende *et al.* 1996). However, whether HLA-A3 is the true ligand of KIR3DL2 remains a matter of controversy. Of particular interest is the fact that the Bw4 motif is carried

by some HLA-A alleles. These are thought to interact with KIR3DL1/DS1 in the same manner as HLA-B alleles carrying the Bw4 motif (Norman *et al.* 2007).

1.1.2.5 Hierarchy of interactions: HLA-C with KIR2DL

There is increasing evidence for the importance of HLA-C ligands in the modulation of NK cell function. Interaction between HLA-C alleles and KIR2DL1 are believed to be more inhibiting to NK cell function than interactions between HLA-C1 alleles and KIR2DL2/2DL3. This may be explained by receptor ligand binding kinetics. However *in vitro* binding studies have yielded equivocal results. Binding measurements using fusion proteins indicate weaker binding for KIR2DL3-Fc fusion protein with HLA-C1 that between KIR2DL1-Fc fusion protein with HLA-C2, however surface plasmon resonance analysis demonstrated identical results for both interactions. KIR2DL1 interacts with HLA-C2 at a more acute angle than KIR2DL2/2DL3 with HLA-C1. As a consequence, KIR2DL1 makes no contact with peptide in the MHC groove, whereas KIR2DL2/2DL3 makes contact with peptide epitopes. This may have an impact on binding kinetics making interactions between KIR2DL2/2DL3 and HLA-C1 weaker or more variable (Parham 2005).

1.1.2.6 KIR haplotypes

KIR genes are organised within the LRC into haplotypes, which have been shown to exhibit extensive variation in the number and type of KIR genes present. All known KIR haplotypes are flanked at their centromeric end by KIR3DL3 and at their telomeric end by KIR3DL2, together with the centric KIR3DP1 and KIR2DL4. These constitute the framework genes

(Martin *et al.* 2000; Wilson *et al.* 2000; Vilches *et al.* 2002b), which limit two regions of variable KIR gene content where the remaining KIR genes are located. All KIR genes are arranged in a head to tail fashion approximately 2.4 Kb apart from each other (Hsu *et al.* 2002a). Many KIR haplotypes have been defined by family segregation studies (Gomez-Lozano *et al.* 2002; Shilling *et al.* 2002; Uhrberg *et al.* 2002).

Based on their gene content two kinds of KIR haplotypes, A and B, have been described. Originally these haplotype groups were distinguished using restriction fragment length polymorphism (RFLP), based on the presence of a ~24 Kb HindIII fragment (present in group B haplotypes and later correlated to the presence of the KIR2DL5 gene) (Vilches *et al.* 2002b) (Uhrberg *et al.* 1997). However, these haplotype groups are currently distinguished by the number of activating and inhibitory KIR genes present. According to this new KIR haplotype group definition, group B haplotypes possess different combinations of KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 genes, where as group A haplotypes possess a single activating gene, KIR2DS4, as well as four inhibitory genes encoding proteins representing the main HLA class I specificities, KIR2DL1, KIR2DL3, KIR3DL1 and KIR3DL2 (Marsh *et al.* 2003).

1.1.2.7 KIR and disease

KIR genes may predispose to human diseases; association studies have highlighted the involvement of various factors including haplotype diversity, individual gene expression and HLA ligand diversity. Although many association studies are available there is only one

functional study (Ahlenstiel *et al.* 2008) to date demonstrating a direct 'cause and effect' relationship. Cross sectional functional studies are difficult to perform because of the inability to generate specific antibody tools are against KIR. The relationship between KIR/HLA genes and various human diseases are now discussed.

Autoimmune diseases

Various associations have been made between KIR haplotype and KIR genes and autoimmune diseases. A higher incidence of psoriasis vulgaris occurs in haplotype B individuals (Suzuki *et al.* 2004) suggesting the involvement of activating receptors in disease progression. Several other groups have also demonstrated an association with activating receptor 2DS1 (Luszczek *et al.* 2004). Likewise the possession of activating KIR2DS2 has been linked to a higher incidence of rheumatoid vasculitis (Yen *et al.* 2001), type I diabetes mellitus (van der Slik *et al.* 2003), and scleroderma (Momot *et al.* 2004). Martin and colleagues demonstrated that subjects with activating KIR2DS1 and/or KIR2DS2 genes are susceptible to developing psoriatic arthritis only when HLA ligands for their homologous inhibitory receptors, KIR2DL1 and KIR2DL2/3, are missing (Martin *et al.* 2002). They proposed that absence of ligands for inhibitory KIR could potentially lower the threshold for NK (and/or T) cell activation mediated through activating receptors, thereby contributing to the pathogenesis of psoriatic arthritis.

Infectious diseases

KIR and HLA ligand interactions have been shown to be important. Clearance of hepatitis C virus has been observed in individuals that possess homozygous 2DL3 and HLA-C1 alleles (Khakoo *et al.* 2004). It was hypothesised that NK cells were less inhibited in this combination, as HLA-C1 alleles were weaker at inhibiting NK cell function that HLA-C2 alleles. As a consequence there was more aggressive NK cell mediated activity against hepatitis C virus. Similarly in HIV, individuals with HLA-Bw4 and 3DS1 progressed more slowly to AIDS than those without. The mechanism for this involves ligation of activating KIR3DS1 by HLA-Bw4 ligands leading to activation and virus clearance (Martin *et al.* 2007). Finally a case study of a patient suffering from multiple infections has been reported where expression of KIR2DL1 was observed on their entire NK cell population. The most frequent infection was CMV and it is possible that overexpression of this inhibitory KIR may lead to an immune deficiency associated with primary CMV infection (Gazit *et al.* 2004)

Pregnancy

NK cells are found in abundance in the uterine circulation and are believed to be very important in feto-maternal tolerance. HLA-C alleles have been shown to have an important role in reproductive success (Hiby *et al.* 2004). Foetal expression of HLA-C2 is associated with an increased risk of pre-eclampsia which is believed to reflect a strong inhibitory signal between trophoblast and decidual NK cells serving to limit trophoblast invasion and subsequent placental vascularisation. An association between HLA-C2 alleles and recurrent miscarriages has been observed (Hiby *et al.* 2008).

Transplantation

In haematopoietic stem cell transplantation for acute myeloid leukaemia, an absence of cognate HLA ligands in the recipient for a corresponding donor, was associated with enhanced engraftment, a reduction in acute graft versus host disease, eradication of malignant cells and successful reconstitution of the immune system. Furthermore, a donor possessing activating KIR2DS2 has been shown to be protective against CMV reactivation in the patient post-transplant (Cook *et al.* 2005).

1.2 ADAPTIVE IMMUNITY

The adaptive immune response is antigen-specific and may take days or longer to develop. Cell types with critical roles in adaptive immunity are antigen-presenting cells including macrophages and dendritic cells. Antigen-dependent stimulation of T cell subtypes, B cell activation and antibody production, and the activation of macrophages and NK cells all play important roles in adaptive immunity. The adaptive immune response also includes the development of immunological memory, a process that continues to develop throughout life and enhances future responses to a given antigen.

1.2.1 T cell memory

The initial activation and expansion phase of both $CD4^+$ and $CD8^+$ T cell responses is invariably followed by a death phase during which the majority (~90%) of effector cells are eliminated. A small proportion of cells do survive however, and this population develops into long-term memory cells (Callan *et al.* 2000; Jenkins *et al.* 2001). Immunological memory is a cardinal feature of the adaptive immune response and serves to provide a mechanism for long-lasting, continuous defence against pathogens. Memory cells exist at a higher frequency than their naïve precursors and are able to respond more rapidly upon a subsequent pathogen encounter because their sensitivity to antigen and costimulatory requirements are lower than for naïve T cells (Sprent *et al.* 2002).

The precise factors which determine clone survival and selection to become long-lived memory cells following the contraction phase of an immune response remain a matter of intense debate. The process may be stochastic, in that all cells have the ability to differentiate

into memory cells following activation, but competition for environmental factors, such as survival cytokines, ensures that only 5-10% escape deletion and survive (Jenkins *et al.* 2001). Alternatively, T cell fate may be deterministic, decided at the time of T cell priming (Mercado *et al.* 2000; Kaech *et al.* 2001; van Stipdonk *et al.* 2001; Badovinac *et al.* 2002) via the interaction with the APC and the milieu of inflammatory cytokines present (Iezzi *et al.* 1999). This latter model proposes that only a small subpopulation of cells which receive the 'correct' signals upon priming will survive to become part of the memory pool.

The lineage of memory T cell development is still not fully understood and it is unclear whether memory cells are direct descendents of effector cells, or if they arise from a second lineage. Data from experiments in transgenic mice suggest that the memory T cell population is not generated from a subset of effector cells that 'divide-out', but rather, is formed directly from the effector cells themselves. Other studies have shown activated T cells seem to be programmed to develop into memory T cells. Therefore it is important to consider that memory T cell development might occur in a non-linear fashion and it can result in qualitatively different memory T cell subsets.

Whatever the selection process, cells that survive to become the memory population escape apoptosis, although, again, the mechanisms by which this occurs are unclear. The upregulation of proteins that inhibit apoptosis, for example the lysosomal protease inhibitior Spi2A (Liu *et al.* 2004) and the anti-apoptotic molecules Bcl-2, for $CD8^+$ cells (Grayson *et al.* 2000), and Bcl-X_L, for $CD4^+$ T cells are thought to be involved however (Garcia *et al.* 1999).

Furthermore, recent work has suggested that IL-7 and expression of the IL-7α-chain receptor (IL-7Rα)(CD127) are also critical to this process (Kaech *et al.* 2003).

Once selected, memory cells persist for many years due to their capacity for self renewal. Studies using heavy glucose have shown that memory populations maintain their numbers by continual division at a rate of between 1.5 and 4.7% per day (Macallan *et al.* 2004). This process does not require interaction with antigen (Lau *et al.* 1994; Tanchot *et al.* 1997; Garcia *et al.* 1999) or MHC molecules (Murali-Krishna *et al.* 1999; Swain *et al.* 1999). Instead it is believed that turnover is driven by cytokines, in particular IL-15 and IL-7 for $CD8^+$ cells (Goldrath *et al.* 2002; Tan *et al.* 2002), and IL-7 for $CD4^+$ T cells (Geginat *et al.* 2001) and is termed homeostatic proliferation.

$CD4^+$ T cell lineages

T helper cell lineage commitment was originally viewed as a unidirectional process with nonreversible termed differentiation of T helper 1 (Th1) and T helper 2 (Th2) cells. Each T helper cell subset expresses its lineage-specific transcription factors and mutually exclusive cytokines. The discovery of two new subsets of T helper cells, regulatory T (Treg) cells and T helper 17 (Th17) cells, and their capacity to produce cytokines that would be considered hallmarks of opposing lineages suggest that the commitment of T helper cell lineages is more complex than previously appreciated (Zhou *et al.* 2009). The differentiation of naïve $CD4^+$ T cells into lineages with distinct effector functions is governed predominantly by cytokines in the microenvironment and, to some extent, by the strength of the interaction of the TCR with antigen (Boyton *et al.* 2002). Naïve $CD4^+$ T cells can differentiate into Th1, Th2, Th17, Treg,

or T follicular helper (Tfh) cells. As illustrated in Figure 1-3 these differentiation programs are controlled by cytokines produced by innate immune cells, such as IL-12 and IFNγ, which are important for Th1 cell differentiation, and IL-4, which is crucial for Th2 cell differentiation. TGF-β together with IL-6 and IL-13 induces Th17 cell differentiation (Veldhoen *et al.* 2006), whereas Treg differentiation is induced by TGF-β, retinoic acid, and IL-2. Tfh cell differentiation requires IL-21 (Nurieva *et al.* 2008; Vogelzang *et al.* 2008). Specific transcription factors that orchestrate the differentiation program of each T helper cell subset have been identified: T-bet for Th1 cells, GATA3 for Th2 cells, RORγt for Th17 cells, and Foxp3 for iTreg cells (Zhou *et al.* 2009). The effector T cells had been thought to be terminally differentiated lineages, but it now appears that there is considerable plasticity allowing for conversion to other phenotypes. Although Th1 and Th2 cells display more stable phenotypes, Treg cells and Th17 cells can readily switch to other T helper cell programs under certain cytokine conditions. For example, Tregs can become IL-17-producing cells upon stimulation of IL-6 and IL-21. Treg cells can also switch to Tfh cells, and this requires B cells and CD40-CD40L interaction. Th17 cells may also convert into IFNγ-producing Th1 cells or IL-4-producing Th2 cells when stimulated by IL-12 or IL-4, respectively. Evidence also suggests that Th2 cells can switch to IL-9-producing cells in response to TGF-β, although it is unclear whether these “Th9” cells truly represent a distinct lineage.

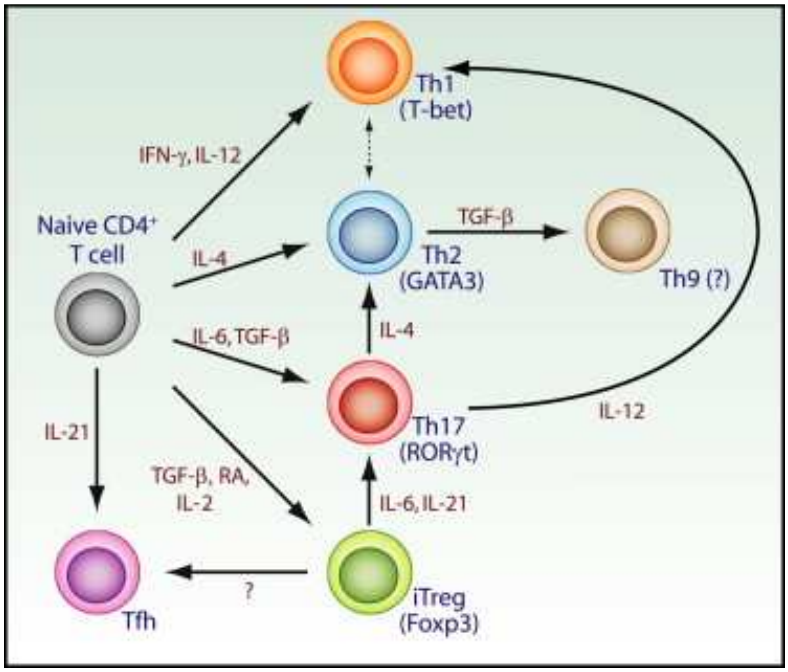


Figure 1-3: The cytokine milieu determines CD4$^+$ T cell differentiation and conversion. (Zhou *et al.* 2009).

Memory cells: Subsets, phenotypes and function

Because of their importance to understanding many of the fundamental immunological questions, a large amount of research has been devoted on methods to identify naïve and memory T cell subsets. Much of this work has focused on distinguishing the populations by their cell surface phenotypes. It is now well documented that naïve T cells can be identified by high expression of the long isoform of the protein tyrosine kinase CD45 (CD45RA), the lymph node homing molecules CD62L and CCR7, and the costimulatory molecules CD28 and CD27 (Hamann *et al.* 1997; Young *et al.* 1997; Sallusto *et al.* 1999; Appay *et al.* 2002;

Rufer *et al.* 2003). In addition, naïve cells have been shown to express low levels of the integrin CD11a and lack expression of the short isoform of CD45 (CD45RO), CD57 and granzyme and perforin effector molecules (Yamashita *et al.* 1993; Zimmerman *et al.* 1996; Hamann *et al.* 1997). Defining the phenotype of memory T cells has proved more difficult however. Originally the differential expression of the CD45 isoform was used to identify subsets of T cells (i.e. $CD45RA^{-}CD45RO^{+}$ versus $CD45RA^{+}CD45RO^{-}$ for memory and naïve cells, respectively) (Young *et al.* 1997), although it has since been demonstrated for both $CD8^{+}$ (Dunne *et al.* 2002) and more recently $CD4^{+}$ (Amyes *et al.* 2003; Weekes *et al.* 2004; Amyes *et al.* 2005) T cell subsets, that antigen experienced cells can re-express CD45RA. For this reason, the use of the CD45 isoform, together with other surface markers for example CD28, is now considered by many to be a way of identifying cells which have recently encountered antigen (Dunne *et al.* 2002; Carrasco *et al.* 2006).

Most recently, expression of the chemokine receptors CCR7 and CD62L have been suggested as a better way to define T cell populations. Using these markers together with CD45 and certain functional characteristics of T cells, Sallusto *et al* were able both to distinguish between naïve and memory T cell subsets and also to identify 2 distinct memory subpopulations (Sallusto *et al.* 1999). These populations were termed central memory (T_{CM}) and effector memory (T_{EM}). T_{EM} cells display a $CD45RA^{-}$, $CD62L^{+}$ and $CCR7^{+}$ phenotype, allowing their homing to secondary lymphoid organs. These cells have little or no immediate effector function but retain the capacity to proliferate and differentiate in response to antigen. In contrast, the T_{EM} subset generally persists in non-lymphoid tissues, due to a lack of CCR7 and heterogeneous CD62L expression. These cells display immediate effector function with $CD8^{+}$ T_{EM} cells carrying large amounts of perforin and both $CD4^{+}$ and $CD8^{+}$ T_{EM} cells able to

rapidly produce IFNγ, IL-4 and IL-5 upon antigenic stimulation (Sallusto *et al.* 1999). Following their characterisation, Sallusto *et al* proposed a linear model of T cell differentiation. They suggest that naïve T cells ($CD45RA^+$ $CCR7^+$ $CD62L^+$) are activated upon antigen encounter and differentiate first into T_{CM} ($CD45RA^-CCR7^+$ $CD62L^+$) and then into T_{EM} ($CD45RA^-$ $CCR7^-$ $CD62^{+/-}$) upon subsequent antigen encounter. Using this model Sallusto *et al* hypothesised that T_{EM} cells represent the first line of defence against invading pathogens whilst T_{CM} subsets are involved in maintaining the peripheral effector pool though the generation of successive waves of new effector cells.

Although these terms are now widely employed to define memory populations, recent data questions this straightforward distinction by demonstrating the presence of T cells negative for CCR7 expression in lymph nodes (Chen *et al.* 2001; Ellefsen *et al.* 2002), production of effector cytokines by cells from both subsets (Ravkov *et al.* 2003), and the conversion of human T_{EM} cells to T_{CM} memory cells (Schwendemann *et al.* 2005). Furthermore, whilst similar populations have been observed in mice (Masopust *et al.* 2001; Reinhardt *et al.* 2001), the two subsets do not appear to behave in the same way as human memory populations (Kaech *et al.* 2002; Wherry *et al.* 2003). However, these differences may simply reflect differences in the number of pathogens encountered between the human and murine immune systems rather than unique differentiation programmes between the two species.

Because of these data, the T_{CM}/T_{EM} model has now been refined by the inclusion of the costimulatory molecules CD28 and CD27. This model is particularly favoured by groups studying the differentiation of human virus-specific responses. Appay *et al* have proposed a

linear model of $CD8^+$ T cell maturation using expression of these markers rather than defining subsets by attributing functional or protective properties (Appay *et al.* 2002). In this model, cells progress from a $CD45RA^+$ $CD27^+$ $CD28^+$ (naïve) phenotype via a $CD45RA^-CD27^+$ $CD28^+$ (early antigen experienced) to a $CD45RA^{+/-}$ $CD27^+$ $CD28^-$ (intermediate antigen experienced), and then to a highly differentiated $CD45RA^{-/+}$ $CD27^-$ $CD28^-$ (late antigen experienced) phenotype (Figure 1-4). The $CD4^+$ T cell population may also be subdivided into early, intermediate and late differentiated subsets (Amyes *et al.* 2003; Day *et al.* 2003; Lucas *et al.* 2004; Yue *et al.* 2004; Amyes *et al.* 2005), however there is one key distinction. This is that $CD4^+$ T cells appear to lose expression of CD27 before CD28, in contrast to $CD8^+$ cells which lose CD28 prior to CD27 (Amyes *et al.* 2003) (Figure 1-4).

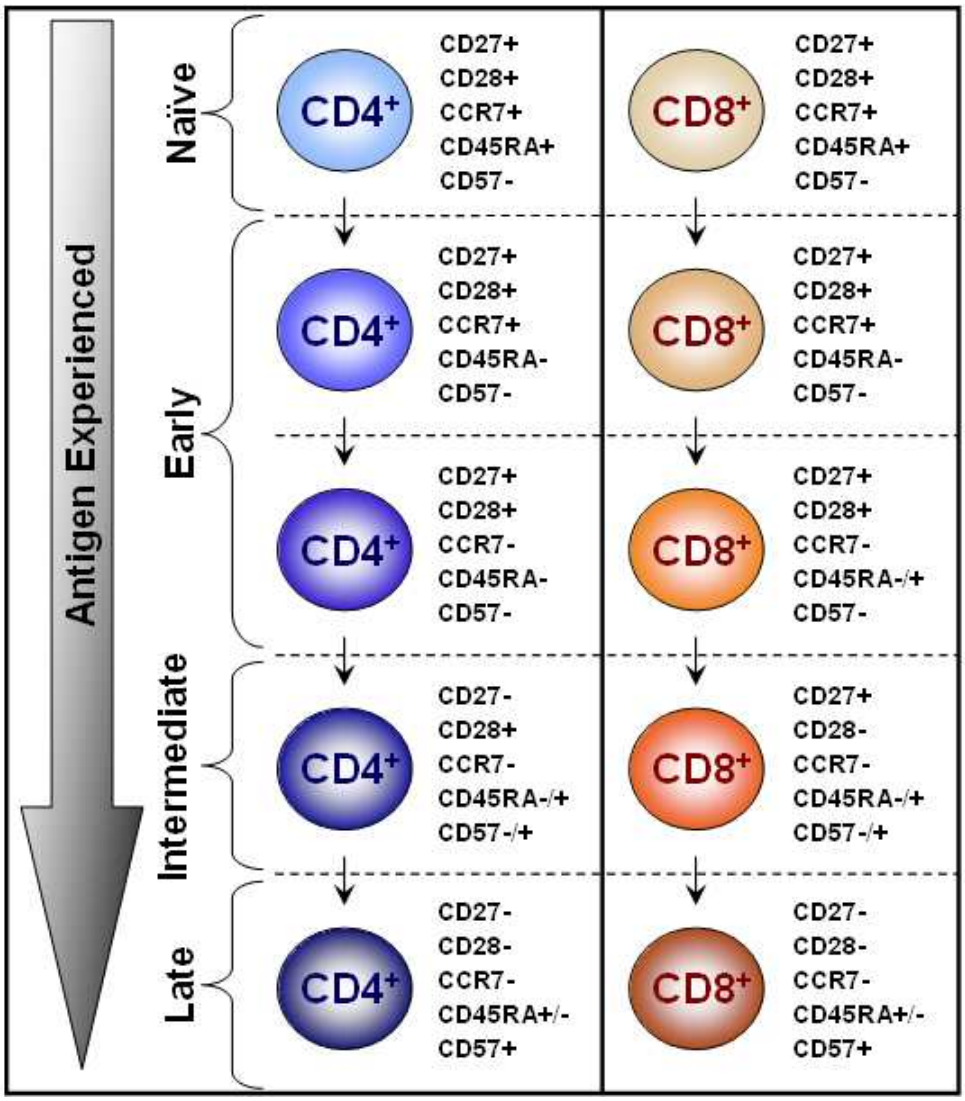


Figure 1-4: Phenotypic evolution pathway of $CD8^+$ and $CD4^+$ T cells following antigen encounter.

Interestingly Appay *et al* have shown that different viral infections are characterised by enrichment of $CD8^+$ T cells with different memory cell phenotypes, consistent with the different stages of differentiation (Appay *et al.* 2002), HCV and EBV specific $CD8^+$ T cells preferentially display an early antigen experienced phenotype, HIV-specific cells display an intermediate phenotype whilst CMV-specific cells accumulate in the late antigen experienced pool (Appay *et al.* 2002). Other groups have confirmed these observations (Roos *et al.* 2000; Hislop *et al.* 2002; Urbani *et al.* 2002); and also shown that a similar phenomenon exists for virus specific $CD4^+$ T cells. HCV, EBV and HIV specific $CD4^+$ T cells all appear to be less differentiated than CMV-specific $CD4^+$ T cells (Amyes *et al.* 2003; Day *et al.* 2003; Yue *et al.* 2004; Fletcher *et al.* 2005)

The above examples do not form an exclusive list of all cell surface markers that can be used to distinguish subpopulations of human cells. A number of others have also been used including CD57, a cell surface marker which is associated with highly differentiated $CD4^+$ and $CD8^+$ T cells, in particular HIV- and CMV-specific cells (Wang *et al.* 1995; Kern *et al.* 1999a; Weekes *et al.* 1999; Brenchley *et al.* 2003; Amyes *et al.* 2005; Palmer *et al.* 2005). Expression of the IL-7α-chain receptor may also be useful in dividing subsets of $CD8^+$ virus-specific T cells. A number of groups have reported that expression of IL-7Rα is lost by T cells specific for persistent viruses such as HIV, EBV, HCV and CMV, whilst influenza-specific cells retain expression (Boutboul *et al.* 2005; van Leeuwen *et al.* 2005; Golden-Mason *et al.* 2006).

1.2.2 KIR expression on T cells

KIRs can be expressed by both $CD8^+$ and $CD4^+$ T cells and may have a role in modulating their function (Anfossi *et al.* 2004; van Bergen *et al.* 2004). KIR expression on T cells is only observed on cells with a memory phenotype or increasing age (van Bergen *et al.* 2004). Acquisition of KIR by T cells with a memory phenotype or with increasing age may indicate their role in dampening the T cell mediated response. Indeed a recent study in the context of HIV disease identified increased expression of KIR on $CD8^+$ T cells in humans infected with HIV who have a high viral load (Alter *et al.* 2008). They demonstrated an antigen-specific inhibition of TCR responses on $CD8^+$ T cells with KIR which was not dependent on the nature of KIR ligand. Despite this evidence the role of KIR and KIR ligands in modulating T cell function remains unclear.

1.3 CYTOMEGALOVIRUS (CMV)

Cytomegalovirus is a ubiquitous member of the herpes family of viruses, a diverse group of large DNA viruses that share common virion morphology, a basic mode of replication and the capacity to establish latent and recurrent infections. Herpes viruses are strictly species-specific and there are currently eight known herpes viruses which infect humans. Although structurally similar, the viruses are grouped into three subfamilies (α,β and γ) on the basis of differences in their genomic homologies and biological properties. Cytomegalovirus, also known as human herpes virus 5 (HHV-5), is the prototypic member of the β herpes virus subgroup which also includes herpes viruses 6 and 7. Herpes virus infections are common and although they usually cause asymptomatic infection, they can be associated with significant morbidity and mortality especially in conditions of immunosupression. Thus, a greater understanding of the immunobiology of herpes viruses is an important area of research.

Table 1-3: Herpes virus subtypes and diseases

	Subfamily	Virus	Site of latency	Oncogenic potential	Pathophysiology
Alpha herpes viruses	**HHV-1**	Herpes simplex type 1 (HSV-1)	Neuron	No	Oral and/or genital herpes
	HHV-2	Herpes simplex type 2 (HSV-2)	Neuron	No	Oral and/or genital herpes
	HHV-3	Varicella Zoster Virus (VZV)	Neuron	No	Chicken pox/ shingles
Beta herpes viruses	**HHV-5**	Cytomegalovirus (CMV)	Monocyte, lymphocyte and others	No	Infectious mononucleosis, retinitis, glioblastoma[1]
	HHV-6	Human Herpes Virus 6 (HHV6)	T cells and others	No	Roseola
	HHV-7	Human Herpes Virus 7 (HHV7)	T cells and others	No	Roseola
Gamma herpes viruses	**HHV-4**	Epstein Barr Virus (EBV)	B cells	Yes	Infectious mononucleosis, Burkitts Lymphoma
	HHV-8	Kaposi's Sacroma - related virus (KSHV)	unknown	Yes	Kaposi's Sacroma

[1] CMV has been shown to be associated with glioblastoma with >90% tumours expressing HCMV nucleic acids and proteins, and 80% patients with newly diagnosed glioblastoma having detectable HCMV DNA in their peripheral blood (Mitchell et al. 2008)

1.3.1 CMV epidemiology and disease

CMV infections are highly prevalent throughout the human population. Between 50 and 70% of the population in western societies are seropositive for the virus and in some parts of Africa, seroprevalence approaches 100%. Primary infection can occur at any age, although the virus is usually first encountered in early childhood through contact with infected bodily secretions, for example tears, saliva, breast milk or blood. In the immunocompetent, primary infection is usually asymptomatic and following infection the virus resides within the host for its lifetime generally without overt disease. Clinical manifestations of CMV disease occur almost exclusively in the immunocompromised, in particular, HIV infected individuals that progress to AIDS and pharmacologically immunosuppressed recipients of stem cell or solid

organ transplants. In these situations, *de novo* or recurrent infections are associated with serious morbidity or mortality, and CMV remains the most important viral pathogen affecting transplantation (Khanna *et al.* 2006). CMV infection can also be a problem if acquired by the immature immune system. Transplacental transmission during pregnancy or neonatal infection of premature newborns can lead to both neurological damage and deafness. In addition, a role for CMV in other disease conditions, such as atherosclerosis (Adam *et al.* 1987; Nieto *et al.* 1996; Horvath *et al.* 2000) and glioblastoma (Mitchell *et al.* 2008) have been suggested.

1.3.2 CMV biology

As illustrated in Figure 1-5, the CMV virion can be structurally divided into three regions, the nucleocapsid, an icosahedral structure containing the viral DNA genome; the tegument, an amorphous layer containing a number of viral proteins; and the envelope, a lipid bilayer containing a number of different viral glycoproteins. The linear double stranded DNA genome of CMV is approximately 220-240 kb in length depending upon the strain, and these can be readily identified by restriction enzyme mapping of genomic DNA (Chandler *et al.* 1986; Retiere *et al.* 1998). Whilst the different strains share a high degree of sequence homology (approximately 95%), studies on clinical isolates have shown that a large number of genetically distinct strains of human CMV exist (Rasmussen *et al.* 2002). In addition, the prolonged passage of CMV in cell culture experiments can lead to mutations and loss of certain regions of the genome (Cha *et al.* 1996).

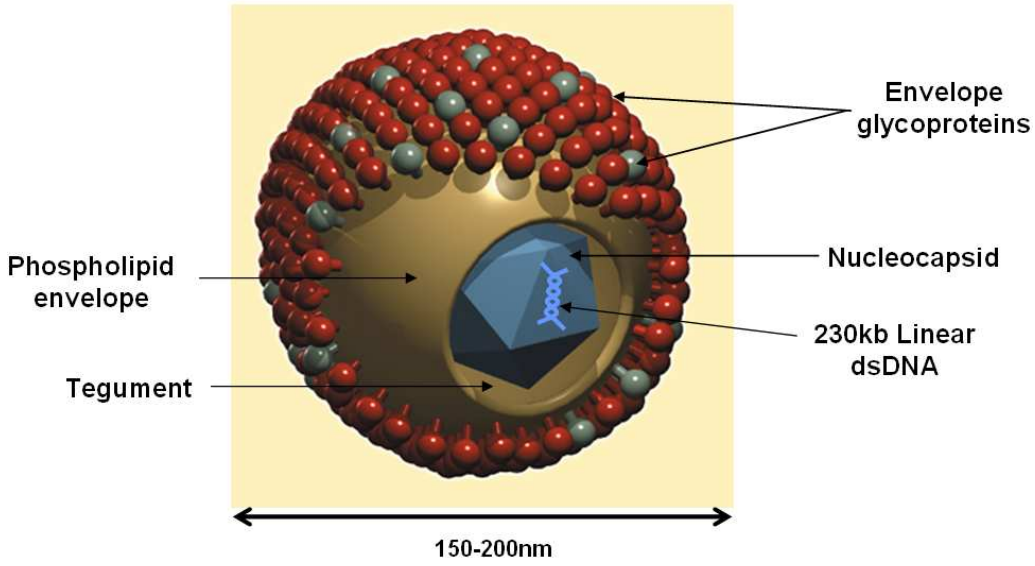


Figure 1-5: CMV virion structure. CMV virions are composed of a double-stranded DNA genome that is contained within an icosahedral nucleocapsid. This is surrounded by a structural layer known as the tegument, which is rich in structural proteins such as pp65. The virion is enclosed by an envelope, which has a lipid bilayer that expresses a number of viral glycoproteins such as the major glycoprotein B.

The human CMV genome is organised into unique long (U_L) and unique short (U_S) regions which are separated by internal repeat regions, and encodes for over 160 proteins (Britt *et al.* 2004). As with other herpesviruses, CMV gene expression can be separated into sequentially expressed kinetic classes. These are the immediate early (α), early (β_1 and β_2) and late (γ_1 and γ_2) phases based on the time of synthesis after infection. The immediate early phase is routinely defined as 2-4 hours post infection and is characterised by the transcription of DNA binding proteins, for example IE-1 and IE-2, important for optimising the cellular environment for the production of viral DNA. This is followed by the early phase (E), during which proteins such as transcription factors and DNA polymerase necessary for viral DNA

replication are produced. Examples are ppUL54 (DNA polymerase), UL57 (DNA binding protein) and UL44 (DNA binding protein). Finally, the late (L) phase occurs approximately 36-48 hours post-infection and is associated with the production of structural proteins such as UL32 (pp150), UL86 (pp65), and viral glycoproteins for example UL55 (gB), and culminates in the release of infectious virions (Britt and Alford, 1996).

CMV proteins are named according to the region of the genome encoding them and are numbered sequentially. The first gene identified in the UL region is designated UL1 and that in the US region is termed as US1 (Figure 1-6). Whilst a number of CMV proteins have been characterised, the functional properties of the majority of CMV derived proteins remain to be determined.

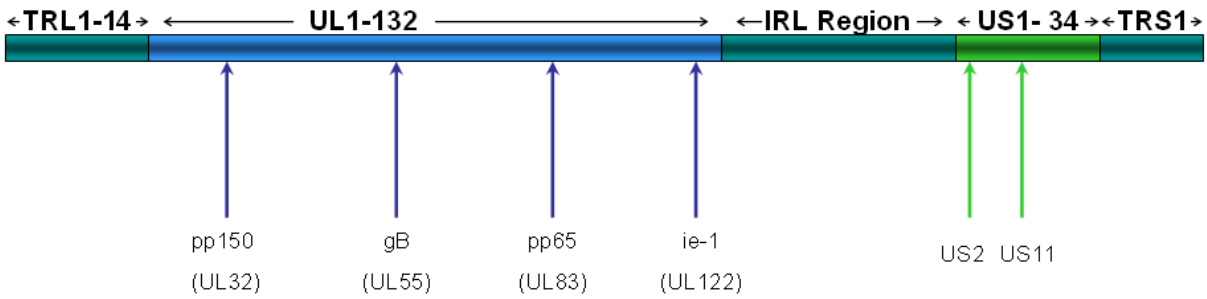


Figure 1-6: The CMV genome. The human CMV genome is organised into unique long (U_L) and unique short (U_S) regions which are separated by internal repeat regions (IRL) and bounded by terminal repeats (TRL and TRS when attached to the long and short regions, respectively), CMV encodes for over 160 proteins which are named sequentially and according to the region of the genome from which they are derived. For example, the first gene identified in the UL region is designated UL1 and that in the US region is termed as US1. In addition, proteins may also named according to other functional properties, for example glycoprotein B (gB/UL55) phosphoprotein 65 (pp65/UL83).

The processes by which CMV infects and persists in the human host remain controversial. The virus is capable of infecting a broad array of different cell types (such as epithelia, endothelia and smooth muscle), as shown by in vitro studies (Sinzger *et al.* 1995; Sinzger *et al.* 1999) and the multiple organ complications of CMV disease. However, cells of the myeloid lineage are thought to be the primary reservoirs (Taylor-Wiedeman *et al.* 1991; Kondo *et al.* 1994). The mechanism of viral attachment and entry into cells is yet to be documented but viral glycoproteins, in particular glycoprotein B (gB/UL55), are likely to be important (Cranage *et al.* 1986) perhaps through an interaction with the epidermal growth factor receptor EGFR (Wang *et al.* 2003) and certain integrins expressed on the surface of the host cell (Wang *et al.* 2005).

It is also unclear whether CMV undergoes true molecular latency, involving a change in the pattern of viral gene expression and a stop in the synthesis of productive virions or whether CMV is a truly persistent infection, with productive virions being produced at very low levels/below level of detection, due to chronic immunosuppression by the host. It is well documented that viral DNA is periodically shed throughout the lifetime of the immunocompetant host (Toro *et al.* 1996). Whether this is due to a reactivation from latency caused by stimulation, for example cellular differentiation (Taylor-Wiedeman *et al.* 1994; Soderberg-Naucler *et al.* 2001) or allogenic stimulation (Soderberg-Naucler *et al.* 1997) or due to a failure in the immune control of a persistent infection, remains to be determined. It is possible that both latency and persistence are involved in CMV infection (Sinclair *et al.* 2006).

1.3.3 The immune response to CMV

It is widely held that the host immune system is the most important factor in controlling CMV infection and disease. This is shown not only by the almost exclusive manifestations of CMV disease in the immunosuppressed or immature, but also by the mechanisms the virus employs to disrupt the host immune system to avoid recognition (reviewed in (Reddehase 2000; Mocarski 2002). It is crucial that a fuller understanding of the immune response towards the virus is gained for the development of improved therapies and the chance of a successful vaccine. A large amount of research has been undertaken, using both human and animal models, in particular murine CMV (mCMV), to fully elucidate the immunobiology of CMV infection. Immune control is dependent upon all the different compartments of the immune system and the roles they play are discussed below.

1.3.3.1 Murine CMV (mCMV)

Herpes viruses are strictly species-specific making the study of human CMV in animal models difficult. However, there are a number of well established animal models used to study other CMV infections. These include cercopithecine herpes virus 8 (CeHV8) (Rhesus Monkey), murine herpes virus 1 (mCMV) (mouse), murine herpes virus 2 (MuHV2) (rat) and cavid herpes virus 2 (guinea pig) (CavHV2). The most widely utilised of all animal models is that of murine CMV. Like human CMV, mCMV has 3 gene families, α, β and γ, which are expressed at the immediate early, early and late phases of the replication cycle and a number of proteins with similar functions have been described for both viruses for example IE-1 is encoded by UL123 and m123 in human and murine CMV, respectively (Reddehase 2000). However, a number of differences exist between the two viruses. Primary mCMV infection in immunocompetent mice leads to localised infection in the salivary glands and not disseminated disease, as occurs in humans, and mCMV does not infect the murine placenta to contribute to congenital infection (Krmpotic *et al.* 2003). Furthermore, the immunoevasion genes of murine and human CMV do not have any sequence homology, indicating specific adaption to the respective host (Reddehase 2002). Consequently, the caveat of using mCMV is that it may not accurately represent the disease process in humans. Despite these differences however, mCMV can be very useful in studying certain aspects of cellular and humoral immunity to CMV.

1.3.3.2 The innate immune response to CMV

Since primary infection with human CMV is asymptomatic, it is difficult to investigate the very early stages of infection *in vivo*. *In vitro* studies however, have suggested that type 1 interferons (IFNα and IFNβ) play a role in the innate response to CMV. The synthesis of

these cytokines is induced following CMV infection (Zhu *et al.* 1998; Boyle *et al.* 1999; Simmen *et al.* 2001) through an interaction between glycoprotein B and TLR2 (Boehme *et al.* 2006). Binding of type I IFNs to their cognate receptor initiates a signalling cascade resulting in the upregulation of more than 100 interferon stimulated genes (ISGs). The products of these are responsible for carrying out the antiviral activities attributed to interferons (Stark *et al.* 1998; Garcia-Sastre *et al.* 2006).

Perhaps the most important component of the innate response to CMV however, are the NK cells. The significance of this cell population is highlighted by the susceptibility of mice depleted of NK cells to experimental infection (Polic *et al.* 1998) and the observation that murine resistance to mCMV maps to a single locus termed Cmv1(Scalzo *et al.* 1990), which encodes the activating NK receptor Ly49H (Brown *et al.* 2001; Daniels *et al.* 2001). In humans, severe and recurrent HCMV infections are associated with naturally occurring NK cell deficiencies (Biron *et al.* 1989). Levels of NK cell cytotoxicity have been shown to correlate with both patient recovery from CMV reactivation following stem cell transplantation (Quinnan *et al.* 1982), and asymptomatic congenital CMV infection (Cauda *et al.* 1987). In the latter study, NK cells isolated from congenitally infected individuals had the ability to lyse CMV-infected target cells *in vitro* (Cauda *et al.* 1987).

NK cell recognition and killing of target cells is mediated through activating and inhibitory NK receptors, and their interaction with MHC-related proteins expressed on the surface of potential target cells (Lanier 2005). Both human and murine CMV actively downregulate the

expression of MHC related proteins, and this represents one way that infected cells can become susceptible to NK cell lysis (Lodoen *et al.* 2005) Once an infected cell is identified, NK control of viral infection primarily involves the production of the antiviral cytokine IFNγ (Orange et al., 1995) and direct lysis of infected cells via the granule exocytosis pathway (Tay *et al.* 1997) Both mechanisms are crucial for control of mCMV replication in the liver and spleen (Loh *et al.* 2005).

1.3.3.3 The adaptive immune response to CMV

Cell-mediated immunity

In comparison to humoral immunity, there is a large body of evidence to suggest that viral control is mediated by components of the cell mediated immune response. Disruption of cell populations that promote cytolysis has by far the greatest effect on CMV survival and disease. The main cell types involved are $CD8^+$ and $CD4^+$ T cells, although recent evidence suggests a role for gamma delta (γδ) T cells. The numbers of γδ T cells are greatly increased following exposure to CMV, but their particular function in the context of infection remains to be determined (Dechanet *et al.* 1999).

CMV-specific $CD8^+$ T cells

CMV-specific $CD8^+$ T cells have been well characterised and are known to play a critical role in CMV-specific cell-mediated immunity. Murine studies have shown that virus specific $CD8^+$ T cells prevent lethal mCMV infection, limit the viral load in latency and reduce the risk of viral reactivation and disease (Reddehase *et al.* 1987; Polic *et al.* 1998; Steffens *et al.*

1998). In humans, evidence for their importance comes largely from various clinical settings where a deficiency of functional CMV specific $CD8^+$ CTLs correlates with the occurrence of CMV disease in the immunosuppressed (Quinnan *et al.* 1984; Reusser *et al.* 1991; Li *et al.* 1994; Reusser *et al.* 1999). Perhaps the most convincing evidence for the involvement of $CD8^+$ T cells comes from an important study by Riddell and colleagues in which adoptive transfer of CMV-specific $CD8^+$ T cells was shown to restore protective immunity in immunocompromised bone marrow transplant patients (Walter *et al.*, 1995). In this study donor-derived CMV-specific $CD8^+$ T cell clones were transferred into patients. No toxic effects were observed and there was no evidence of CMV viraemia or disease in any of the 14 patients. In addition, molecular analysis of TCR genes in 2 donors showed that the infused clones had persisted for at least 12 weeks (Walter *et al.* 1995).

CMV-specific $CD8^+$ T cells proliferate rapidly following infection and dominate the primary response (Sester *et al.* 2003). Their role in controlling viral infection involves the direct lysis of infected cells and secretion of antiviral cytokines, including IFNγ. Interestingly, it appears that numbers of CMV-specific $CD8^+$ T cells do not decline as dramatically as other viral specific $CD8^+$ T cells following primary infection. Instead, an extremely large CMV-specific $CD8^+$ T cell response is evident in asymptomatic human and murine carriers (Khan *et al.* 2002; Karrer *et al.* 2003) which continues to expand throughout life. In the elderly, CMV-specific $CD8^+$ T cells dominate the repertoire to such an extent that they may impair the ability to respond to other antigens (Khan *et al.* 2004).

Successful CMV-specific immunity is dependent on CD8+ T cell response to a broad range of epitopes. It was originally believed that CMV-specific $CD8^+$ T cell responses are mainly focused towards IE-1 or pp65 antigens (McLaughlin-Taylor *et al.* 1994; Wills *et al.* 1996; Gillespie *et al.* 2000; Kern *et al.* 2002; Gillespie *et al.* 2007), however more recent work has identified responses to structural, early and late proteins, for which a number of epitopes have been described (Kern *et al.* 1999b; Frankenberg *et al.* 2002; Kern *et al.* 2002; Burrows *et al.* 2003; Elkington *et al.* 2003; Kondo *et al.* 2004). Elkington *et al* used computer based algorithms to predict HLA-class I epitopes from 14 CMV derived proteins and tested their ability to induce IFNγ responses (Elkington *et al.* 2003). Their results suggested that CMV specific immune control in healthy virus carriers is dependent on a strong $CD8^+$ T cell response directed against a broad range of antigens. Interestingly, this work also showed that immunomodulatory proteins can become targets of CMV specific $CD8^+$ T cells themselves (Elkington *et al.* 2003).

CMV-specific $CD4^+$ T cells

CMV-specific $CD4^+$ T cells have been studied in much less detail than $CD8^+$ T cells. This, presumably, is because $CD8^+$ T cells are considered to be the direct effector cells in controlling viral infection, $CD4^+$ T cell responses are generally of much smaller magnitude and because of the difficulties in the production of MHC Class II tetramers. However, there is increasing evidence to suggest that $CD4^+$ T cells do indeed play an important role in CMV immunity, and appear to be more important than previously thought. In mice, work by Jonjic *et al* using mice depleted of $CD8^+$ T cells, showed that clearance of mCMV from salivary glands was dependent on the $CD4^+$ subset (Jonjic *et al.* 1990) suggesting a direct role for this subset in controlling infection. More recently, clearance of the virus from the brains of

infected mice has also been shown to be due to the function of $CD4^+$ T cells (Reuter *et al.* 2005). In humans, CMV-specific $CD4^+$ T cells have a significant influence the outcome of disease in primary infection. Using a cohort of CMV seronegative individuals who received a CMV seropositive renal transplant to study primary infection, Sester *et al* showed that the size of the CMV specific $CD4^+$ T cell pool was significantly decreased in individuals displaying clinical symptoms of infection (Sester *et al.* 2001). Furthermore, a delayed reconstitution of the $CD4^+$ subset correlated with the occurrence of CMV-associated morbidity, even in the presence of functional CMV-specific $CD8^+$ T cells (Gamadia *et al.* 2003; Gamadia *et al.* 2004).

An interesting observation in respect to CMV-specific $CD4^+$ T cells is that, like CMV-specific $CD8^+$ T cells, the magnitude of response is very large. Using intracellular staining for IFNγ, Sester *et al* analyzed the size of the response in 50 healthy seropositve donors and showed that between 0.1 and 16% of total $CD4^+$ T cells were CMV-specific in these individuals. Furthermore, using a cohort of seropositive renal transplant patients the same group showed that up to 40% of the $CD4^+$ T cell pool could be specific for the virus (Sester *et al.* 2002). Other groups have also reported large frequencies of virus-specific $CD4^+$ T cells (Waldrop *et al.* 1997; Dunn *et al.* 2000; Bitmansour *et al.* 2001; Kern *et al.* 2002) and that the response is much greater than the $CD4^+$ T cell response to other virus-specific cells. Asanuma *et al.*, also using intracellular staining for IFNγ, investigated the $CD4^+$ T cell response to varicella zoster virus (VZV) herpes simplex virus (HSV) and CMV in 12 donors. They reported that the mean percentage of the CD4+ T cell pool specific for each virus were 0.11% , 0.22% and 1.21% respectively (Asanuma *et al.* 2000).

The kinetics of the $CD4^+$ T cell response during primary infection have also been investigated. Using renal transplant patients, Rentenaar *et al* showed that virus-specific $CD4^+$ T cells appear approximately 1 week after the detection of viral DNA in the peripheral blood, interestingly *before* the detection of virus specific $CD8^+$ T cells or IgM or IgG antibodies. Following the initial rise, $CD4^+$ T cells in peripheral blood reduce dramatically, presumably as they migrate to sites of CMV replication in peripheral tissues (Rentenaar *et al.* 2000).

The way in which CMV-specific $CD4^+$ T cells contribute to CMV-specific immunity involves numerous mechanisms. In addition to supporting CMV-specific antibody production $CD4^+$ T cells also appear to be essential for both the maintenance and function of CMV-specific $CD8^+$ T cells (Gamadia *et al.* 2001; Komanduri *et al.* 2001), because the persistence of adoptively transferred $CD8^+$ CMV-specific T cells is dependent upon the presence of $CD4^+$ specific T cells(Walter *et al.* 1995). IFNγ and TNFα produced by the $CD4^+$ T cells seem to be critical in the control of CMV infection (Gamadia *et al.* 2003). In mice, the mechanism of viral clearance from the salivary glands of $CD8^+$ T cell depleted mice involves IFNγ production by $CD4^+$ T cells, while in humans IFNγ production by $CD4^+$ T cells inhibits virus replication *in vitro* (Davignon *et al.* 1996). Furthermore, Tu showed that viral persistence in a cohort of young children was associated with a deficiency in IFNγ production by $CD4^+$ T cells (Tu *et al.* 2004) suggesting that IFNγ production by $CD4^+$ T cells is also important to CMV control *in vivo* in humans. In addition to the traditional helper roles displayed by $CD4^+$ T cells, recent work has reported a subset of $CD4^+$ CMV-specific T cells which display cytotoxic activity *in vitro.* This suggests that the activity of $CD4^+$ T cells may extend to the elimination of virus

through the direct lysis of infected cells (Elkington *et al.* 2004; Zaunders *et al.* 2004; Elkington *et al.* 2005; van Leeuwen *et al.* 2006).

A number of studies have focused on identifying the antigen specificity of the CMV-specific $CD4^+$ T cell pool. Initially the approach taken to detect CMV-specific $CD4^+$ T cells, involved stimulating PBMC from healthy virus carriers *in vitro* with recombinant CMV proteins and using proliferation as a readout of T cell recognition. Using this approach, IE-1, pp65, gB and gH were suggested to be important antigenic targets and a number of $CD4^+$ T cell epitopes were reported (Alp *et al.* 1991; Davrinche *et al.* 1993; Beninga *et al.* 1995; Beninga *et al.* 1996; Davignon *et al.* 1996; Gautier *et al.* 1996; Hopkins *et al.* 1996; Khattab *et al.* 1997). However, a number of these studies lacked the robustness of using a large cohort of donors**.** The development of intracellular cytokine staining (Waldrop *et al.* 1997) offered a quicker and more direct method of detecting-antigen specific cells. Using this method a number of groups have confirmed the presence of cells specific for pp65, ie-1, gB, gH, (Kern *et al.* 2002; Li *et al.* 2004; Elkington *et al.* 2005; Harcourt *et al.* 2006). The relative contribution of these proteins to the $CD4^+$ T cell response however, remains under investigation. Immunodominant proteins have been suggested; pp65 (Kern *et al.* 2002), ie-1 (Davignon *et al.*, 1995) and gB (Elkington *et al.* 2004) have all been proposed as the most immunogenic proteins for $CD4^+$ T cell responses. However, these conclusions were based simply upon the observations that responses were detectable in the majority of donors tested and/or that antibodies to the proteins are the most abundant in the sera of CMV infected individuals, and therefore remain equivocal.

Interestingly, a study by Beninga *et al* attempted to address the question of the hierarchy of immunodominance. Using 14 different CMV proteins they observed that $CD4^+$ T cell lines from all donors responded to pp65, cell lines from 3/5 donors responded to gB and gH and cell lines from 1/5 of donors responded to ie-1, ie-2, and UL69. However, the proliferative responses used as a read out are likely to have biased the results because of the cell culturing processes involved (Beninga *et al.* 1995).

It is evident therefore that a number of proteins are involved in this response but detailed studies of this area are clearly necessary to fully define the antigen specificity of the CMV-specific $CD4^+$ T cell population. Sylwester *et al* began to address this by using overlapping 15mer peptides encompassing all 213 known or predicted HCMV open reading frames (ORFs) in a flow cytometric assay using CD69 and IFNγ as readouts to identify peptide-specific responses (Sylwester *et al.* 2005). This study provided the first glimpse of the total human T cell response to HCMV and provides insight into immunodominance and cross-reactivity in such viral infections.

1.3.3.4 Involvement of inhibitory NKR in the response to CMV

The special relationship between NK cells and CMV has been appreciated ever since it was reported that both humans and mice lacking functional NK cells are particularly susceptible to infection with CMV (Bancroft *et al.* 1981; Biron *et al.* 1989). NK cells express several inhibitory receptors such as KIR, the CD94/NKG2A killer lectin-like receptor (KLR) and CD85j (ILT2 or LIR-1) (Colonna *et al.* 1999; Lopez-Botet *et al.* 1999; Moretta *et al.* 2004), that are also expressed by some T lymphocytes (Vivier *et al.* 2004). The spectra of class I

HLA molecules covered by inhibitory KIR and, indirectly, by CD94/NKG2A are partially overlapping. Both receptor systems complement each other to monitor the surface expression of most class I molecules, which are also broadly recognised by CD85j. The heterogeneous distribution of NKR in distinct NK cell subsets enables the system to react against variable alterations of HLA class I expression, provided that activating signals overcome the inhibitory threshold.

KIR

The possibility that CMV-infected cells might preserve HLA-C to escape KIR-mediated surveillance, as originally proposed for HIV (Cohen *et al.* 1999), remains unclear. HLA-C appeared resistant to US2 and US11 when expressed in a trophoblast cell line (Schust *et al.* 1998). In contrast HLA-Cw7 was downregulated in $US2^+$ and $US11^+$ transfected cells (Llano *et al.* 2003), and it has also been reported that $US11^+$ targets were sensitive to $KIR2DL^+$ NK cells (Huard *et al.* 2000), supporting the idea that HLA-C expression was inhibited.

1.3.3.5 Involvement of activating NKR in the response to CMV

The nature of the cellular ligands for triggering human NK cell receptors has been only partially unravelled. Some of them appear to be constitutively expressed by target cells (i.e. HLA class I molecules), others are inducible under stress conditions and can be detected in virus-infected anmd tumour cells (i.e. MICA/B), whereas a third category remains unknown.

1.4 SUMMARY AND AIMS

From reviewing the current literature it is clear that the mechanisms underlying KIR expression and function are not fully known. The NK cell receptor repertoire in humans is far more evolved and complex than in mice, making it impossible to draw any specific conclusions from mouse model experiments that may be transferrable to humans. Both KIR and HLA are highly polymorphic and inherited independently of each other and so the interactions within an individual are highly complex. Many association studies have been carried out, and it is obvious that KIRs play an extremely important role in human health. A better understanding of the pattern of KIR expression in healthy donors will undoubtedly help us gain a clearer picture of the changes that occur with disease progression and the functional effect this has. Up to this point studying KIR expression has been difficult due to the high homology between receptors. Commercial KIR-specific antibodies are cross-reactive and unable to discriminate between activating and inhibitory receptors. Since this work was carried out novel antibodies have been generated, which when used in combination with commercial antibodies, can discriminate between some of the 2DKIRs (David *et al.* 2009), however generating a single KIR-specific reagent is still the holy grail.

In this thesis therefore, I investigated interactions between 2DKIRs and their HLA-C ligands, and then sought to generate a KIR-specific antibody. I also characterised the pattern of KIR expression on lymphoid cells in healthy donors, and finally investigated the changes that occurred upon CMV infection.

The aims of the project were:

1) To investigate the binding of activating KIR2DS2 to its predicted Cw*0702 ligand.
2) To attempt to generate a specific antibody against activating KIR2DS2.
3) To perform a detailed FACS study on the pattern of KIR expression on lymphocyte subsets, particularly T cells.
4) To investigate the effect of CMV on the pattern of KIR expression.
5) To determine whether KIRs play a role on CMV-specific T cells, and investigate their function.

Chapter 2

Materials and methods

2.1 CELLULAR BIOLOGY

2.1.1 Tissue culture media and reagents

RPMI-1640 supplemented with 2mM L-glutamine (Sigma) was stored at 4°C.

Foetal calf serum (FCS) (PAA) was stored in 50ml aliquots at -20°C.

Human serum (HuS) (PAA) that was free from viruses and mycoplasma, and derived from a male type AB, was stored in 50ml aliquots at -20°C.

Penicillin-streptomycin (GIBCO/Invitrogen) containing 5000 IU/ml penicillin and 5000µg/ml streptomycin was stored as a 100x stock solution in 10ml aliquots at -20°C.

Phosphate buffered saline (PBS) was made by dissolving 1 Dulbecco A tablet (Oxoid) per 100ml of water that had been filtered through an Elgastat purifier and ion remover (referred to as SDW). PBS contains: 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 2mM KH_2PO_4 at a pH of 7.4. Aliquots of 50ml were dispensed into bottles and sterilised by autoclaving for 20 minutes at 121°C.

Recombinant interleukin-2 (IL-2) was reconstituted from lyophilised powder (PeproTech) in PDW to give a concentration of 10^5 IU/ml and then sterilised through a 0.2µm filter and stored at -20ºC in 200µl aliquots.

MLA-144 supernatant (MLA) was obtained from cultured MLA-144 cells (see section 2.1.7) by sterile filtration through Millipore Steritop™ 0.22µm vacuum driven disposable bottle top filters, and 60ml aliquots were stored at -20°C.

Lymphoprep™ was purchased from Axis-Shield in 300ml bottles.

Dimethyl sulphoxide (DMSO) was purchased from Sigma-Aldrich in 100ml bottles.

2.1.2 Subjects

Healthy members of the institute were recruited and consented as donors for this study. In addition, with local ethical approval (LREC No: 2002/073), healthy individuals aged 60 and over were recruited from the local West Midlands community in collaboration with the department of Geriatric Medicine. DNA was extracted from whole blood from each donor (see section 2.5.2) and sent to the Anthony Nolan trust for HLA typing. A latex agglutination kit (CMVScan, Becton Dickinson, Oxford, UK) was used to determine CMV serostatus using plasma samples. All experiments were approved by the South Birmingham Local Research Ethics Committee (07/Q2702/24). All donors provided written informed consent for the collection of blood samples and subsequent analysis.

2.1.3 Lymphocyte isolation from peripheral blood

Peripheral blood samples were obtained by venepuncture and collected into heparinised syringes and processed as soon as possible. Buffy coat samples were obtained from the West

Midlands Blood Transfusion Service. Both were then diluted 1:1 with RPMI-1640 (Invitrogen, UK), layered on top of 15mls of Lymphoprep™ (Axis-Shield UK, Huntingdon, UK) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. PBMCs were harvested from the interphase and washed twice in RPMI-1640 and then resuspended in RPMI-1640 supplemented with 10% FCS, 2mM L-glutamine and 100U/ml penicillin-streptomycin (Sigma-Aldrich, UK) before counting using a FastRead 102™ counting slide (Immune Systems Limited, UK). Cells were then used immediately or cryopreserved for later use. Plasma samples were also collected following density gradient centrifugation and stored at -20°C for CMV serotyping at a later date.

2.1.4 Generation and maintenance of B95.8 transformed lymphoblastoid cell lines (LCLs)

5 x 10^6 PBMCs were resuspended in 1ml of supernatant from the B95.8 cell line (provided by Alison Leese). Cells were incubated for 1 hour with gentle agitation every 15 minutes, before being washed twice in LCL media (RPMI-1640 plus 10%FCS, 2mM L-glutamine and 100U/ml penicillin-streptomycin solution). Cells were resuspended in 2mls of LCL media containing 0.5µg/ml cyclosporin A and plated out into 2 wells of a 48-well plate. B cell transformation could be observed within one week and transformed cells were expanded until they could be maintained in 25cm^2 tissue culture flasks (IWAKI) in 10ml LCL media.

2.1.5 Generation and maintenance of fibroblasts

Primary human fibroblasts from donors in the Institute of Cancer Studies had already been established from skin punch biopsies. Briefly, skin biopsies were sterilely transferred to a 100mm Petri dish and minced finely. Tissue pieces were distributed into a 12-well tissue culture plate and fibroblast growth medium (DME medium supplemented with Hepes, 10%

FCS, and 100U/ml penicillin-streptomycin solution) was added. Cells were fed by half medium change until they reached 80% confluence. At this point the cells were passaged by removing the medium, washing with PBS and incubating in PBS containing 0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA) until cells detached from the plate. Cells were collected by centrifugation and seeded into 25cm^2 flasks. Human foetal foreskin fibroblasts (HFFFs) (a kind gift from Dr. Naeem Khan) had previously been established and stored in liquid nitrogen.

2.1.6 Maintenance and passage of human cells in culture

All cell cultures were kept at 37° C with 5% CO_2 in a Galaxy R humidified incubator (RS Biotech Irvine, UK).

LCLs

LCLs were maintained in LCL media and were fed once weekly by half medium change. Flasks were maintained at a cell density between 10^5 and 10^6 cells/ml. Once established, LCLs were frozen in aliquots of 5×10^6 cells and transferred into liquid nitrogen for long-term storage.

Fibroblasts

Both primary human fibroblasts and HFFFs were maintained in flasks in fibroblast growth medium. Cells were passaged when they reached around 80% confluence. Aliquots of $5x10^6$ cells were frozen down and stored in liquid nitrogen for later use.

MLA-144

The gibbon cell line MLA-144 is an established line derived from a spontaneous lymphosarcoma of gibbon (Rabin, 1981 #424). This cell line releases IL-2 and so supernatant was used as a source of T cell growth stimuli. Cells were routinely maintained in standard media, but for the production of MLA supernatant, cells were cultured in standard media for two weeks in 150cm^2 flasks without further feeding, after which time supernatant was harvested as described in section 2.1.3.

Cytotoxic T lymphocyte (CTL) clones

Clones were maintained in established clone media (RPMI-1640 supplemented with 10% B Cell Serum (BCS), 1% HuS, 30% MLA, 2mM glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin and 50U/ml IL-2) in 24-well plates and were fed twice weekly by half medium exchanges. Once cell numbers exceeded 10^6/ml clones were split into further wells.

2.1.7 Cryopreservation of cells and revival of cryopreserved cells

Cells were pelleted and resuspended in freezing media (90% FCS + 10% DMSO) at a concentration of 1-10 x 10^6 cells/ml and then aliquoted into sterile 1ml cryovials (Nunc). These were transferred to a "Mr Frosty" (Nalgene) and placed at -80°C to ensure a 1°C/minute decrease in temperature for the cryovials. The following day, cryovials were transferred to liquid nitrogen freezers for long-term storage.

Revival of cells was carried out by placing frozen cryovials into a 37°C waterbath for rapid thawing, followed by washing the cells with RPMI-1640 + 10% FCS. Cells were pelleted by

centrifugation before resuspending in appropriate media and transferred into a culture flask/plate.

2.1.8 Mycoplasma testing

Cell cultures were routinely screened for mycoplasma contamination by using a MycoAlert® kit (Cambrex), according to the manufacturer's instructions.

2.2 VIRUSES AND ANTIGENS

2.2.1 AD169

AD169 is a laboratory strain of CMV which during extensive passage in vitro suffered a spontaneous 15-kb deletion in one end of the long unique (UL) region commonly referred to as the UL/b_ sequence. This region is predicted to encode 23 ORFs designated as UL128–UL150 (Dolan *et al.* 2004). Consequently, both *UL141* and *UL142* are missing from strain AD169. *UL141* encodes an endoplasmic reticulum resident glycoprotein that acts to prevent surface expression of CD155 (also called poliovirus receptor), a ligand for NK cell-activating receptors CD226 (DNAM-1) and CD96 (TACTILE) (Bottino *et al.* 2003; Fuchs *et al.* 2004; Tomasec *et al.* 2005). *UL142* helps to protect HCMV-infected fibroblasts from NK cell attack by decreasing surface expression of most MICA alleles (Wills *et al.* 2005; Chalupny *et al.* 2006). Finally, UL144, a TNFR homologue which inhibts $CD4^+$ T cells is also absent from the AD169 virus strain. AD169 is also unique in that it is unable to enter latency and nearly always assumes lytic growth upon infection.

The AD169 strain of human CMV (a kind gift from Dr. Andreas Moosmann) was propagated in a human foreskin fibroblast cell line at a multiplicity of infection of 0.1. Supernatants were harvested 5 days after 100% cytopathic effect (CPE) was observed by microscopy. Supernatants were frozen in 1ml aliquots and stored at –80°C. Virus titres were determined by plaque assays in 12-well plates using 10-fold dilutions of virus.

2.2.2 Modified Vaccinia Ankara (MVA)

An MVA construct containing pp65 was provided by Dr. Naeem Khan. Briefly, cDNA was made from RNA extracted from CMV-infected human fibroblasts. The PCR product for pp65 was digested with restriction enzymes and ligated with a modified version of the vaccinia virus shuttle vector pSC11. Recombinant MVAs were generated by transfecting the pSC11 plasmid into 10^6 primary chicken embryo fibroblasts (CEF). The CEF were previously infected with wild-type MVA at an MOI of 0.1 in 25cm^2 tissue culture flasks. Recombinant MVA plaques were then selected by ß-galactosidase screening. After six rounds of plaque purification, the viruses were expanded by infecting serially greater numbers of BHK-21 cells and were harvested after visible CPE occurred. Virus stocks were resuspended in PBS and then subjected to three freeze-thaw cycles and sonication. Cell debris was removed by low-speed centrifugation prior to storage of the virus in aliquots at –80°C. Virus titers were determined by plaque assay on CEF and/or BHK-21 cells. Typical titers were $5x10^7$ to $5x10^8$ pfu/ml. A control MVA was also generated, which incorporated empty pSC11 plasmid sequences.

2.2.3 Infection of cells with viruses

2.2.3.1 Infection of fibroblasts with CMV

Cells were seeded into 150cm^2 tissue culture flasks at least 24 hours prior to infection. Media was removed from the flasks and virus was added in 5ml of fibroblast growth medium. Cells were incubated for 2 hours (37°C, 5% CO_2) and flasks were rocked every 15 minutes. Following incubation, virus was removed from the cells and fresh media added.

2.2.3.2 Infection of LCLs with MVAs

LCLs were counted and aliquoted into 15ml propylene tubes. Cells were centrifuged and the pellets resuspended in 100µl of LCL growth medium to which an MVA construct had been added (MOI of 2:1). Cells were incubated for 2 hours (37°C, 5% CO_2) with gentle agititation every 15 minutes. Following incubation, cells were washed and resuspended in growth medium.

2.2.4 Generation of CMV lysate

HFFF monolayers at 80% confluence were infected with the AD169 strain of human CMV at an MOI of 3 (pfu/cell) as described above. Cells were incubated at 37°C, 5% CO_2 and the supernatant was harvested and replaced with fresh media at 2-3 day intervals until full CPE was seen. In addition some cells were harvested at 6 hours post infection by removing the medium and using a cell scraper to detach the monolayer. Harvested supernatants and cells were stored at -80°C until all had been collected. Supernatants were then defrosted and centrifuged at 18,368 x g for 2 hours at room temperature. The pellets were resuspended in a small volume of RPMI-1640. The suspension was sonicated and subjected to 3 rounds of a freeze/thaw cycle using liquid nitrogen. Lysate was aliquoted and stored at -80°C until use. As a control mock lysate was also generated using the same method but with uninfected cells.

2.2.5 Peptides

Peptides were synthesised by Alta Biosciences (University of Birmingham, UK) and dissolved in DMSO. Peptide concentration was determined by using biuret reagent (Sigma, UK) (see section 2.2.6). A description of peptides used can be found in Tables 2-1 and 2-2.

Table 2-1: Table of peptides used for tissue culture

Peptide	Protein	Peptide sequence	HLA restriction
A1	pp65 (UL83)	KYQEFFWDANDIYRI (509-523)	HLA-DR1/3
A2	pp65 (UL83)	AGILARNLVPMVATV (489-503)	HLA-DRB1*0701
A3	pp65 (UL83)	PQYSEHPTFTSQYRIQ (361-376)	HLA-DR11
A4	pp65 (UL83)	FTSQYRIQGKLEYRHT (369-384)	HLA-DR11

Table 2-2: Table of peptides used for stimulation of CMV-specific T cell responses

	Peptide sequence	HLA restriction	CD8/CD4
Mix 1 : IE-1	ATTFLQTMLRK	A68	CD8
	KEVNSQLSL	B40	CD8
	QIKVRVDMV	B8	CD8
	DELRRKMMY	B44	CD8
	ELRRKMMYM	B8	CD8
	ELKRKMIYM	B8	CD8
	KRKMMYMCY	B27	CD8
	FPKTTNGCSQA	B55	CD8
	CVETMCNEY	A1/B18	CD8
	CRVLCCYVL	B7	CD8
	YVLEETSVML	A2 (contains 2 epitopes: YVL & VLE)	CD8
	RRIEEICMK	B27	CD8
	EEAIVAYTL	B44	CD8
	VLEETSVML	A2	CD8
Mix 2 : pp65	DTPVLPHETR	A68	CD8
	QPSLILVSQY	B35	CD8
	YTPDSTPCHR	A68	CD8
	CPSQEPMSIY	B35	CD8
	VYALPLKML	A24	CD8
	IPSINVHHY	B35	CD8
	FVFPTKDVALR	A68	CD8
	FPTKDVAL	B35	CD8
	QYVKVYLESF	A24	CD8
	RPHERNGFTVL	B7	CD8
	QAIRETVELR	B35	CD8
	QYDPVAALF	A24	CD8
	YSEHPTFTSQY	A1	CD8
	(HPTFTSQY)	(also spans a B35 epitope)	
	TPRVTGGGAM	B7	CD8
	NLVPMVATV	A2	CD8
	RIFAELEGV	A2	CD8
	PDVYYTSAFVFP	DR7	CD4
	IIKPGKISHIMKL	DR4	CD4
	PQYSEHPTFTSQYRI	DR11	CD4
	FTSQYRIQGKLEYRH	DR11	CD4
	AGILARNLVPMVATV	DR	CD4
	KYQEFFWDANDIYRI	DR52	CD4
Mix 3	VTEHDTLLY	A1 (pp50)	CD8
	DYSNTHSTRYV	DR7 (gB)	CD4
	VFETSGGLVVFWQGI	DR7 (gB)	CD4
	CMLTITTARSKYPYH	DR4(gB)	CD4
	HELLVLVKKAQL	DR11 (gH)	CD4

2.2.6 Biuret assay

A standard curve was generated with serial dilutions of bovine serum albumin (BSA) dissolved in DMSO. 20µl of each dilution was transferred into duplicate wells of a 96-well plate along with 20µl of each sample of unknown peptide concentration. 180µl of biuret reagent was added to each well and the plate was incubated for 30 minutes at room temperature. Plates were then centrifuged and 100µl of each well was transferred to a 96-well flat bottom plate. The absorbance was measured at 540nm using a Victor plate reader (Wallac, Finland). Concentrations of the unknown samples were then determined from the standard curve.

2.3 FLOW CYTOMETRIC ANALYSIS

2.3.1 Analysis of surface antigens

The cell surface phenotype of PBMCs, T cell clones and peptide lines was examined by flow cytometry. Between $1x10^5$ and $1x10^6$ cells were used for each set of antigens examined. Additional cells were used for colour compensation and isotype controls. Cells were washed in ice cold FACS buffer (PBS, 2% FCS) and aliquoted into wells of a 96-well V bottomed plate. Centrifuging the plate at 400 x g for 3 minutes at 4°C pelleted the cells and the supernatant was discarded. Cells were resuspended in 50µl of FACS buffer containing a Live/Dead® Fixable Dead Cell Stain (Invitrogen Molecular Probes) which stains dead cells so they can later be excluded from analysis. Cells were then washed in ice cold FACS buffer, and incubated in HuS for 15 minutes to prevent non-specific binding. Cells were washed x3 in FACS buffer and then resuspended in 50µl FACS buffer containing the primary antibody. Cells were incubated at 4°C for 30 minutes before being washed x3 in FACS buffer. If the primary antibody was directly conjugated to the fluorophore then the pellet was resuspended in FACS buffer, transferred to FACS tubes and analysed on the flow cytometer. If the primary antibody was un-conjugated samples were incubated in 20µl mouse serum (DakoCytomation) for 20 minutes at 4°C before being washed x3 in FACS buffer and incubated with the conjugated secondary antibody diluted in 50µl of FACS buffer. Cells were incubated for a further 30 minutes at 4°C before being washed and transferred to FACS tubes for analysis.

2.3.2 Analysis of intracellular cytokines and components

To assess the production of intracellular interferon gamma (IFNγ) following antigenic stimulation the Intraprep intracellular staining kit was used according to manufacturer's instructions. Approximately 1 x 10^6 PBMCs were used for each condition and were aliquoted in 15ml propylene tubes in 0.5ml growth media. Cells were stimulated with either CMV lysate (50µl), peptide (2µg/ml) or 0.5µg/ml staphylococcal enterotoxin (SEB) (Sigma-Aldrich, UK) as a positive control. Samples were incubated (37°C, 5% CO_2) for 6 hours with 10µg/ml of Brefeldin A (Sigma) added to each sample 1 hour after stimulation to prevent IFNγ being exported via the golgi apparatus. Following incubation, cells were washed, transferred to a 96-well V bottomed plate, and stained for surface antigens using both conjugated and unconjugated antibodies (as described in section 2.3.1). Cells were then fixed and permeabilised using the IntraPrep kit (Beckman Coulter) according to the manufacturer's instructions. 1µl of α-IFNγ mAb (BD Biosciences) was then added to the wells and samples were incubated for a further 30 minutes (4°C). Cells were washed x3 in FACS buffer and transferred to FACS tubes for analysis.

Table 2-3: Table of antibodies for flow cytometry

	Antigen	Clone	Fluorophore	Isotype	Source
Conjugated antibodies	CD3	UCHT1	PC5	IgG1	Beckman Coulter
	CD3	SK7	AmCyan	IgG1	BD Biosciences
	CD3	S4.1	APC	IgG2a	Caltag
	CD4	RPA-T4	FITC	IgG1	BD Pharmingen
	CD4	RPA-T4	R-PE	IgG1	BD Pharmingen
	CD4	13B8.2	PC5	IgG1	Beckman Coulter
	CD4	SK3	PerCP-Cy5.5	IgG1	BD Biosciences
	CD4	S3.5	Pacific Orange™	IgG2a	Caltag
	CD8	B9.11	PC5	IgG1	Beckman Coulter
	CD8	RPA-T8	Pacific Blue™	IgG1	BD Pharmingen
	CD8	3B5	Qdot® 655	IgG2a	Invitrogen Molecular Probes
	CD14	M5E2	Pacific Blue™	IgG2a	BioLegend
	CD19	HIB19	Pacific Blue®	IgG1	eBioscience
	CD27	M-T271	FITC	IgG1	BD Pharmingen
	CD28	CD28.2	ECD	IgG1	Beckman Coulter
	CD45RA	HI100	Alexa Fluor® 700	IgG2b	BioLegend
	CD56	C5.9	R-PE	IgG2b	DakoCytomation
	CD56	HCD56	Pe/Cy7	IgG1	BioLegend
	CD158a	HP-3E4	FITC	IgM	BD Biosciences
	CD158e1	DX9	FITC	IgG1	BD Biosciences
	CD158e1	DX9	Biotin	IgG1	BioLegend
	CD158i	FES172	PE	IgG2a	Beckman Coulter
	CCR7	150503	APC	IgG2a	R&D Systems
	IFNγ	25723.11	FITC	IgG2b	BD Biosciences
	IFNγ	25723.11	PE	IgG2b	BD Biosciences
	IFNγ	4S.B3	Alexa Fluor® 700	IgG1	BioLegend
Primary antibodies	CD158b	GL183	Unconjugated	IgG1	Beckman Coulter
Secondary antibodies	α-mouse IgG	-	R-PE	Goat	DakoCytomation
	α-mouse IgG1	-	PC5	Goat	Caltag
	α-mouse IgG	-	Pe/Cy7	Goat	Santa Cruz Biotechnology
	α-mouse IgG	-	Pacific Blue™	Goat	Invitrogen Molecular Probes
	α-mouse IgG	-	APC	Goat	Southern Biotech
Streptavidin	Streptavidin	-	R-PE	-	Invitrogen Molecular Probes
	Streptavidin	-	APC	-	Caltag
	Streptavidin	-	APC/Cy7	-	BioLegend
Isotypes	Negative mouse IgG1 control	-	Unconjugated	-	DakoCytomation
	Negative mouse IgG2a control	-	Unconjugated	-	DakoCytomation
	Negative mouse IgG2b control	-	Unconjugated	-	DakoCytomation

2.3.3 Colour compensation and isotype controls

Flow cytometer settings were optimised for each set of cells analysed. Isotype control antibodies were used for the different antibody isotypes used and the negative population was set on the basis of the isotype controls. Colour compensation settings were adjusted for each experiment. Single antibody stained cells were mixed with equal numbers of unstained cells.

For experiments analysed on the BD LSR II cytometer, colour compensation was carried out using BD™ CompBeads (BD Biosciences) where appropriate. Analysis was performed using BD FACSDiva software.

2.4 IMMUNOLOGICAL ASSAYS

2.4.1 Production of T cell clones

CMV-specific T cells were isolated using different methods (described in sections 2.4.1.1 – 2.4.1.3) and then cloned by limiting dilution. Once established, clones were amplified in order to produce large enough numbers to address their characteristics.

2.4.1.1 FACsorting

Cell sorting was employed to isolate $CD4^+$ $CD28^-$ T cells. PBMCs were stained with α-CD4 phycoerythrin (PE) and α-CD28 fluorescein isothiocyanate (FITC) and then washed with sterile PBS (containing 2% FCS and no azide) prior to sorting using a FACS Vantage cell sorter (Becton Dickinson). Single $CD4^+$ $CD28^-$ cells were sorted into 96-well plates containing 200µl of cloning mix. A 96-well plate with only cloning mix and no T cells added was used as a control. After 14 days growing microcultures were expanded to 2ml cultures.

2.4.1.2 Cytokine Secretion Assay (CSA)

The IFNγ secretion assay (Miltenyi Biotec, Germany) was used to detect CMV-specific T cells based on antigen-triggered induction of cytokine production. In this assay the secreted IFNγ is bound to the cell surface and then stained as an artificial surface molecule and analysed by flow cytometry. The assay was performed according to the manufacturer's instructions. Briefly, PBMCs at 10^7cells/ml were incubated with 50µl of CMV lysate overnight (37°C, 5%CO_2) or peptide (5µg/ml) or SEB (1µg/ml) for 3 hours. Cells were washed with MACS buffer (PBS, 0.5% BSA, 2mM EDTA) and incubated with cytokine

capture reagent on ice before addition of PBMC medium. Cells were then incubated for 45 minutes under constant rotation (37°C, 5% CO_2). Samples were washed with MACS buffer before being stained with IFNγ detection antibody and then washed again before incubation with α–PE microbeads. Samples were either passed through an autoMACS separator (Miltenyi Biotec, Germany) or through MACS MS separator columns (Miltenyi Biotec, Germany) and the positive fraction containing IFNγ secreting cells was collected. Pre- and post-sort samples were stained with α-IFNγ, α-CD4, and α-CD8 monoclonal antibodies (mAbs) and analysed for purity by flow cytometry.

2.4.1.3 Generation of polyclonal CTL lines

PBMCs were isolated as described from fresh blood. For lines using total PBMC, after the second wash with RPMI-1640 the cells were ready for peptide stimulation. For CD4 lines, $CD8^+$ cells were depleted using CD8 dynabeads (Dynal, UK). Briefly, beads were washed twice in cold PBS prior to incubation with cells. Cells were then incubated with CD8 beads at 4°C on a tube rotator for 30 minutes (at a ratio of 4 beads: 1 $CD8^+$ T cell, assuming that 33% of the PBMC population are $CD8^+$ T cells). Afterwards the separation was carried out using a Dynal magnet. The purity of the separation was determined by staining the negatively selected fraction with an α-CD8 FITC conjugated mAb. Subsequent flow cytometric analyses revealed excellent purity with less than 2% of the negatively-selected fraction being $CD8^+$ cells. In cases where lower levels of purity were observed, the selection process was repeated.

Cells were resuspended in 100µl of RPMI-1640 containing 5µl/ml of peptide and incubated for 1-2 hours at 37°C. Cells were then transferred to a 24-well plate and diluted in RPMI-

1640 containing 2mM L-glutamine, 100U/ml penicillin-streptomycin and 5% autologous plasma. The culture was restimulated with γ-irradiated autologous LCL pulsed with peptide at a ratio of 2:1 after 7 days. Polyclonal CTL lines were screened by IFNγ enzyme-linked immunosorbent assay (ELISA) (as described in section 2.4.2) for antigen specificity before cloning by limiting dilution.

2.4.1.4 Limiting dilution assay (LDA)

A cloning mix was prepared which comprised cloning media (RPMI-1640 supplemented with 10% HuS (Invitrogen), 2mM glutamine, 100 U/ml penicillin-streptomycin solution and 50U/ml recombinant IL-2 (Chiron, Netherlands)) with 10^6/ml feeder cells (γ-irradiated (40 greys)) PBMCs pooled from 3 buffy coats) and 10^5/ml antigen presenting cells (APCs) (γ-irradiated autologous or HLA matched LCL pulsed with the appropriate antigen).

Antigen-specific cells separated as described above were counted and plated out in 96-well U bottomed plates at dilutions of 0.3, 3 and 30 cells/well in 100µl of the cloning mix. Cells were incubated for 3 days (37°C, 5% CO_2) before 100µl/well of cloning media supplemented with 60% MLA was added. Cells were then incubated for a further 2-3 weeks until cell growth could be identified.

2.4.1.5 Buffy boost protocol for expansion of T cell clones

Once established, clones were screened for antigen specificity using IFNγ ELISA (see section 2.4.2). CMV-specific clones were then expanded using the buffy boost protocol. Briefly, a mix was prepared comprising established clone media (RPMI-1640 supplemented with 10%

BCS, 1% HuS, 30% MLA, 2mM glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin and 50U/ml IL-2) plus10^6/ml feeder cells (γ-irradiated (40 greys) PBMCs pooled from 3 buffy coats) and 10^5/ml APCs (γ-irradiated autologous, or HLA matched, LCL pulsed with the appropriate antigen). Approximately 10^5 cells of each established clone (or one well from the 96-well cloning plates) were placed into one well of a 24-well plate containing 2 ml of buffy boost mix.

2.4.2 IFNγ ELISA

IFNγ ELISA was used to screen clones for antigen specificity using production of IFNγ as a marker of recognition. Autologous or HLA matched LCL served as APCs and were pulsed with CMV lysate or peptide or infected with MVA constructs expressing individual CMV proteins (see section 2.2.2). APCs were also left untreated, pulsed with mock lysate, irrelevant peptide or infected with control MVA (MVA-pSC11). $5x10^4$ /well APCs were plated out in a 96-well U bottomed plate in PBMC growth media. T cell clones were washed in RPMI-1640 and between $1x10^2$ and $2x10^3$ of each T cell clone were added to the appropriate wells containing APCs. Plates were then incubated for 16 hours (37°C, 5% CO_2).

On the same day, F96 maxisorp ELISA plates (Nunc, Denmark) were coated with α-human IFNγ mAb (Pierce, UK) diluted to 0.75µg/ml in coating buffer (0.1M $Na_2H_2PO_4$) and incubated overnight (4°C). Wells were then blocked by addition of 200µl of blocking buffer (PBS 1% BSA 0.5% Tween 20 (v/v)) and incubated at room temperature for 1 hour. Plates were washed x3 with washing buffer (PBS 0.05% Tween 20 (v/v)) and 50µl of supernatants from the wells containing APCs/T cell clones were added to each well. IFNγ standards were

prepared in PBMC growth media using two fold serial dilutions of IFNγ (Sigma) from 2ng/ml to 31.25pg/ml. 50µl of each standard were added in triplicate to the ELISA plates. Plates were incubated for 3 hours at room temperature, after which they were washed x3 in washing buffer and then 50µl of biotinylated α-IFNγ (Pierce, UK) diluted to 0.375µg/ml in blocking buffer was added to each well. Plates were incubated for 1 hour at room temperature followed by 3 washes. 50µl of ExtrAvidin (Sigma-Aldrich, UK) diluted 1:1000 in blocking buffer was added to each well and the plates incubated for 30 minutes. Following this, plates were washed x8 in washing buffer before 100µl of 3,3'-5,5'-tetramethylbenzidine (TMB) (Tebu-Bio, UK) was added to each well. Reactions were terminated after 20 minutes by the addition of 100µl 4M H_2SO_4/well. The concentration of IFNγ in each well was quantified by determining the absorbance at 450nm, using a Victor plate reader (Wallac, Finland), and comparing the values to a standard curve constructed using the IFNγ standards.

2.4.3 Chromium release assay

To determine specific cytotoxicity of generated T cell clones standardised chromium release assays were performed. Autologous and HLA mismatched LCLs served as target cells. LCLs were infected with MVAs (MOI of 2:1) as described above and incubated for 16 hours following infection. Alternatively, targets were loaded with 50µl CMV lysate for 12 hours prior to the assay. In addition, LCLs were loaded with relevant or irrelevant peptide for 90 minutes prior to the assay. Following antigen-loading targets were incubated with 100µCi sodium chromate ($Na_2{}^{51}CrO_4$) (Amersham-Pharmacia Biotec, UK) for 90 minutes at 37°C, 5% CO_2 with agitation. Targets were washed, counted and resuspended (25000 cells /ml) in growth medium. 100µl of each target were then added to wells of a 96-well V bottomed plate. T cell clones were washed, counted and resuspended at the correct concentration before

being added in 100µl to the plate. T cells (effectors) were used at 2 different effector/target ratios, 2.5:1 and 5:1 – each carried out in triplicate. Maximum release was determined by incubation of radioactively labelled target cells with 1% SDS, and spontaneous release by incubation with medium alone. Plates were incubated for 6 and 18 hours at 37°C, 5% CO_2. After this time γ emission was quantified in 100µl culture supernatant using Packard Cobra gamma counter (Global Medical Instrumenatation, Minnesota, USA.) Results were represented as percentage lysis and were calculated using the following formula;

$$\% \text{ lysis} = \frac{\text{Gamma count of sample} - \text{spontaneous gamma release}}{\text{Gamma count of maximum release} - \text{spontaneous gamma release}} \times 100$$

2.5 MOLECULAR BIOLOGY

2.5.1 Media and buffers

LB media

LB (Luria Broth) media was prepared by dissolving 20g/L of Lennox L Broth base powder (Invitrogen) in SDW and sterilised by autoclaving for 20 minutes at 15psi and 121°C.

Luria agar (LB agar)

LB agar was prepared by dissolving 20g/L of LB powder (Invitrogen) and 7.5g/L of LB Select Agar (Invitrogen) in SDW and sterilised by autoclaving for 20 minutes at 15psi and 121°C.

SOB/SOC media

SOB media was prepared by dissolving 28g/L DIFCO-Bacto-SOB medium (Becton-Dickinson) in SDW and sterilised by autoclaving for 20 minutes at 15psi and 121°C.

SOC was made by adding 20ml of 20% glucose filter sterilised through a 0.2µM filter (Millipore) to 1L of SOB media.

Antibiotics

Ampicillin was made up as a 1000X stock at 100mg/ml in distilled water and filter sterilised through a 0.2µm filter (Millipore). This was then stored in aliquots at -20°C.

Tris/acetate buffered EDTA (TAE)

TAE was prepared as a 50X stock by dissolving 242g of Tris base (Sigma), 57.1ml glacial acetic acid and 18.2g EDTA in 1L of SDW.

Tris-EDTA (TE)

A 1X TE stock was prepared as 10mM Tris-HCl pH8.0 with 1mM EDTA in SDW. This was sterilised by autoclaving for 20 minutes at 15psi and 121°C prior to use.

2.5.2 DNA extraction from PBMCs

DNA was extracted from PBMCs using the DNeasy™ Tissue Kit (QIAgen, UK) according to the manufacturer's instructions. Briefly, up to $5x10^6$ cells were washed in PBS and pelleted by centrifugation at 145 x g, then resuspended in 200µl PBS. 20µl Proteinase K and 200µl lysis buffer were added to the mix to lyse cells and digest protein. Proteinase K is also used to inactivate nucleases that might degrade the DNA during purification. The sample was incubated at 70°C for 10 minutes, then 200µl ethanol (96-100%) were added to provide the appropriate binding conditions and the sample was applied to a DNeasy column where DNA binds to a silica gel membrane. The column was washed and spun at 11,015 x g x3 before the DNA was eluted in 200µl of dH_2O. The purified DNA was quantified using a Nanodrop machine (Thermo Scientific), and stored at -80°C until needed.

2.5.3 RNA extraction from PBMCs

Total RNA was extracted from PBMCs using the RNeasy® Mini Kit (QIAgen, UK) according to the manufacturer's instructions. Briefly, between $2x10^6$ and $5x10^6$ cells were washed in PBS and pelleted by centrifugation at 17,900 x g. Cells were resuspended, lysed and homogenized in a high denaturing guanidine-isothiocyanate buffer which functions to inactivate all RNases ensuring isolation of intact RNA. Ethanol was then added to provide the appropriate binding conditions and the sample applied to an RNeasy. Subsequent washing steps were carried out to eliminate contaminants before the RNA was eluted in 30µl of dH_2O. The purified RNA was quantified using the Nanodrop machine, and stored at -80°C until needed.

2.5.4 cDNA synthesis

5µg RNA was incubated with an oligo(dT)$_{12-18}$ primer and 10mM deoxyribonucleotide triphosphate (dNTP) mix at 65°C for 5 minutes and then chilled on ice for a further 2 minutes. The RNA/primer mix was used for reverse transcription in a 20µl reaction at 42°C for 50 minutes containing 50mM Tris-HCl (pH 8.3), 75mM KCl, 5mM $MgCl_2$, 10mM dithiothreitol (DTT) and 50 units of Superscript II (Life Technologies, UK). Reactions were terminated by incubation at 70°C for 15 minutes. The presence of cDNA was verified by PCR amplification using primers specific for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.5.5 Standard PCR amplification

50µl reactions were set up containing <0.1µg of DNA template (e.g. 0.5µl of Qiagen plasmid miniprep), dNTPs (10mM), forward and reverse primers (~20pM) (see Table 2-4), DNA polymerase and the enzyme buffer recommended by the manufacturer. All reactions were carried out using a thermocycler (Perkin Elmer) according to the following programmes :-

KIR proteins

94°C for 2 minutes

94°C for 30 seconds
61°C for 30 seconds
72°C for 30 seconds
} 35 cycles

72°C for 7 minutes.

HLA proteins

95°C for 3 minutes

95°C for 1 minute
61°C for 1 minute
72°C for 1 minute
} 30 cycles

72°C for 10 minutes

Table 2-4: Table of primers

Amplicon	Forward primer	Reverse primer
KIR2DS2	AGTCGTCATATGCCACATCATGAGGGAGTCCACAG	AGCTACGGATCCGGTTTTGGAGCTTGGTTCAG
KIR2DL2 SDM 1	GGCCCACCCAGGTCGCCTGGTGAAATCAG	-
KIR2DL2 SDM 2	GCACAGAGAAGGGAAGTTTAAGGACAC	-
KIR2DL2 SDM 5	GGAGGCCCATGAATGTAGGTTCTCTGC	-
KIR2DL2 SDM 7	CTTGTTTCTGTCATAGGAAACCCTTC	-
HLA-Cw*0702	AGTCGTCATATGTGCTCCCACTCCATGAGGTATTTC	AGCTACGGATCCTGGCTCCCAGCTCAGGGTGAGGGG
E.coli optimised HLA-Cw*0702	GGGAATTCCATATGGGTTCTCATTCTATGAGATATTTCGA TACTGCTGTGTCCCGGCCCGGCCGCGGAG	-
T7	TAATACGACTCACTATAGGG	TATGCTAGTTATTGCTCAG

—— Restriction site – CATATG = BamHI; GGATCC = NdeI

2.5.6 Site-Directed Mutagenesis (SDM)

PCR products were modified using a QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene, CA) according to manufacturer's instructions. Briefly, a reaction mix of <0.1µg of DNA template, dNTPs (10mM), 0.125µg multiple primers (all forward) (see Table 2-4), QuikChange® Multi enzyme blend and QuikChange® Multi reaction buffer. The reaction was carried out as the standard PCR but using the following cycling conditions :-

95°C for 2 minutes

95°C for 1 minute
55°C for 1 minute
65°C for 8 minutes
} 35 cycles

68°C for 10 minutes

Amplification products were *Dpn* I digested at 37°C for 1 hour to remove parental DNA.

2.5.7 Bacterial strains

The bacterial strains of *Escherichia coli* (*E.coli)* used in this work are detailed in Table2-5 below :-

Table 2-5: Table of E.coli strains used

***E.coli* strain**	**Genotype**	**Source**
DH5α	F- endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG lacZdeltaM15 hsdR17	Stratagene
BL-21 DE3 pLysS	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3) pLysS(cm^R)	Novagen
One Shot® Top 10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-	Invitrogen

2.5.8 Generation of competent bacteria

Competent cells were prepared using the "Simple Efficient Method" (Inoue *et al.* 1990). 3ml of LB medium (10mg/ml Tryptone, 5mg/ml Yeast Extract, 10mg/ml NaCl) was inoculated with either DH5α or BL-21 DE3 pLysS strains of *E.coli*. The cells were incubated overnight at 37°C shaking at 200rpm. 10^3 cells of the overnight culture were streaked onto an LB agar plate containing no antibiotics and incubated until colonies had reached a diameter of about 5mm. 5–6 of such colonies were used to inoculate 125ml of SOB media (20mg/ml Tryptone, 2.5mg/ml Yeast Extract 10mM NaCl, 3mM KCl), supplemented with 10mM $MgCl_2$ and 10mM $MgSO_4$, in a 1 litre flask. The cultures were grown at 18°C with shaking until the optical density (OD) at 600nm reached approximately 0.6. At this point the cells were harvested by centrifugation at 4°C. Cell pellets were gently resuspended in 80ml of cold SEM medium (15mM Pipes, 10mM $CaCl_2$, 150mM KCl, 30mM $MnCl_2$) and spun again. The cell pellet was resuspended a second time in 20ml SEM media, this time containing 7% DMSO, aliquoted into sterile eppendorf tubes at 200µl per tube and snap frozen immediately in liquid nitrogen to be stored at -80°C.

2.5.9 Transformation of competent bacteria

1µl of plasmid was added to competent cells of the appropriate *E.coli* strain and incubated on ice for 45 minutes. This was followed by a heat shock of 42°C for 1 minute and then brief chill on ice. 200µl of LB medium was added to each tube and cells were incubated at 37°C for an additional 45 minutes. 100µl of each tube was added to a petri-dish containing LB agar medium supplemented with 100µg/ml of ampicillin. The cells were spread evenly across the

whole surface area of the agar and after 10 minutes plates were inverted and incubated overnight at 37°C. The next day plates were checked for colonies.

2.5.10 Preparation of plasmid DNA from transformed bacteria

Bacterial colonies were picked and transferred into 15ml Falcon tubes containing 5ml of LB media containing 100µg/ml of ampicillin. Cultures were then grown with constant shaking overnight at 37°C. The following day the bacterial culture was pelleted by microfuge centrifugation and plasmid DNA then extracted using a QIAprep® Miniprep Kit following manufacturer's instructions. The kit is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica membrane in the presence of high salt. DNA is then washed and eluted in Tris buffer. The recovered bacteria were resuspended in 250µl resuspension buffer containing RNase. 250µl lysis buffer was added, mixed by inversion and allowed to stand for 5 minutes. 350µl neutralisation buffer was added and samples mixed immediately by inversion. A precipitate formed which was cleared by centrifuging at 17,900 x g for 10 minutes and the supernatant poured into a separation column. After centrifugation the pellet was discarded and 750µl wash buffer added. After standing for 1 minute, columns were centrifuged and the flow-through again discarded. Columns were centrifuged for a second time to ensure all wash buffer was removed and DNA eluted into a fresh 1.5ml eppendorf tube with 30-50µl elution buffer. The purified plasmid DNA was stored at -20°C for future use.

2.5.11 Restriction endonuclease digestion of DNA

DNA was digested with specific restriction endonucleases (Roche Applied Science and New England Biolabs) using appropriate buffers as supplied by the manufacturers. Reactions were carried out at 37°C for 1 hour.

2.5.12 Agarose gel electrophoresis

Separation of DNA fragments generated by PCR amplification was performed by agarose gel electrophoresis. 1.5% agarose in 1x TBE (0.09M Tris-Borate, 2mM EDTA) was cast into a gel and loaded with DNA samples diluted in 5x sample loading buffer (25%w/v sucrose, 0.01% bromophenol blue, 0.01% xylene cyanol in 5x TBE buffer). Gels were run for approximately 40 minutes at 140 volts. Bands were visualized with ethidium bromide staining using an ultra-violet transilluminator and the size was estimated with reference to a 100bp or 1kb molecular markers (Life Technologies, UK).

2.5.13 Purification of DNA from agarose gels

DNA fragments were excised from agarose gels with a scalpel and DNA extracted using a GENECLEAN® SPIN Kit (Qbiogene, Inc). The kit is designed on the principle that DNA adsorbs to a silica gel membrane in the presence of high salt while contaminants pass through. Impurities are washed away and the DNA eluted in Tris buffer. Briefly, the gel slices were added to 400µl GLASSMILK® in a spin filter and incubated at 55°C for 5 minutes (or until gel slice had melted). A maximum of 300mg of gel can be added to each filter. Samples were then centrifuged at 17,900 x g for 1 minute and the flow-through discarded. 500µl wash buffer was added to the filter and centrifuged again for 30 seconds. Again the flow-through was discarded and samples were centrifuged to eliminate any residual wash buffer. 30µl

elution buffer was added to the column and the sample allowed to stand for 1 minute. Finally columns were placed in fresh collection tubes, centrifuged, and the eluate containing the DNA stored for future use at -20°C.

2.5.14 Alkaline phosphatase treatment of DNA

Digested plasmid vector DNA was treated with shrimp alkaline phosphatase (SAP) (Roche Applied Science) in order to prevent recircularisation during subsequent ligation steps. The reaction was carried out as per manufacturer's instructions, using 1 unit of SAP per reaction for 1 hour at 37°C.

2.5.15 Ligation

DNA fragments with complementary overhangs were ligated to pGMT7 plasmid vectors through the action of T4 DNA ligase (Roche Applied Science). DNA insert and vector DNA were mixed at a 10:1 ratio in a total volume of 20µl and incubated at 24°C for 6 hours, and then left at 4°C overnight.

2.5.16 Sequencing

Sequencing of DNA in a PGMT7 vector was carried out using an ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). 1µg of DNA was mixed with 3.2pmol/µl of T7 primer (forward or reverse) (see Table 2-4) and 8µl of Terminator Ready Reaction mix in a total volume of 20µl. The sequencing reaction was then carried out in a GeneAmp 9600 PCR system (Hewlett-Packard) using the following protocol :-

96°C for 10 seconds
50°C for 5 seconds
60°C for 4 minutes
} 25 cycles

4°C until precipitation.

Sequencing sample precipitation was then carried out by adding 2µl of 3M sodium acetate (pH4.8) and 50µl of absolute ethanol to the reaction mixture, vortexing and incubating at room temperature for 30 minutes. Afterwards sample tubes were spun at 17,900 x g for 20 minutes, the supernatant aspirated and the pellets rinsed with 250µl of 70% ethanol. Pellets were then air dried and stored at -20°C until needed. Samples were resuspended in 10µl of HiDi formamide buffer and boiled for 5 minutes prior to sequence analysis, carried out commercially by the Department of Genomics, Department of Biosciences, University of Birmingham.

2.6 TETRAMER PRODUCTION

2.6.1 Protein synthesis

KIR and HLA proteins were synthesized using standard methods (Garboczi *et al.* 1992; Altman *et al.* 1996). Plasmids encoding KIR and HLA-C heavy chains (modified by substitution of the transmembrane and cytoplasmic domains by a BirA target sequence) and beta2 microglobulin (β_2M) were used to transform chemically competent BL-21 (DE3) pLysS strains of *E.coli*. Cells were plated overnight onto LB-agar plates using ampicillin as a selection agent. The next day colonies were picked and grown in 4ml LB with ampicillin selection (100µg/ml) to test expression. Cells were grown at 37°C with shaking until the OD at 600nm reached 0.4-0.6; at this point pre-induction samples were taken as controls, and protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to 500µM. Following 3 hours incubation at 37°C, post-induction samples were taken. Samples were then centrifuged, and cell pellets were resuspended in reducing SDS-PAGE loading buffer containing DTT prior to electrophoresis. For large-scale expression, *E. coli* BL21 DE3 cells were transformed with the appropriate plasmid, and colonies were incubated in 10ml LB with ampicillin selection and grown at 37°C with vigorous shaking until cloudy. Cultures were then transferred into 2L flasks containing 1L LB and incubated at 37°C for 4-6 hours induction with IPTG to 500µM at an OD_{600} between 0.4 and 0.6. After 3 hours the cultures were incubated at 4°C overnight.

2.6.2 Protein purification

Cultures were centrifuged, and cell pellets were resuspended in 50ml of ice-cold PBS. Cell membranes were disrupted by several rounds of sonication after which the sample was washed in detergent buffer (50mM Tris.HCl, 0.5% Triton X-100, 100mM NaCl, 1mM EDTA, 1mM DTT, 0.1% sodium azide & 1mM phenylmethylsulphonyl fluoride (PMSF)) by homogenisation with a glass homogeniser and centrifuged for 10 minutes at 26,892 x g in a centrifuge pre-cooled to 4°C. The detergent wash and subsequent spin was repeated twice followed by a wash in resuspension buffer lacking detergent (50mM Tris.HCl, 0.5% 100mM NaCl, 1mM EDTA, 1mM DTT, & 1mM PMSF). The supernatant was discarded and the pellet was solubilised overnight in 20ml denaturing buffer (8M urea, 50mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.5, 10mM EDTA & 2mM DTT) on a rotator. Insoluble debris was then removed by centrifugation at 21,782 x g for 15 minutes. The supernatant was transferred to a fresh tube and dispensed into 1ml aliquots before snap freezing in liquid nitrogen. The frozen protein preparations were stored at –80°C. The yield of protein was determined by protein assay using known amounts of BSA as standards.

2.6.3 Sodium-Dodecyl-Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

SDS PAGE was carried out using the Bio-Rad minigel system. Glass plates were first wiped clean in ethanol and then with sterile water. Spacers were attached to the sides and the plates were clamped together and attached to a stand. Two gel solutions were prepared: a separating gel (12% acrylamide, 0.375M Tris.HCl pH8.8 and 0.1% SDS) and a stacking gel (4% acrylamide, 0.125M Tris.HCl pH6.8 and 0.1% SDS). 10µl of Tetramethylethylenediamine (TEMED) and 50µl of 15% ammonium persulphate (APS) was added to the separating gel, which was mixed and then injected into the glass plates quickly. Once the separating gel had

set, 5µl of TEMED and 25µl of APS was added to the stacking gel. Once mixed, this was injected onto the top of the separating gel and the combs were inserted. After the stacking gel had set the comb was removed and wells were washed with running buffer (25mM Tris.HCl pH8.3, 250mM glycine, 0.1%SDS) to flush out any unpolymerized acrylamide. Samples were then prepared. Each sample was mixed with an equal volume of loading dye (0.0625M Tris.HCl pH 6.75, 2% SDS, 10% glycerol, 5% mecapto-ethanol 0.001% bromophenol blue) and heated at 90°C for 5 minutes to allow proteins to denature. The samples were then loaded onto the gel and run for 20 minutes at 200V. Size determination was aided by also running a protein molecular weight marker on each gel. Gels were stained in Coomassie blue solution (25% methanol, 7% acetic acid, 0.25% coomassie blue) for 30 minutes and then destained (30% methanol, 10 % acetic acid) for 3 hours before drying.

2.6.4 Protein quantification – Bradford assay

Protein quantity was estimated by performing a simple protein assay using known amounts of BSA as standards. Briefly, dilutions of BSA ranging from 5µg/ml to 400µg/ml were added in triplicate to separate wells in a 96-well flat bottomed plate. Then 100µl of 1 in 5 dilution of Bio-Rad protein assay reagent (Bio-Rad) was added to each well, and the plate was left at room temperature for 10min. After this incubation, absorbances at 595nm were read using a plate reader and average values were calculated to derive a standard curve. Dilutions of the refold fractions were simultaneously tested and absorbances measured. The standard curve was used to calculate the protein concentration of each fraction tested.

2.6.5 Generation of protein monomers

Proteins were renatured by dilution refolding, as opposed to dialysis methods, using the following buffer: 100mM Tris-HCL, 2mM EDTA, 0.4M L-arginine-HCL, 0.5mM oxidized glutathione, 5mM reduced glutathione, and 0.1mM PMSF, pH 8.3.

KIR Proteins

15mg KIR protein was added in 3 additions, an hour apart, to 250ml refold buffer at 4°C over a period of 3 hours. The mixture was left for a further 24 hours stirring at 4°C before continuing with the next stage.

HLA Proteins

Heavy chain and β_2M were refolded around the appropriate peptide (see Table 2-6) for 48 hours at 4°C in refold buffer (as above). 6mg β_2M were added to 250ml refold buffer and left stirring for 30 minutes. Then 1.25mg of appropriate peptide were added followed by the first pulse of 7.5mg HLA-C heavy chain. The following day, another 7.5mg heavy chain were added in the morning, and again in the evening. On day 3, the last 7.5mg heavy chain were added, and the mixture was left for a further 24 hours before continuing. Each 7.5mg addition was divided into 3 aliquots, added to 0.5µl DTT and diluted in 10ml refold buffer before being added dropwise to the refold to prevent protein precipitation.

The refolding solution was incubated overnight and then concentrated down to a final volume of 4-8ml using a stirred cell and then ultrafiltration device (Amicon). The buffer was exchanged into a biotinylation buffer (100mM Tris.HCl pH 7.5, 5mM $MgCl_2$, 20mM NaCl, 0.1mM PMSF) using a PD-10 column (Amersham Pharmacia, Bucks UK). Biotinylation was

conducted overnight in the dark at room temperature in the following reaction mix: 5mM d-biotin, 5mM ATP, 5µg BirA enzyme. The BirA enzyme was produced in *E.coli* as described previously (O'Callaghan C *et al.* 1999).

Refolded protein was purified by size exclusion chromatography using Superdex S75 or S200 columns equilibrated with 50mM NaCl, 20mM Tris-HCl, pH 8.0, using an AKTA FPLC. The heavy chain protein is highly susceptible to protease degradation. In cases of such degradation one observes a lower molecular weight heavy chain band on SDS gels. Therefore monomer integrity was analysed by SDS-PAGE. To minimize any protease activity, inhibitors (1µg/ml leupeptin and pepstatin, Sigma UK) were used at all stages. Fractions constituting the main peak of protein eluting at an expected volume were subjected to trichloroacetic acid (TCA) precipitation. Briefly, samples were incubated on ice, centrifuged, and pellets were washed in ice-cold acetone, followed by resuspension in either reducing or non-reducing SDS-PAGE buffer prior to electrophoresis.

Table 2-6: Table of peptides used to refold HLA monomers

HLA allotype	Peptide sequence	Source protein
HLA-Cw*0401	QYDDAVYKL	Consensus peptide
HLA-Cw*0401	QYDPVAALF	CMV peptide – pp65
HLA-Cw*0702	RYRPGTVAL	MHC binder – histone H3.3 40-48
HLA-Cw*0702	CRVLCCYVL	CMV peptide - IE-1
HLA-Cw*0702	AYADFVYAY	MHC binder – Cw7 consensus
HLA-Cw*0702	FAMPNFQTL	MHC binder – Cw3 consensus
HLA-Cw*0702	IPFPIVRYL	MHC binder – Cw6 consensus
HLA-Cw*0702	KYFDEHYEY	MHC binder – CKShs2 11-19
HLA-Cw*0702	KYPDFVDAL	MHC binder – Cw7 consensus
HLA-Cw*0702	YQFTGIKKY	MHC binder – unknown Cw6 natural ligand
HLA-Cw*0702	YRHDGGNVL	MHC binder – unknown Cw6 natural ligand

2.6.6 Protein ELISA to check conformation of folded monomers

To confirm monomers were refolded in the correct conformation antibody recognition was tested.

KIR Proteins

Protein was added at various dilutions (neat to 1:1000000) to a F96 maxisorp ELISA plate in coating buffer and left at 4°C overnight. The following morning the plate was washed x4 in PBS/Tween, and the protein blocked by addition of 5% milk. This was left for 1 hour at 37°C. The plate was then washed again x4 with PBS/Tween and primary antibody (α-KIR)

was diluted in milk, added to the plate and left for 1 hour at 37°C. Following the incubation the plate was washed x4 in PBS/Tween and the secondary antibody (α-mouse-HRP (horseradish peroxidise) (DakoCytomation)) diluted 1:2000 in milk added to the plate. This was left for a further hour at 37°C before being washed for a final time with PBS/Tween. SIGMA*FAST*™ OPD (*o*-Phenylenediamine dihydrochloride) tablets were dissolved and 100µl added per well. OPD is a soluble substrate for the detection of peroxidase, and is used in enzyme immunoassays as it is highly sensitive. This was left for 20 minutes at room temperature, in the dark. To stop the reaction 100µl sulphuric acid (H_2SO_4) was added and absorbance read at 490nm.

HLA Proteins

A F96 maxisorp ELISA plate was coated with W6/32 mAb (α-HLA class I) at ~1µg/ml and left for 1 hour at 37°C (or overnight at 4°C). Wells were then blocked by addition of 200µl of blocking buffer and incubated at room temperature for 1 hour. Plates were washed x3 with washing buffer and HLA proteins then added to wells at a range of dilutions from 1µg to 0.0016 µg. Plates were incubated for 1 hour at 37°C, after which they were washed x3 in washing buffer and then 50µl of extravidin-peroxidase diluted 1000x with blocking buffer was added to each well and the plates incubated for 30 minutes. Following this, plates were washed x8 in washing buffer before 100µl of TMB was added to each well and the plates incubated for 10 minutes before the absorbance was read at 405nm.

2.6.7 Generation of tetrameric complexes

The quantity of refolded protein was estimated by performing a simple protein assay using the Bradford Assay (see section 2.6.4). In addition an ELISA was performed to confirm that the

sample was biotinylated. Serial dilutions of each fraction were added to wells in a 96-well plate and left for 1 hour at 37°C to allow the proteins to adhere to the wells. Each well was then washed x4 with PBS containing 0.05% Tween and then twice with PBS only. 100µl of a 1/1000 dilution of extravidin-peroxidase conjugate (Sigma, UK) in PBS with 0.1% BSA was added to each well. This would bind to any wells with biotinylated proteins but not to non-biotinylated proteins. After 15 minutes incubation at room temperature, the wells were washed again x4 with PBS containing 0.05% Tween and then twice with PBS only. Afterwards 100µl of TMB substrate solution was added to each well. The plate was left to allow for colour development. The appearance of blue colour was indicative of biotinylated protein.

Tetrameric complexes were then generated by addition of PE- or Allophycocyanine (APC)-conjugated streptavidin (Molecular Probes) in a molar ratio of 1:4 to the biotinylated monomer over 3-4 days. This was equivalent to adding 0.312µg of streptavidin-PE or 0.087µg streptavidin-APC to 1µg of monomer. Each tetramer was hereby designated according to the HLA type and first three letters of the presented peptide e.g. the tetrameric complex composed of HLA-Cw*0702, β_2M and RYRPGTVAL peptide is referred to as Cw7-RYR tetramer.

2.7 LUMINEX

2.7.1 ONELAMBDA – HLA beads

Binding of refolded KIR2DS2 and KIR2DL2 proteins to a broad panel of HLA-A, HLA-B and HLA-C allotypes was assessed using commercially available LABScreen single-Ag bead sets (One Lambda). Cumulatively, the three sets encompass 29 HLA-A, 50 HLA-B, and 16 HLA-C allotypes. KIR proteins at a concentration ranging from 10µg/ml to 1mg/ml were incubated with LABScreen microbeads for 30 minutes at room temperature. Beads were then washed three times and streptavidin-PE added to bind biotinylated KIR protein. Fluorescent intensity and identification labels of the individual beads were visualised on a Luminex 100 reader (Luminex). A minimum of 200 events per Ag were collected. The W6/32 (α-HLA class I) and 2M2 (α-β_2M) antibodies were used as positive controls and to account for bead-to-bead differences in the amount of HLA class bound to each bead. Median values were normalised to the 75th percentile and the correction factor was determined for each bead.

2.7.2 Conjugation of KIR and HLA proteins to Bio-Plex COOH microspheres

Bio-Plex COOH microspheres (Bio-Rad) are carboxy-coated beads internally labelled with 2 fluorescent dyes (xMAP technology), and so are spectrally distinct. Beads were coupled to KIR and HLA proteins. Briefly, 12,500 beads were mixed and added to protein monomers. These were left for 15 minutes, washed and resuspended in 500µl PBS. 50µl were then used per test. Coupling was checked by addition of α-KIR or W6/32 Abs and analysis on the Luminex 100 reader. Once it was confirmed that protein had been successfully conjugated to the beads tetramer binding was tested.

2.7.3 Luminex IS 2.3 software

The Luminex 100 reader employs a software package – IS 2.3 to analyse the recorded data. Negative cut-off values for HLA antibody identification using the kits manufactured by both OneLambda, and Tepnel are arbitrary. H&I at NHSBT in Birmingham use an approximate MFI of >1000 to assign positive reactions. However, as these Luminex assays were designed for an alternative use, it may be that the supplied software is not appropriate to use in this analysis. Thus, all raw data was exported in parallel and reanalysed.

2.8 BIACORE

Surface plasmon resonance (SPR) studies were carried out at 25°C in HBS-EP buffer using a BIAcore 3000 machine (BIAcore AB). Briefly, streptavidin was immobilized on CM5 sensor chips at pH5.5 as described previously (Willcox *et al.* 1999) allowing subsequent oriented coupling of HLA monomers. HLA was immobilized onto streptavidin-coated surfaces by injection at 5µl/min. Reactivity towards KIR proteins was tested by injection of each KIR protein over HLA-C and control surfaces of either streptavidin alone or HLA-A2 immobilised to streptavidin.

2.9 STATISTICAL ANALYSIS

Results were analysed using Graphpad Prism. Intergroup comparisons were performed using the Mann-Whitney U test. For multiple comparisons a Dunns multiple comparison test was used. For comparisons between paired results, a Wilcoxon signed rank test was performed. When 3 or more groups were being compared one-way analysis of variance (ANOVA) was used. All *p* values were two-tailed and considered significant if less than 0.05. Relationships were tested by applying linear regression, and then correlation was tested for using nonparametric (Spearman) test and *p* values were two-tailed. Results were presented using Graphpad Prism 4.0.

Multivariate analysis

Data was reanalysed using SPSS to carry out multivariate analysis in order to account for variable factors such as age and CMV serostatus. Linear regressions and ANOVA tests were used. All multivariate analysis was done in consultation with a statistician.

Chapter 3

The interaction of 2DKIRs with their HLA-C ligands

There remains some doubt as to the true nature of the ligands for the activating KIRs. Based on sequence similarity, they would be expected to bind HLA-C with the same affinity as their inhibitory counterparts. However, despite these similarities, the evidence for binding of the same ligands is quite weak. Crystallography of KIR2DS2 indicated that the reason that KIR2DS2 tetramers did not bind HLA-Cw3 was due to subtle displacement of two residues: tyrosine (Tyr) at position 45 and glutamine (Gln) at position 71 (Saulquin *et al.* 2003). However, Stewart *et al.* used KIR tetramers to show increased binding of KIR2DS1 to EBV infected cells and through using blocking experiments they demonstrated that the ligand was HLA-C (Stewart *et al.* 2005). Therefore it was proposed that KIR2DS1 binding to HLA-C is dependent on the upregulation of MHC class I expression on the cell surface following viral infection; – interestingly, infection of cells with CMV could not induce the same effect in this experiment. It remains to be seen if this conclusion is the correct explanation for the differential binding of HLA-C by inhibitory and activating homologues (Stewart *et al.* 2005). KIR2DS2 has been shown to activate T cells when in the presence of the adaptor protein

DAP12 – the latter being crucial in differentiating the KIR from a co-receptor into a receptor capable of independently inducing cell activation (Snyder *et al.* 2004).

Many studies of KIR genotype in relation to the development or natural history of different diseases have been performed, and the activating receptor KIR2DS2 has often emerged as an important factor in the control of disease progression. In some conditions it is associated with disease severity such as rheumatoid vasculitis (Yen *et al.* 2001), Sjogren's syndrome (Lowe *et al.* 2009), type I diabetes mellitus (van der Slik *et al.* 2003), scleroderma (Momot *et al.* 2004) and psoriatic arthritis (Nelson *et al.* 2004). In others such as chronic myeloid leukaemia (CML) (Middleton *et al.* 2009), HIV-1 infection in adolescents (Lazaryan *et al.* unpublished data) and CMV reactivation post-transplant (Cook *et al.* 2005) it appears to have a protective role.

In light of the finding of our group that KIR2DS2 plays a protective role against CMV reactivation I sought to investigate this receptor further. Reagents to measure KIR2DS2 are limited and, apart from primers to genotype DNA, the antibody GL183 is somewhat limited in value as it detects not only KIR2DS2, but also KIR2DL2 and KIR2DL3. My aim for the work presented in this chapter was to try and develop a KIR2DS2-specific antibody to allow investigation of KIR2DS2 expression in healthy individuals. A second aim was to examine KIR2DS2 binding to HLA-C ligands. As already mentioned existing data is somewhat lacking, and a better understanding of which proteins KIR2DS2 binds, with what affinity, and whether or not this is peptide dependent is essential for studying the role of this receptor in CMV and other diseases.

3.1 PRODUCTION OF RECOMBINANT 2D KIR PROTEINS

Antibodies are invaluable reagents for biological research and are also emerging as important therapeutic agents for clinical use. Mouse antibodies have been the mainstay of research to date but are seen as foreign by the human immune system which limits their *in vivo* efficacy. Recombinant and phage display technologies have emerged as useful applications for developing humanised antibodies. Phage display is also used due to the high-throughput screening potential (Schofield *et al.* 2007). I decided to attempt to employ this approach for the development of a KIR2DS2-specific antibody as the 2-domain KIR proteins KIR2DS2 and KIR2DL2 are highly homologous. There are only 10 nucleotide changes between the two proteins, four of which result in an amino acid change. These are shown in Table 3-1 below.

Table 3-1: Nucleotide differences between KIR2DS2 and KIR2DL2

Nucleotide position	Nucleotide substitution	Amino acid change
50	C → G	Pro → Arg
129	G → A	-
137	A → T	Ser → Phe
186	G → A	-
358	T → C	-
445	C → T	Arg → Cys
558	C → A	-
561	T → C	-
602	C → T	Thr → Ile
648	C → T	-

In order to attempt to produce specific antibodies via phage display and investigate KIR interactions with their HLA ligands, recombinant KIR proteins were generated. An *E.coli* expression system using the bacteriophage T7 promoter was employed (Tabor *et al.* 1985; Studier *et al.* 1986). The KIR2DS2 and KIR2DL2 proteins consist of two Ig-like domains in their extracellular region. Prokaryotic expression constructs (pGMT7, encoding KIR2DS2 and KIR2DL2) were designed to encode a 230aa insert encompassing both Ig-like domains along with the stem and part of the transmembrane region (Figure 3-1).

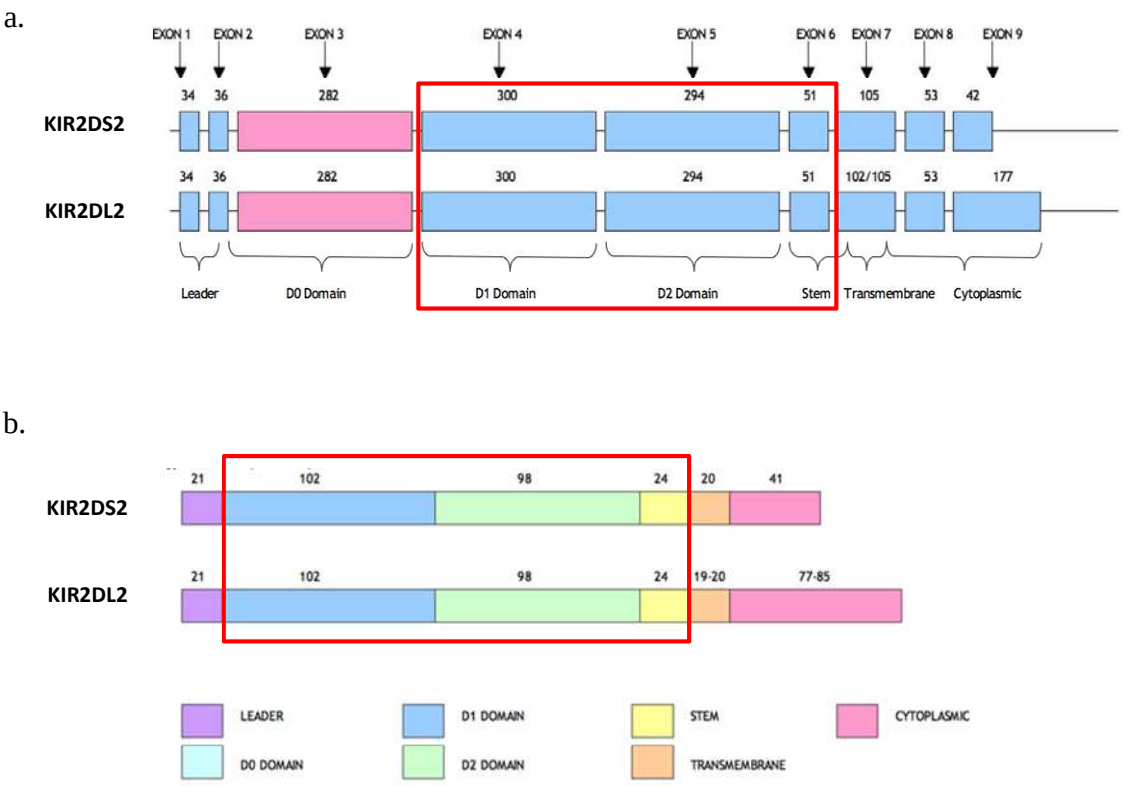


Figure 3-1: Schematic of KIR2DS2 and KIR2DL2 proteins. (a) KIR gene organisation. The coding regions of the exons are represented as blue boxes; their size in base pairs is shown in digits above them. The pseudoexon 3 is shown in pink. (b) KIR protein domain and region lengths. The amino acid length of each region is shown in digits above the corresponding box.

RNA was extracted from a BW5147.3 cell line that had been transfected with a plasmid encoding KIR2DS2 (a kind gift from Prof. Eric Vivier). Total cellular cDNA was synthesised by reverse transcription, from which KIR2DS2 cDNA was amplified via PCR using primers designed to incorporate NdeI and BamHI restriction sites (see Table 2-4). This enabled direct cloning into pGMT7 expression vector after digestion of the PCR product with NdeI and BamHI. Following amplification, a small amount of the PCR product was visualised as a DNA band using agarose gel electrophoresis. An example of this is shown in Figure 3-2.

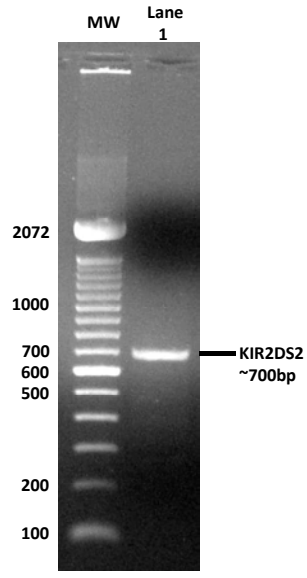


Figure 3-2: KIR2DS2 PCR product. Following PCR, 1µl amplified sample was separated using agarose gel electrophoresis. Photograph shows the KIR2DS2 product.

The remaining PCR product and the pGMT7 vector were subjected to restriction digest using NdeI and BamHI before being visualised on a gel. Products of the correct size were excised from the gel and purified. The digested vector was treated with SAP prior to ligation to prevent recircularisation. Ligation products were then transformed into competent *E.coli* cells, and individual colonies were selected for further expansion before DNA extraction and sequence analysis were performed to validate the KIR2DS2 insert.

Having successfully made the pGMT7-KIR2DS2 construct, primers were designed to incorporate the four nucleotide substitutions that resulted in amino acid changes (see Tables 2-4 and 3-1) in order to express KIR2DL2. A multi-SDM reaction was carried out using the Stratagene QuikChange kit. The PCR product could not be verified on an agarose gel as it contained single stranded DNA after DpnI digest. Therefore DNA was transformed immediately into Top_{10} cells and resulting colonies sequenced to check that all 4 nucleotide substitutions had been successful. Five out of 25 colonies tested contained the substituted DNA sequence and these were subsequently analysed for expression of KIR2DL2 protein.

Clones containing inserts with the correct sequence were taken forward to test for inducible protein expression. In *E. coli* strain BL21(DE3), IPTG is known to induce the expression of T7 RNA polymerase gene and this enzyme can transcribe the KIR2DS2 gene under the control of the T7 promoter leading to expression of KIR2DS2 protein. The level of KIR2DS2 and KIR2DL2 protein expression was assessed using small test expression cultures and SDS-PAGE to visualise induction. Coomassie blue staining of the SDS-PAGE gel indicated

induction of protein producing bands at molecular weights between the 21 and 31 kDa marker bands, consistent with the molecular weight of the KIRs (24kDa) (Figure 3-3).

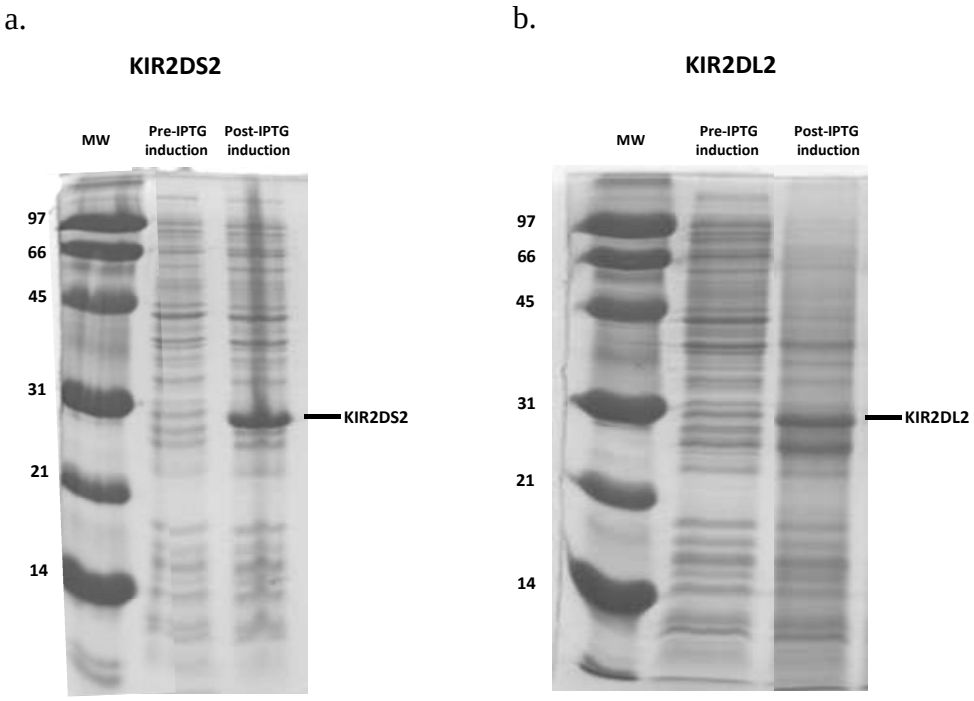


Figure 3-3: SDS-PAGE analysis of 2DKIR test expression. For each protein, molecular weight standards are shown in the first lane with masses indicated in kDa. Pre- and post-induction samples of (a) KIR2DS2 and (b) KIR2DL2 are shown in lanes 2 and 3 of each gel. In post-induction samples an induced protein band can be seen between 21 and 31 kDa.

To obtain high yields of recombinant protein, initial expression conditions were replicated using 2L overnight cultures of pGMT7-KIR2DS2 and pGMT7-KIR2DL2 transformed bacteria. For both KIR2DS2 and KIR2DL2, centrifugation of lysed cultures resulted in a

large pellet of insoluble material, consistent with accumulation of each protein in intracellular inclusion bodies, a phenomenon that occurs for many other Ig-domain proteins, including MHCs, TCRs and LIRs (Garboczi *et al.* 1992; Chapman *et al.* 1999). Inclusion bodies were purified as described previously, by homogenisation in a detergent wash solution incorporating Triton X-100, and were finally solubilised in a denaturing buffer containing urea. SDS-PAGE analysis of purified inclusion body pellets confirmed the presence of KIR2DS2 and KIR2DL2 (Figure 3-4) at relatively high purity.

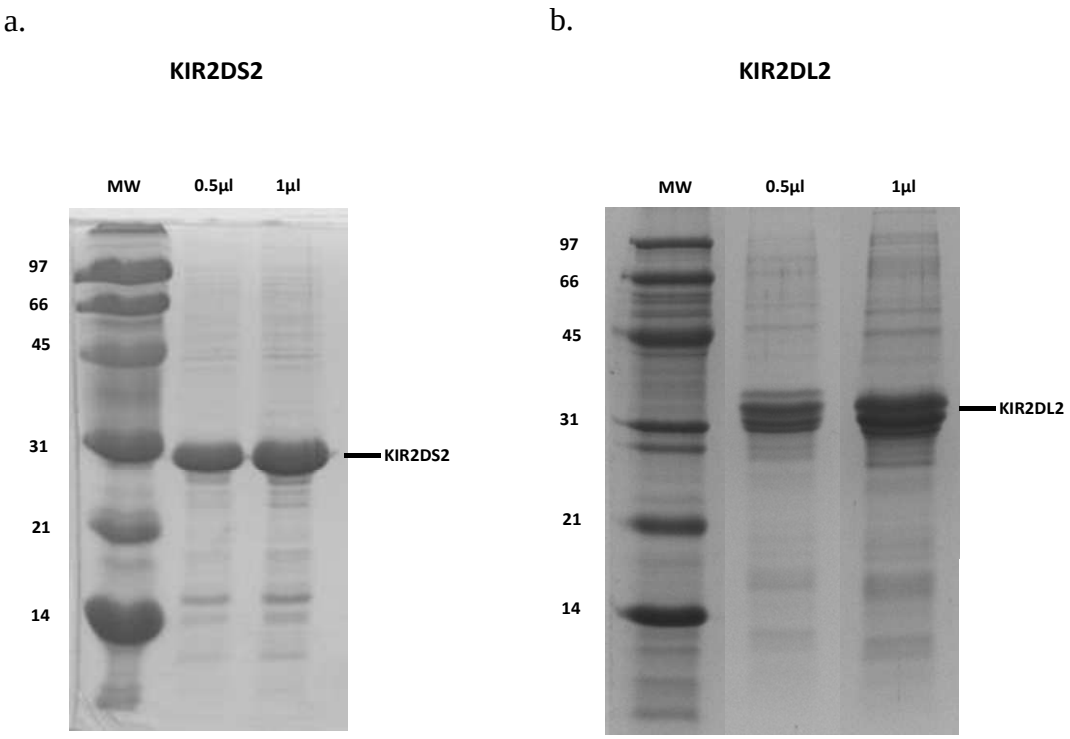
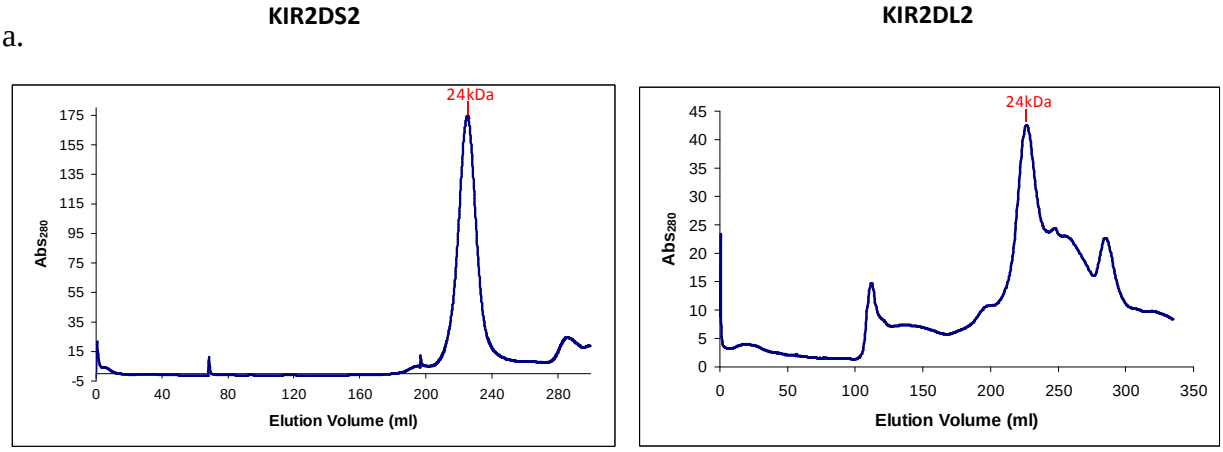


Figure 3-4: SDS-PAGE analysis of KIR2DS2 and KIR2DL2 inclusion body preparations. (a) KIR2DS2, (b) KIR2DL2. For each protein molecular weight standards are shown in the first lane and inclusion body preparations in the second and third lanes (0.5µl and 1µl respectively). The mass of molecular weight standards is shown in kDa.

Dilution refolding was then used to renature natively folded KIR2DS2 and KIR2DL2, a technique that has proven successful for many other Ig-like domain-containing proteins (Snyder *et al.* 1999; Chapman *et al.* 2000; Willcox *et al.* 2002). Renaturation was carried out in the presence of glutathione redox components to allow shuffling of disulphide bonds. During the process, an alkaline pH was maintained to promote formation of the active glutathione thiolate anion and hence disulphide exchange. Also, L-arginine, an additive widely used to enhance in vitro protein refolding (Reddy *et al.* 2005), was incorporated into

the refolding buffer, and refolding was carried out at protein concentrations <100µg/ml to lessen concentration-dependent aggregation effects. Folded protein was concentrated by ultrafiltration, the buffer exchanged on disposable PD-10 columns containing G25 resin, and the BirA enzyme substrate peptide (BSP) tag in pGMT7 vector was biotinylated by the BirA enzyme overnight. Size exclusion analysis of refolded samples of both KIR2DS2 and KIR2DL2 was performed using FPLC. This resulted in a prominent peak of protein eluting at ~220ml, an elution volume consistent with a molecular weight of ~24kDa (Figure 3-5a). SDS-PAGE analysis of peak fractions under reducing conditions revealed a single prominent band equivalent in motility to that of reduced KIR2DS2 and KIR2DL2 in a sample of solubilised inclusion body (Figure 3-4), confirming that the vast majority of protein in these peaks correctly corresponded to extracellular portions of 2DKIR proteins (Figure 3-5b).

a.



b.

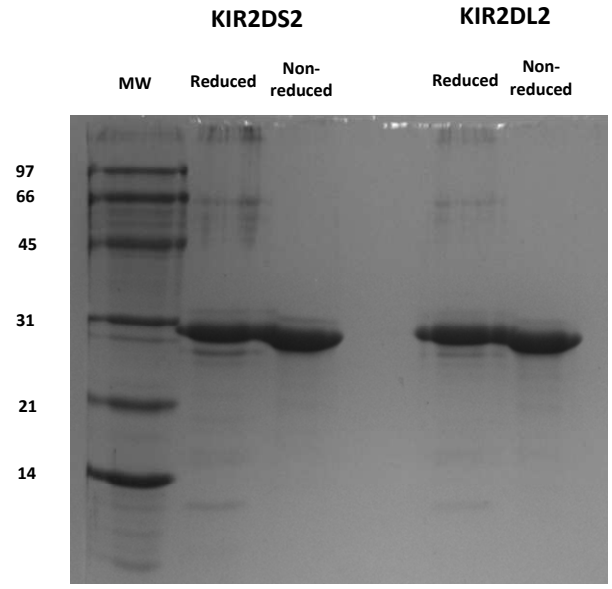


Figure 3-5: Size exclusion chromatography and SDS-PAGE analysis of refolded KIR2DS2 and KIR2DL2. (a) Size exclusion chromatogram showing KIR2DS2 and KIR2DL2 elution profiles. (b) SDS-PAGE analysis of KIR2DS2 and KIR2DL2 samples TCA-precipitated from FPLC fractions. Proteins are shown under both reducing and non-reducing conditions. The mass of molecular weight standards is shown in kDa.

In order to verify correct folding of KIR2DS2 and KIR2DL2 proteins, refolded monomers were subjected to an ELISA to check for GL183 Ab recognition. ELISA plates were coated with proteins overnight and then blocked with milk before the addition of GL183 Ab. The KIR-specific antibodies HP-3E4 and DX9 were used as negative controls, as was an α-HLA-A2 Ab. An α-mouse-HRP secondary antibody was then added, and antibody binding detected with the use of OPD substrate. As can be seen in Figure 3-6, both KIR2DS2 and KIR2DL2 proteins are recognised by the GL183 Ab but not any of the control antibodies, indicating correct folding. Recognition is apparent in the neat sample and at a 1:10 dilution, although lower concentrations of the proteins are undetectable by GL183 Ab. Control HP-3E4, DX9 and α-HLA-A2 Abs failed to recognise KIR2DS2 and KIR2DL2 proteins even at a high concentration, demonstrating protein-specific binding by the GL183 Ab.

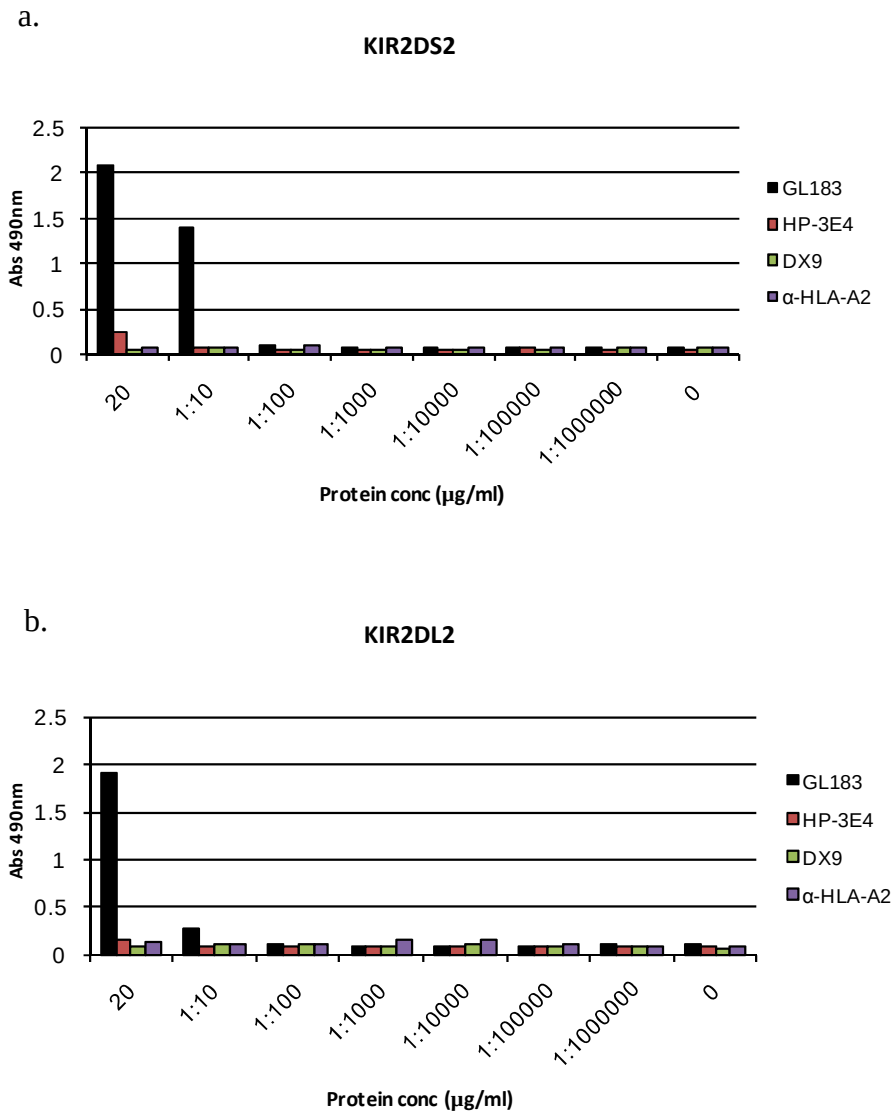


Figure 3-6: Validation of refolded KIR2DS2 and KIR2DL2 by GL183 Ab ELISA. Refolded monomers were coated on an ELISA plate and tested for GL183 Ab recognition. Irrelevant HP-3E4, DX9 and α-HLA-A2 Abs were included as negative controls. Absorbance was read at 490nm.

Finally a biotinylation ELISA was performed to prove that KIR monomers were biotinylated. Antibody production via phage display requires such a tag for selection and purification, and it would also be needed for the generation of KIR tetramers. KIR2DS2 and KIR2DL2 proteins were serially diluted and coated onto an ELISA plate. An HLA-A2 monomer that was previously known to be biotinylated (as confirmed by tetramerisation and subsequent flow cytometric staining) was included as a positive control. Wells were treated with extravidin-peroxidase and TMB substrate solution to detect biotinylated protein. The result of this ELISA, shown in Figure 3-7 confirms that KIR2DS2 and KIR2DL2 monomers were highly biotinylated when compared to the HLA-A2 control.

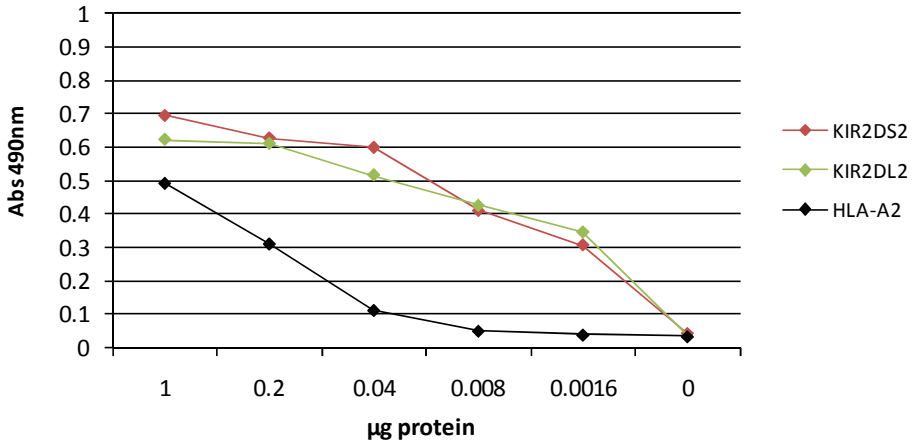


Figure 3-7: Biotinylation ELISA of KIR2DS2 and KIR2DL2. KIR2DS2 and KIR2DL2 monomers were tested for biotinylation. A previously generated HLA-A2 monomer was included as a positive control. Absorbance was read at 490nm.

At this stage activating and inhibitory members of the 2DKIR family had been successfully generated. We were confident of conformation and biotinylation status and therefore these were passed on to Dr. Ayman Antoun to attempt generating KIR2DS2- and KIR2DL2-specific antibodies.

3.2 PRODUCTION OF RECOMBINANT HLA-C1 AND HLA-C2 PROTEINS

HLA-C alleles can be divided into two groups**,** HLA-C group 1 (C1) and HLA-C group 2 (C2), based on their amino acid sequences in the α1 domain. C1-group alleles have the amino acid serine (Ser) at residue 77 and asparagine (Asn) at residue 80, whereas C2-group alleles have the amino acid Asn at residue 77 and lysine (Lys) at residue 80 (Colonna *et al.* 1993). The inhibitory receptors KIR2DL2 and KIR2DL3 (CD158b) recognise C1-group alleles, while KIR2DL1 recognises C2-group alleles. Although the amino acid sequences of activating KIRs (KIR2DS1, KIR2DS2 and KIR3DS1) suggest that they might bind to similar ligands as their inhibitory counterparts (KIR2DL1, KIR2DL2/3 and KIR3DL1 respectively), it has been difficult to show that activating receptors bind to these HLA-C ligands.

In order to investigate HLA-C binding by the 2DKIR proteins, recombinant HLA-C proteins were also made. As KIR2DS2 has high homology to KIR2DL2 which binds to HLA-C1-group alleles, it has been predicted that this group will also be the ligand for the activating KIR (Saulquin *et al.* 2003; Stewart *et al.* 2005). However a C2-group allele also needed to be expressed as a negative control. Plasmids expressing HLA-Cw*0401 (C2-group) and HLA-Cw*0702 (C1-group) were kindly donated by Dr. Katsumi Maenaka. HLA-Cw*0401 was in a vector already containing a biotin tag (necessary for BIAcore analysis and tetramer production). HLA-Cw*0702 however had to be subcloned into a vector containing the Bir A biotin-protein ligase sequence (pGMT7) in order to add a biotin tag. Primers were designed to amplify the extracellular domain of HLA-Cw*0702 and also incorporate NdeI and BamHI restriction sites ready for cloning into the pGMT7 expression vector (see Table 2-4) as for the KIR proteins. Following amplification, a small amount of the PCR product was visualised as a DNA band using agarose gel electrophoresis. An example of this is shown in Figure 3-8.

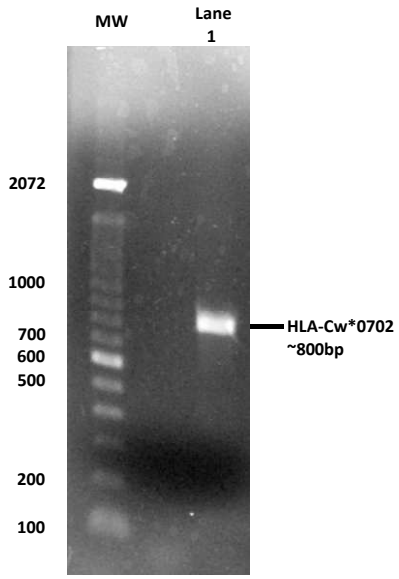


Figure 3-8: HLA-Cw*0702 PCR product. Following PCR, 1µl amplified sample was separated using agarose gel electrophoresis. Photograph shows the HLA-Cw*0702 product.

The remaining PCR product was cloned into pGMT7 via the NdeI and BamHI restriction sites. DNA was transformed into Top_{10} cells and resulting colonies sequenced to check that the HLA-Cw*0702 insert was successfully generated. Clones containing an insert with the correct sequence were then tested to see if protein expression could be induced.

The level of HLA-Cw*0401 and HLA-Cw*0702 protein expression was assessed using small test expression cultures and SDS-PAGE to visualise induction. Coomassie blue staining of the SDS-PAGE gel indicated induction of protein bands at molecular weights between the 31 and 45 kDa marker bands, consistent with the molecular weight of HLA class I heavy chain (~33kDa) (Figure 3-9).

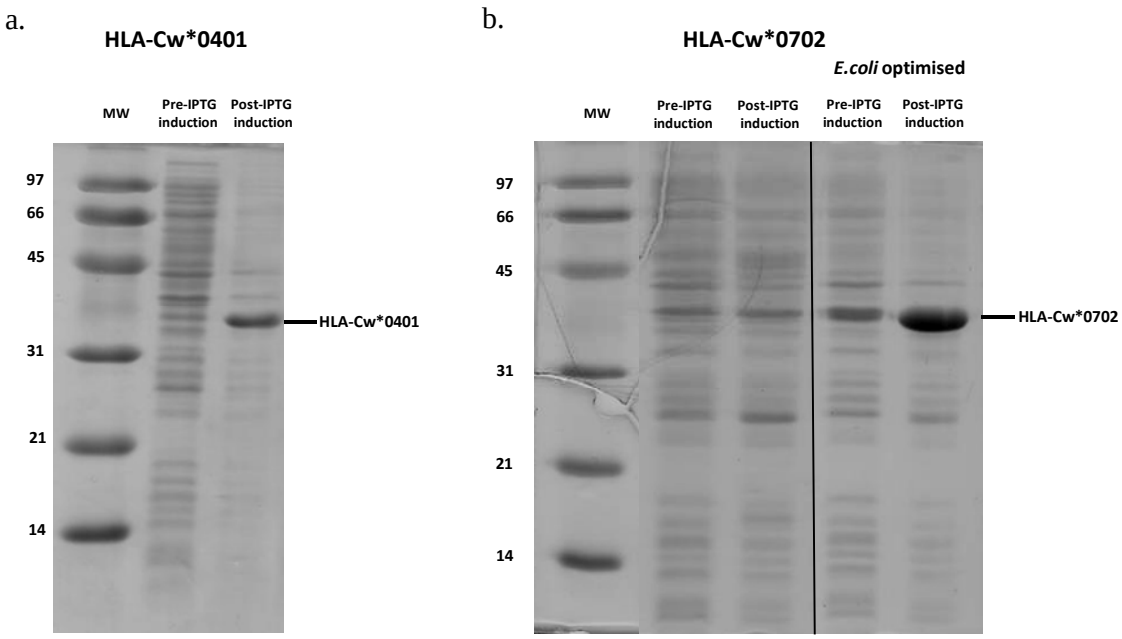


Figure 3-9: SDS-PAGE analysis of HLA-Cw*0401 and HLA-Cw*0702 test expression. For each protein, molecular weight standards are shown in the first lane, masses indicated in kDa. Pre- and post-induction samples of (a) HLA-Cw*0401, (b) HLA-Cw*0702 are shown in lanes 2 and 3 of each gel. In post-induction samples an overexpressed protein band can be seen between 31 and 45 kDa. Lanes 4 and 5 of gel (b) show pre- and post-induction samples of the *E.coli* optimised HLA-Cw*0702 construct.

As shown in Figure 3-9a, there was clear expression of HLA-Cw*0401 post-induction. However the expression of HLA-Cw*0702 was considerably lower (Figure 3-9b, lane 3). A method often used to overcome this is to alter the ORF of the DNA sequence to use alternative codon sequences which encode for the same amino acid, but are known to be more efficiently expressed in *E.coli.* (Williams *et al.* 1988; Makoff *et al.* 1989). This can lead to enhanced expression of a protein with the same amino acid sequence. Some organisms, especially bacteria, have a wide range of GC content and this reflects the types of amino acids they have and the codons they use preferentially for these amino acids. Dong *et al.* showed that tRNA abundances in rapidly growing *E.coli* are correlated with the pool of available codons in a way that optimises translation rate (Dong *et al.* 1996). It is evident that the synonymous codon choices of highly expressed genes have evolved to match tRNA. This assumes that there are selective differences among synonymous codons that become stronger for proteins that are expressed more often. Therefore the sequence of HLA-Cw*0702 in pGMT7 was adjusted to substitute for codons preferentially used by *E.coli b*y redesigning the 5' primer (see Table 2-4). Clones containing the *E.coli* optimised insert were tested for inducible protein expression. Lanes 4 and 5 of the gel shown in Figure 3-9b demonstrate an improved yield of HLA-Cw*0702 protein expressed.

Expression conditions were replicated on a 2L scale to obtain high yields. As for the purification of the KIRs, inclusion bodies were purified and then solubilised in a denaturing buffer. SDS-PAGE analysis of purified inclusion body pellets confirmed the presence of HLA-Cw*0401 and HLA-Cw*0702 (Figure 3-10). Protein was also produced using the original untagged HLA-Cw*0702 plasmid obtained from Dr. Katsumi Maenaka and run on the gel as a positive control

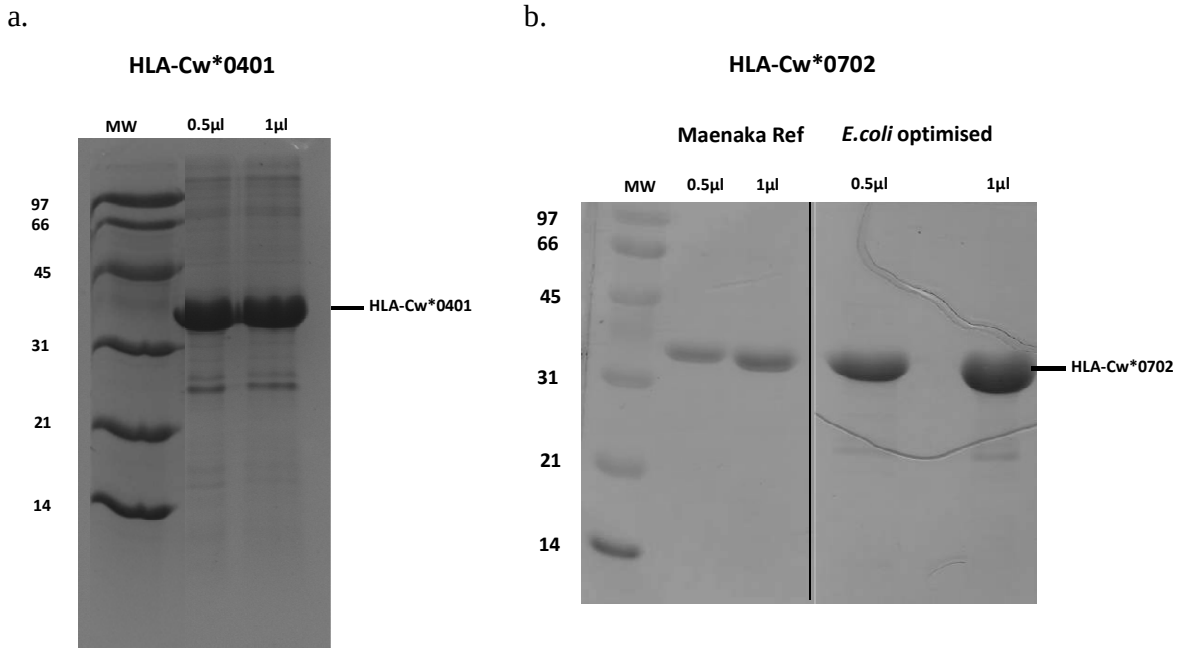
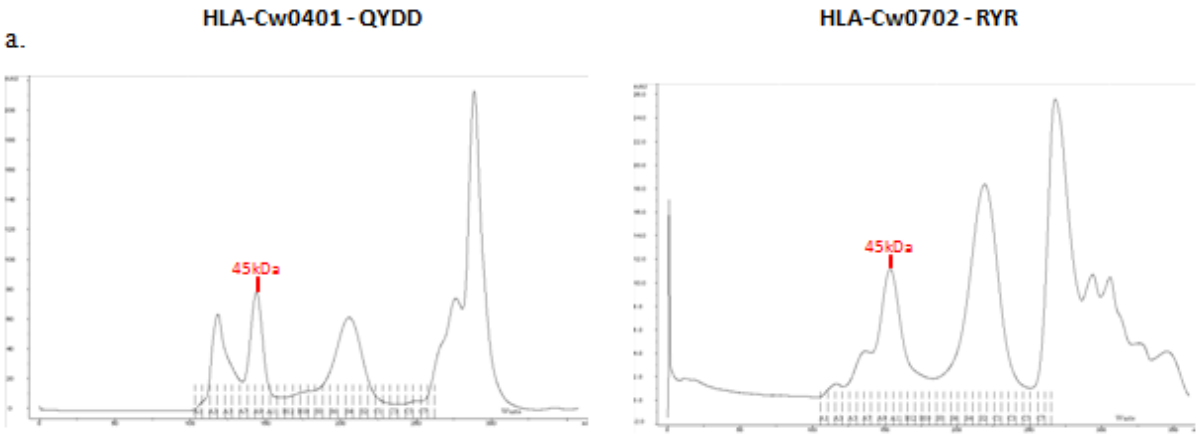


Figure 3-10: SDS-PAGE analysis of HLA-Cw*0401 and HLA-Cw*0702 inclusion body preparations. (a) HLA-Cw*0401, (b) HLA-Cw*0702 – Lanes 2 and 3 show original untagged plasmid as a reference, lanes 4 and 5 show *E.coli* optimised sequence. For each protein molecular weight standards are shown in the first lane and inclusion body preparations in the second and third lanes (0.5µl and 1µl respectively). The mass of molecular weight standards is shown in kDa.

In order to obtain HLA-C ligands in their correct conformation, dilution refolding was carried out using the same principle as for the KIRs. However, this time β_2m and peptide (see Table 2-6) were first added to the refold buffer, followed by heavy chain to generate stable peptide MHC complexes. Refolding was carried out at protein concentrations <100µg/ml to lessen concentration-dependent aggregation effects. Using this strategy, size exclusion analysis of refolded samples of both HLA-Cw*0401 and HLA-Cw*0702 resulted in a peak of protein eluting at ~155ml on an S75 column, an elution volume consistent with a molecular weight of ~45kDa (Figure 3-11a). SDS-PAGE analysis of peak fractions under reducing conditions revealed two bands equivalent in motility to that of reduced HLA-C heavy chains (~33kDa) and β_2m (~12kDa) in a sample of solubilised inclusion body, confirming that the vast majority of protein in these peaks correctly corresponded to the extracellular regions of HLA-C proteins (Figure 3-11b).

a.



b.

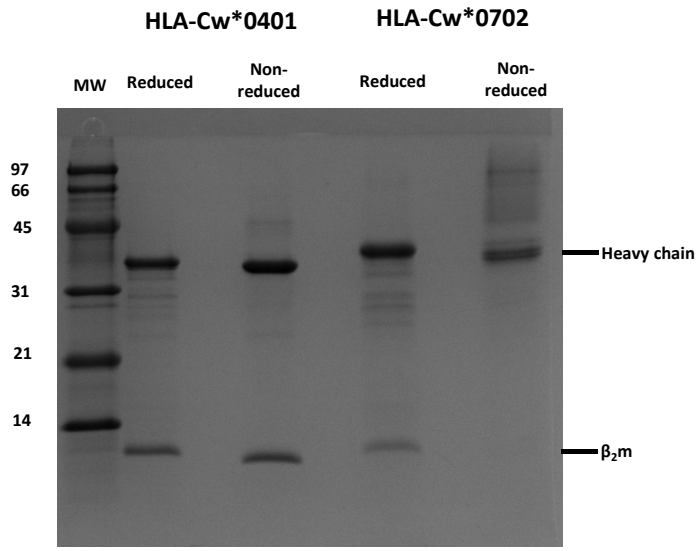


Figure 3-11: Size exclusion chromatography and SDS-PAGE analysis of refolded HLA-Cw*0401-QYDD and HLA-Cw*0702-RYR. (a) Size exclusion chromatogram showing HLA-Cw*0401 and HLA-Cw*0702 elution profiles. (b) SDS-PAGE analysis of HLA-Cw*0401 and HLA-Cw*0702 samples TCA-precipitated from FPLC fractions. Proteins are shown under both reducing and non-reducing conditions. The mass of molecular weight standards is shown in kDa.

In order to verify correct folding of HLA-Cw*0401 and HLA-Cw*0702 proteins, refolded monomers were subjected to an ELISA to check for W6/32 Ab recognition. W6/32 is a mAb used to characterise human HLA class molecules, which recognises a conformation-dependent epitope on the intact MHC molecule, thus requiring the correct conformation of β_2m and heavy chain. ELISA plates were coated with W6/32 Ab and then blocked with blocking buffer. The purified recombinant HLA-C proteins, as well as biotinylated HLA-A2 as a positive control and an inclusion body as a negative control, were added to wells at concentrations ranging from 1µg to 0.0016µg. Extravidin-peroxidase was added to each well to bind to the biotinylated proteins. Binding was then detected by the addition of TMB substrate. As can be seen in Figure 3-12 both HLA-Cw*0401 and HLA-Cw*0702 proteins are recognised by W6/32 Ab indicating correct folding. Wells containing inclusion body or no protein as negative controls failed to be recognised by W6/32 Ab even at a high concentration, demonstrating conformation-specific binding. Recognition of HLA-C protein is apparent in wells containing 1µg and 0.2µg, although lower concentrations of the proteins are undetectable by W6/32 Ab. HLA-A2 was recognised with higher affinity, possibly due to the presence of a higher proportion of biotinylated protein. This may be the case as HLA-C monomers were not subjected to ion exchange for the removal of protein which had not been biotinylated, as previous attempts resulted in the protein precipitating.

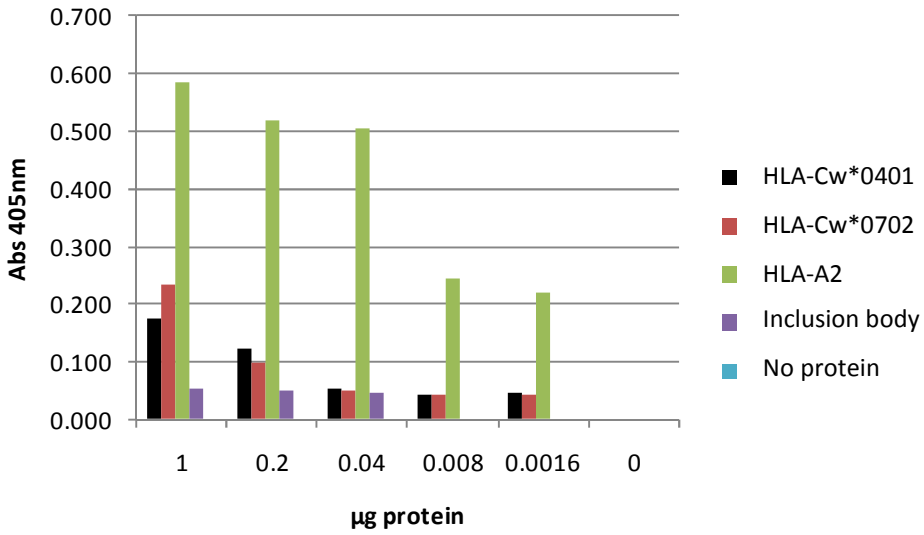


Figure 3-12: Validation of refolded HLA-Cw*0401 and HLA-Cw*0702 by W6/32 Ab ELISA. W6/32 Ab was coated onto an ELISA plate to test recognition of HLA-C monomers. HLA-A2 was used as a positive control, and wells containing inclusion body and no protein were included as negative controls. Absorbance was read at 405nm.

To confirm the biotinylation of the HLA-C monomers, an ELISA was performed as biotinylation is necessary for the generation of tetramers. HLA-C proteins were serially diluted in an ELISA plate and an HLA-A2 monomer that was previously known to be biotinylated and had subsequently been tetramerised and used for staining was included as a positive control. The result shown in Figure 3-13 confirms that HLA-Cw*0401 and HLA-Cw*0702 monomers were well biotinylated when compared to the HLA-A2 control.

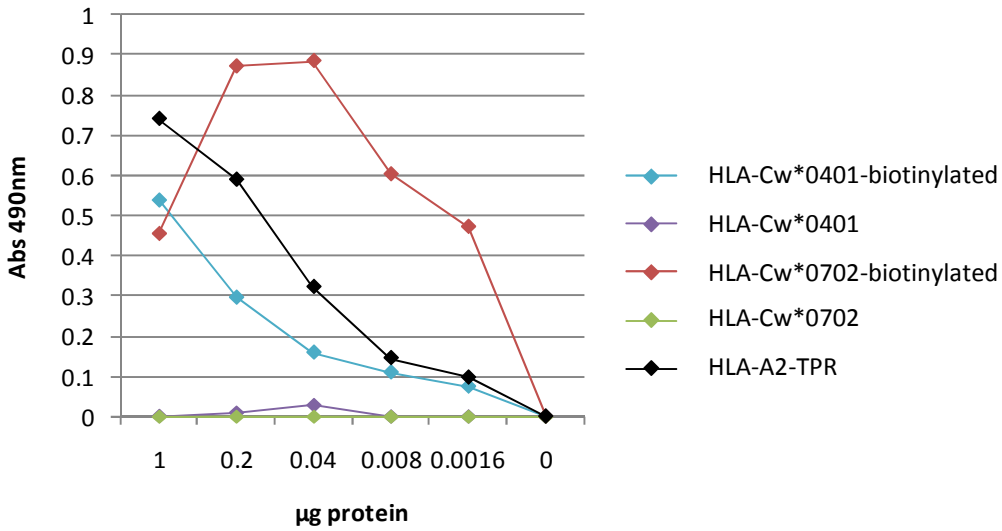


Figure 3-13: Biotinylation ELISA of HLA-Cw*0401 and HLA-Cw*0702. HLA-Cw*0401 and HLA-Cw*0702 monomers were tested for biotinylation. A previously generated HLA-A2 monomer was included as a positive control. Absorbance was read at 490nm.

3.3 INTERACTION OF KIR2DS2 AND KIR2DL2 WITH HLA-C

Having successfully generated both 2DKIR receptors and HLA-C ligand proteins we wanted to investigate their interaction. It is important to be able to show that the recombinant proteins have the same structure as their native counterparts, which is most easily done by confirming that the protein binds its natural ligands. Therefore the proteins were subjected to SPR analysis which offers advantages for analysing weak macromolecular interactions (van der Merwe *et al.* 1994; van der Merwe *et al.* 1996). In the absence of natural ligands mAbs that are known to bind to the native protein are an excellent means of assessing the structural integrity of the recombinant protein. All monomers had been tested and shown to bind relevant mAbs prior to SPR analysis. A BIAcore 3000 was used which is particularly well suited to evaluate the binding of recombinant proteins to natural ligands and mAbs. Biotinylated recombinant HLA proteins were immobilised onto a streptavidin coated binding surface and 2DKIR proteins were then injected over the HLA surface. Injection of both KIR2DS2 and KIR2DL2 resulted in a substantial increase in the signal; however, this appeared to be non-specific as the increase seen for both HLA-Cw*0401 and HLA-Cw*0702 was also observed in the negative controls (HLA-A2 and blank) (Figure 3-14). Injection of KIR2DL2 resulted in a higher level of binding to HLA-Cw*0401 than the blank chip, but in light of the other interactions observed this result was unreliable. Following this, several experiments were carried out in order to try and optimise protein purity in case this was a factor. Inclusion bodies were expressed in Rosetta cells as opposed to BL-21 but results remained unchanged.

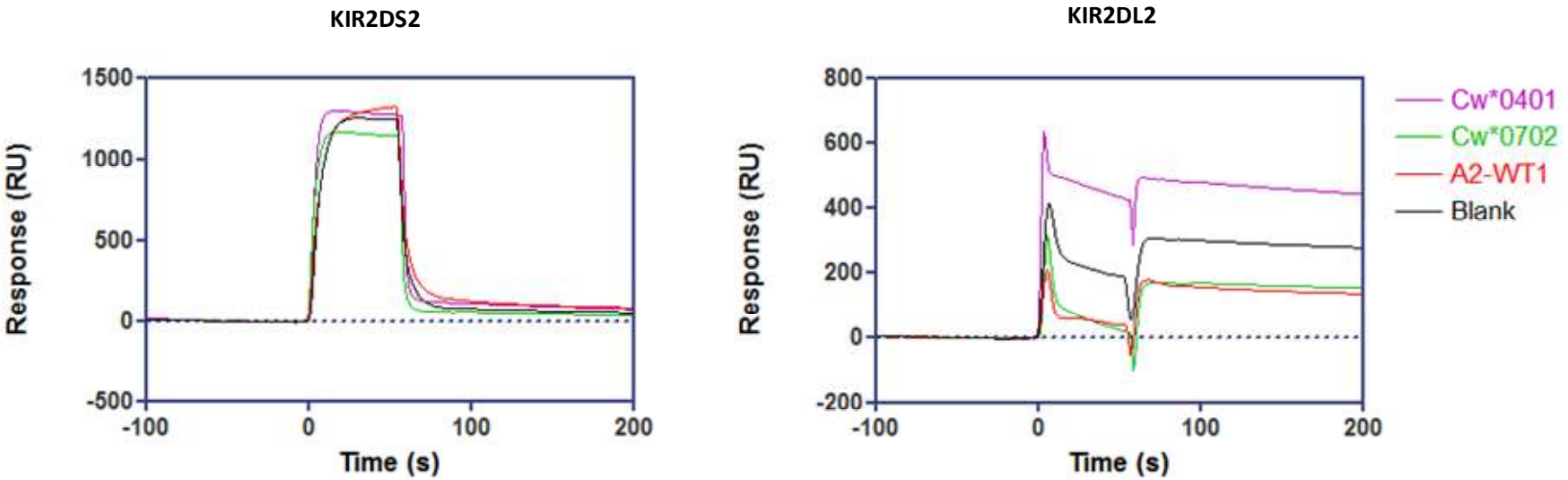


Figure 3-14: Surface plasmon resonance analysis of recombinant 2DKIR proteins binding to HLA proteins. HLA-Cw*0401 and HLA-Cw*0702 monomers were immobilised to a streptavidin coated chip. HLA-A2 and a blank containing no protein were included as negative controls. KIR2DS2 and KIR2DL2 were injected over the HLA surface to test for binding.

Luminex technology was employed as an alternative approach to surface plasmon resonance. Recombinant KIR2DS2 and KIR2DL2 proteins were tested for binding to beads coated with single HLA class I allotypes (One Lambda single Ag beads). Variation in the density of HLA antigens coupled to polystyrene beads for use in the Luminex assay is a key factor which hinders the interpretation of data provided by this technique. Therefore in these assays the binding of 2DKIR proteins to each bead was normalised to the binding of monomorphic α-HLA class I mAbs w6/32 and α-β2m to the same bead (Figures 3-15 and 3-16). Median channel values were normalised to the 75th percentile and the corresponding correction factor applied for each bead.

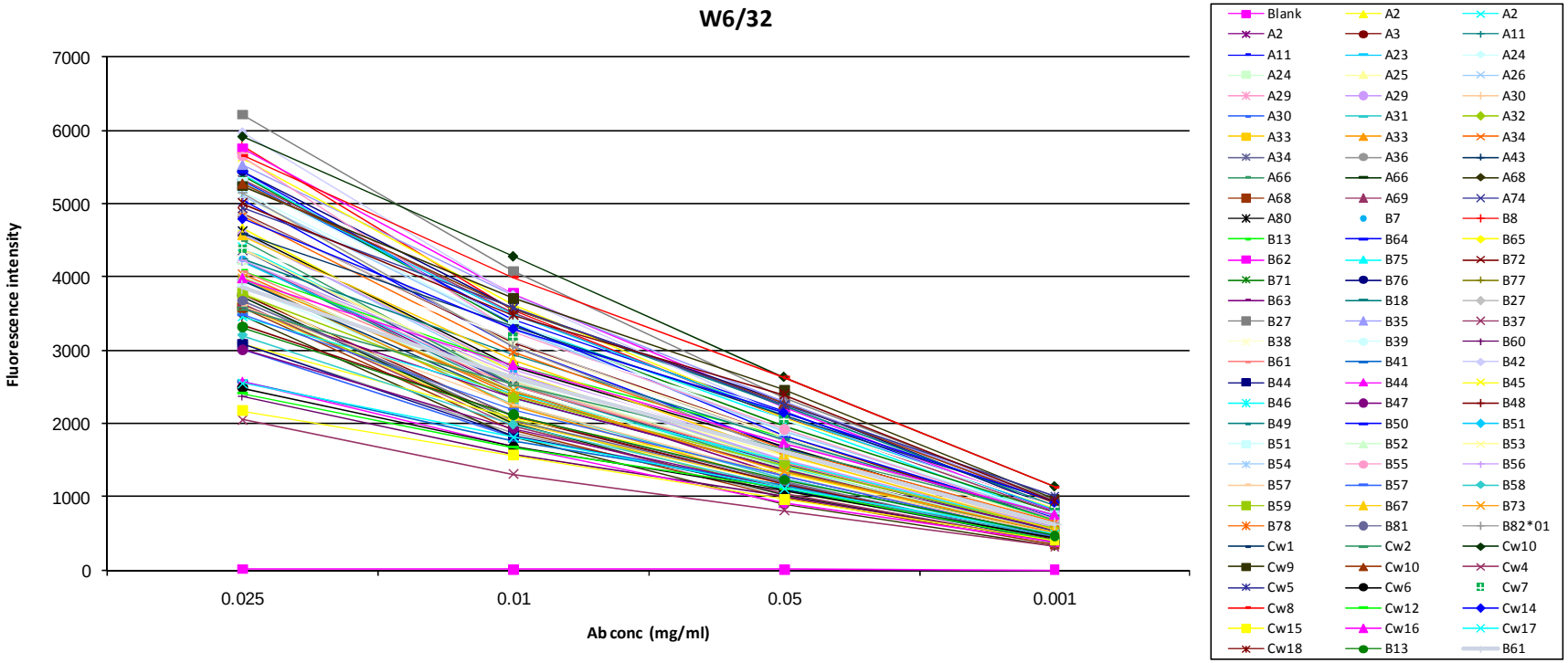


Figure 3-15: Use of W6/32 Ab for correction of variability in HLA antigen density on Luminex beads. W6/32 Ab was used at a range of concentrations to determine HLA antigen density on each bead. Median channel values were then normalised to the 75th percentile and a correction factor obtained for each bead.

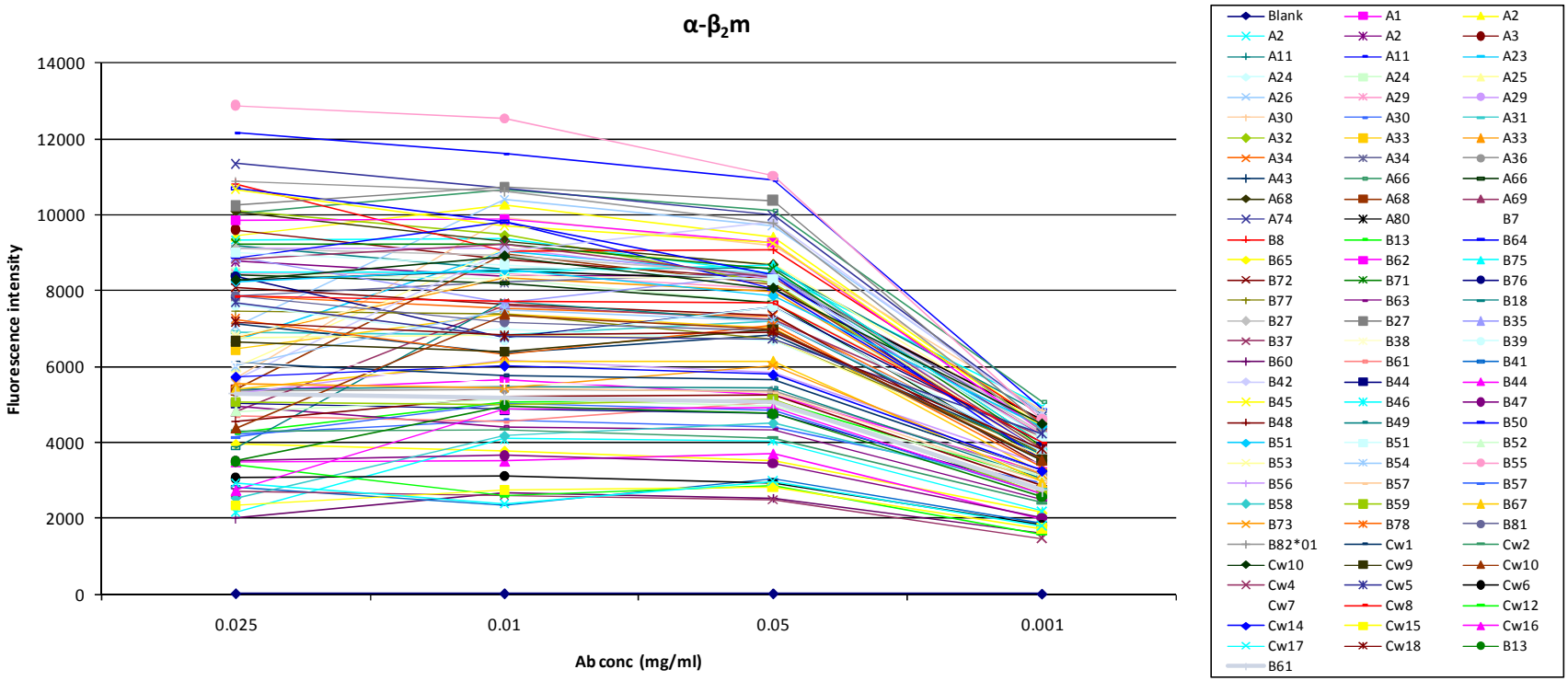


Figure 3-16: Use of 2M2 Ab for correction of variability in HLA antigen density on Luminex beads. α-β_2m Ab 2M2 was used at a range of concentrations to determine HLA antigen density on each bead. Median channel values were then normalised to the 75th percentile and a correction factor obtained for each bead.

The binding of 29 HLA-A, 49 HLA-B and 15 HLA-C allotypes was independently assessed. 2DKIR monomers were incubated with HLA class I coated beads at a concentration range from 1mg/ml to 10µg/ml before being washed and streptavidin-PE added for detection of binding. Samples were then run on a Luminex 100 machine. As can be seen in Figure 3-17 both KIR2DS2 and KIR2DL2 proteins failed to bind any of the HLA coated beads. To rule out any possibility that the streptavidin-PE did not bind the biotinylated KIR proteins, monomers were tetramerised prior to use in the assay. The results when using KIR2DS2 and KIR2DL2 tetramers were unchanged from those achieved using the monomers and streptavidin-PE as a secondary Ab.

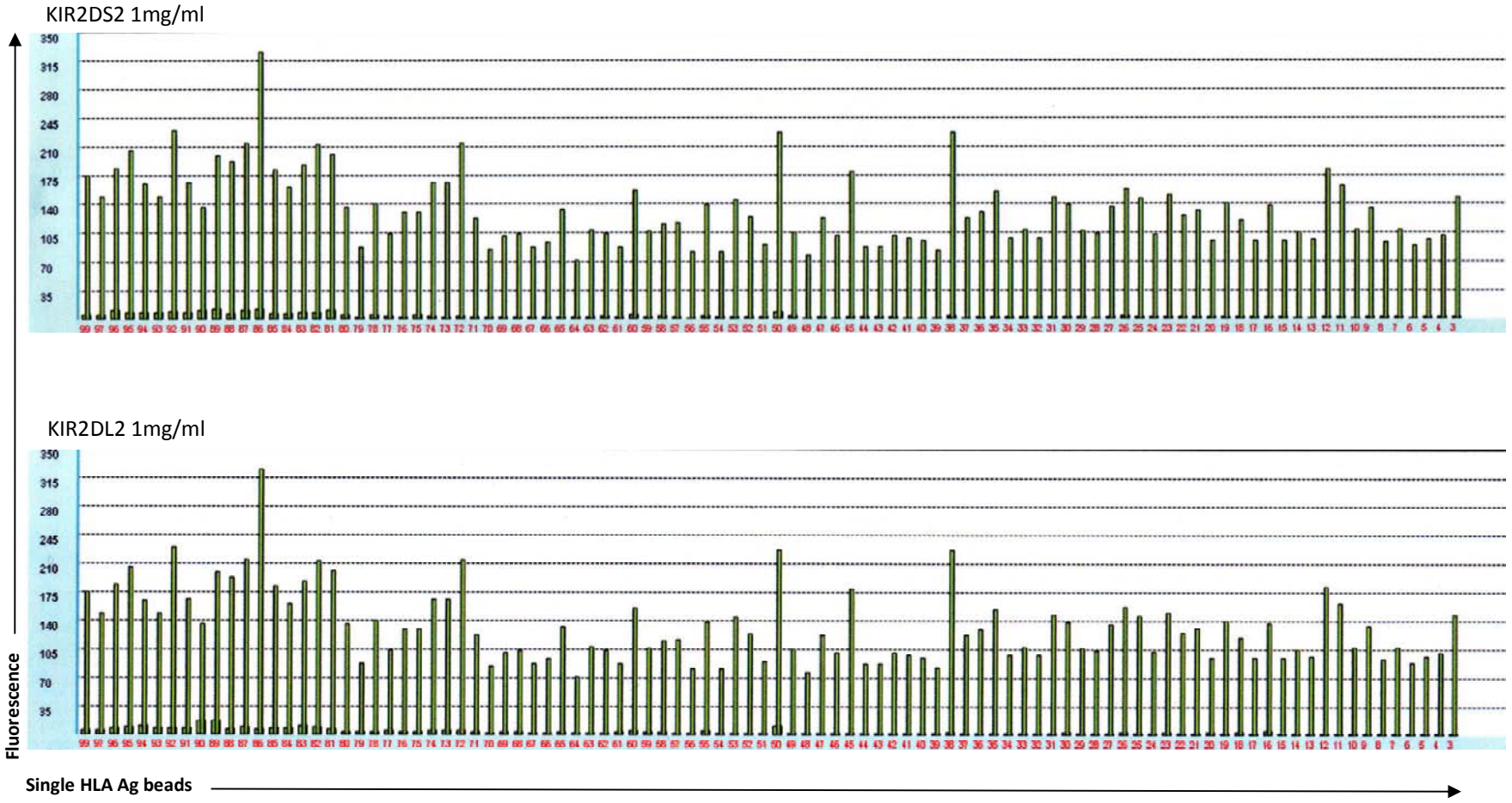


Figure 3-17: Luminex assay using One Lambda single antigen beads. KIR2DS2 and KIR2DL2 tetramers tested for recognition against a panel of 93 beads each coated with a single HLA class I antigen. Green bars represent a negative signal, whilst red bars are indicative of protein binding.

Negative cut-off values for HLA antibody identification using the kits manufactured by OneLambda are arbitrary. H&I at NHSBT in Birmingham use an approximate MFI of >1000 to assign positive reactions. The application of negative cut-off values is a controversial area in the field of HLA antibody identification with a range of negative cut-off values in use in UK laboratories. However analysis of the range of negative cut-off used by UK H&I laboratories quote values in the range of 500-2000MFI to assign positivity. As this assay was not used for the application it was designed for, and HLA antibodies may well bind at a higher affinity, data was reanalysed and a cut-off figure determined using one standard deviation (S.D) away from the mean (Table 3-2a). Using this cut-off several beads may be classed as positive (Table 3-2b), including HLA-C allotypes and some HLA-B allotypes (which may be plausible as they are highly homologous to HLA-C).

Table 3-2: Reanalysis of Luminex data.

a) Data statistics

Mean	132.54
Median	120
Standard Deviation	44.57
+1 S.D.	177.11

b) Beads classed as positive

Bead	HLA
38	B65 (Bw6)
50	B37 (Bw4)
72	B57 (Bw4)
81	Cw1
82	Cw2
83	Cw3
86	Cw4
87	Cw5
88	Cw6
89	Cw7
92	Cw14
95	Cw17
96	Cw18

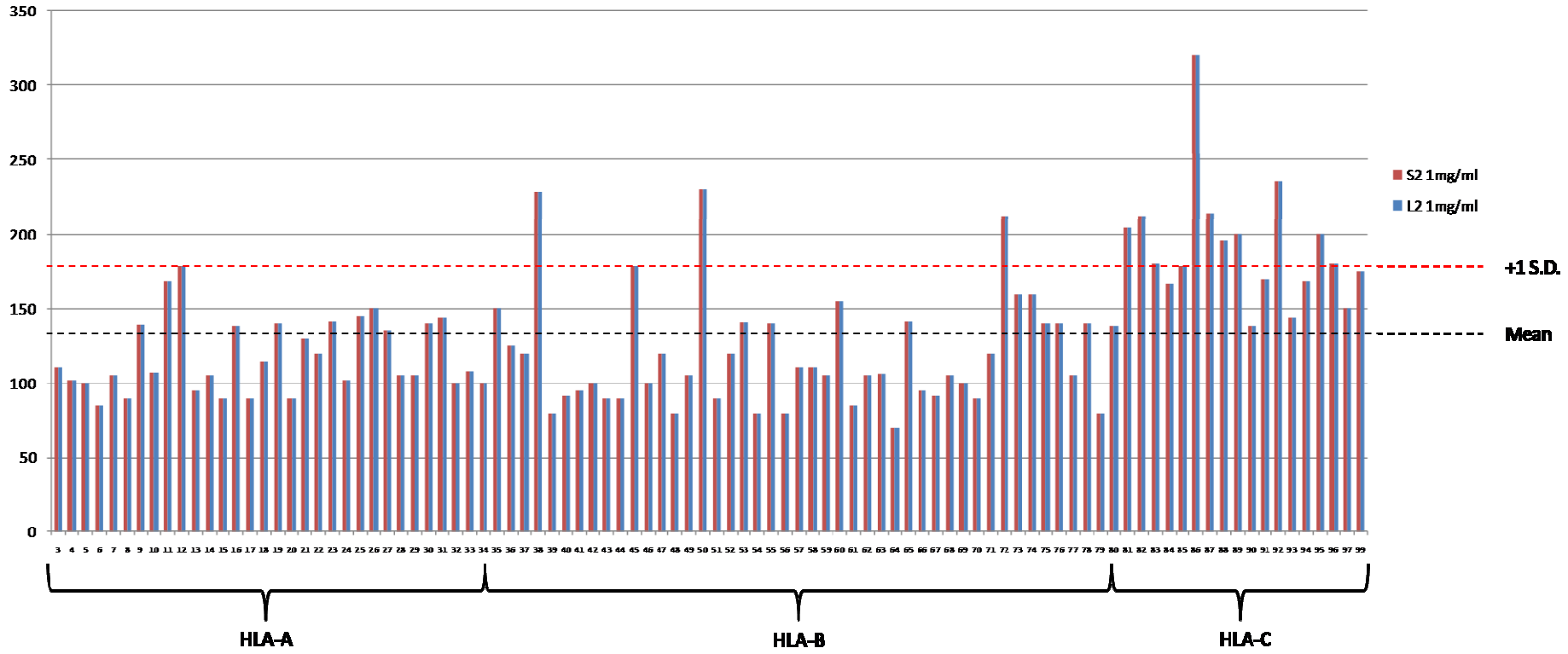


Figure 3-18: Reanalysis of One Lambda Luminex assay. Data from Figure 3-17 was re-graphed and cut-off values assigned post statistical analysis. Bars above the red cut-off (+1 S.D from mean) were considered positive.

To ensure the high concentration of 2DKIR protein used had not caused the beads to form duplets, the remaining samples were run on a flow cytometer. As shown in Figure 3-19 all HLA beads could be seen as singlets. At the same time the sample was checked for PE staining, but in accordance with the Luminex assay all samples were PE-negative.

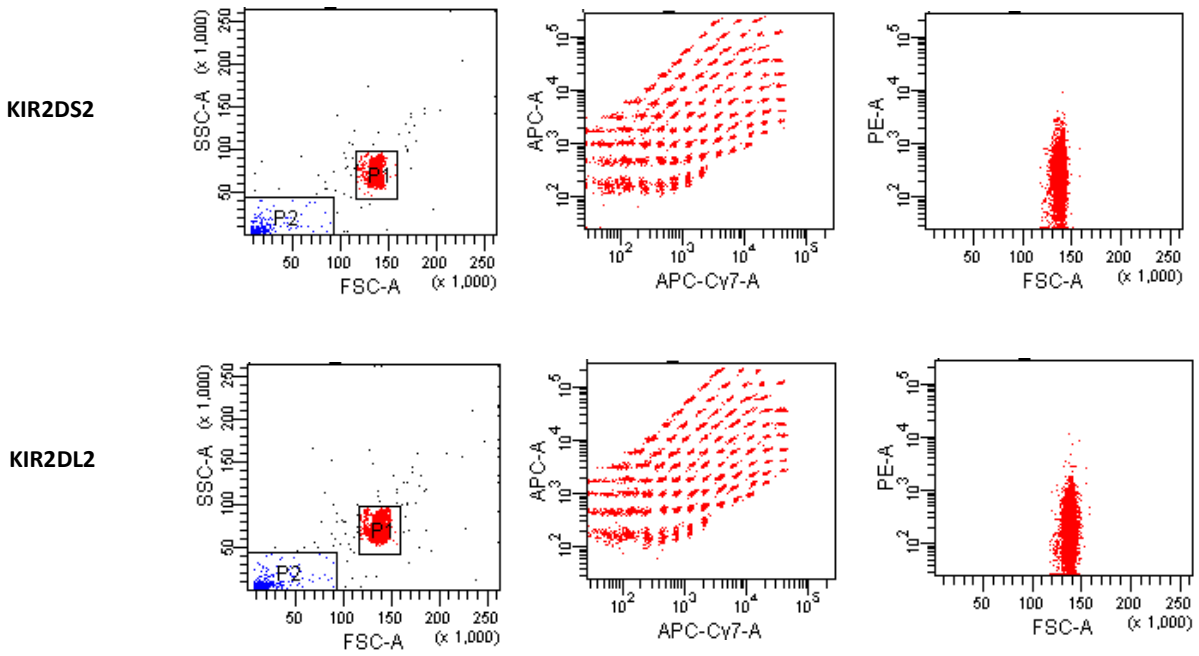


Figure 3-19: Possibility of duplet formation is excluded by flow cytometric analysis. Remaining samples from the Luminex assay were analysed on a flow cytometer to ensure that all beads were singlets and examine PE staining. The red laser excites both the internal red and infrared dyes allowing the proper classification of the microsphere (APC and APC-Cy7 channels). The green laser excites any fluorescence associated with the binding of 2DKIR proteins (PE channel).

To validate the KIR tetramers PBMC were stained. As shown in Figure 3-20 the tetramers stained a small percentage of NK and T cells (both $CD8^+$ and $CD4^+$) from an HLA-C1 homozygous donor. Percentages were lower than expected – presumably all T cells express HLA-C albeit at a much lower level (90% less) than HLA-A and HLA-B (Snary *et al.* 1977; Sodoyer *et al.* 1984; Gussow *et al.* 1987). Only 5 donors were stained and their HLA-C type not tested. It may be that donors did not possess any C1-group alleles, or were heterozygous C1/C2. Also it may be the case that KIR binding is peptide dependent. In the absence of HLA-C isoform-specific antibodies this could not be validated.

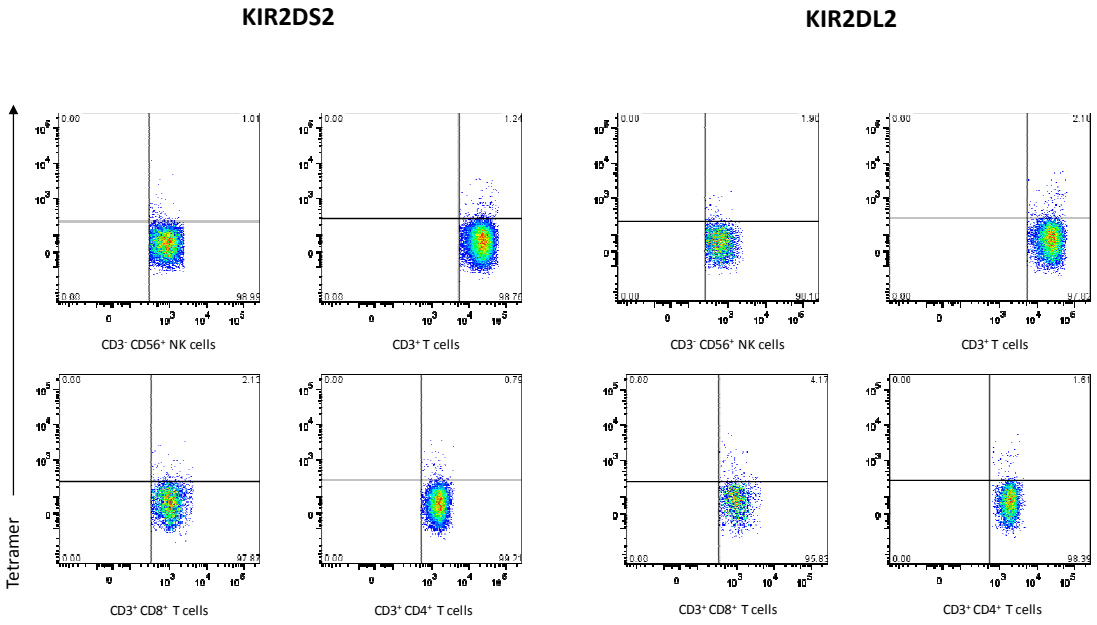


Figure 3-20: KIR tetramer staining. KIR2DS2 and KIR2DL2 monomers were tetramerised by the addition of streptavidin-PE. PBMC from a HLA-C1 homozygous donor were then stained using KIR tetramers and the percentage of $tetramer^+$ cells recorded.

Assuming KIR proteins were correctly folded based on SDS-PAGE gels, antibody ELISAs and biotinylation ELISAs (Figures 3-5, 3-6 and 3-7) they were next checked for stability during the Luminex assay. As before, an antibody ELISA was used to check for GL183 recognition. Samples that had been diluted in Luminex buffer were compared to neat monomer stocks. As controls monomers denatured either by high temperature, urea or SDS were included along with inclusion body and wells containing no protein. Figure 3-21 shows that samples diluted in buffer ready for the Luminex assay were no longer recognised by GL183 Ab. Denatured proteins, inclusion bodies and wells containing no protein were all negative for GL183 binding.

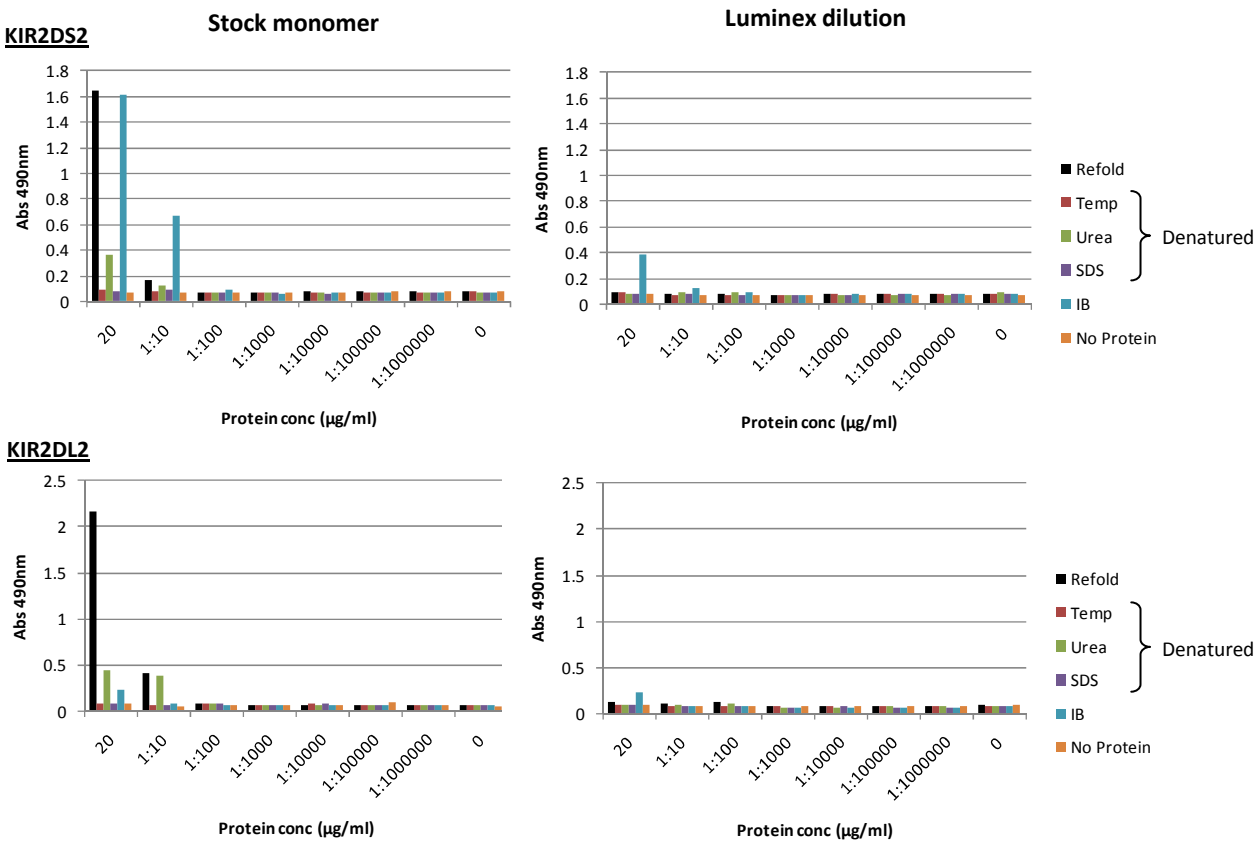


Figure 3-21: Validation of KIR monomers in Luminex buffer. KIR2DS2 and KIR2DL2 monomers were tested for recognition with GL183 Ab. Stock monomer and samples that had been diluted in Luminex buffer were compared. Denatured protein, inclusion body and no protein were used as negative controls.

To overcome this, another Luminex assay was developed. Both 2DKIR and HLA monomers were coupled to spectrally distinct Bio-Plex COOH microspheres. Binding of 2DKIR and HLA tetramers to the beads was investigated. Figure 3-22 shows KIR2DS2 and KIR2DL2 were recognised by Cw7-CRV tetramer and Cw7-RYR (to a lesser extent). There is some slight recognition by Cw4-QYDP after beads had been normalised and background excluded. In disagreement with the belief that KIR2DL2 binds only C1-group allotypes, HLA-Cw*0401 refolded with QYDD peptide was recognised by KIR2DL2 tetramer and there was weak recognition when using KIR2DS2 tetramer. This is interesting as it concurs with the data obtained from BIAcore experiments.

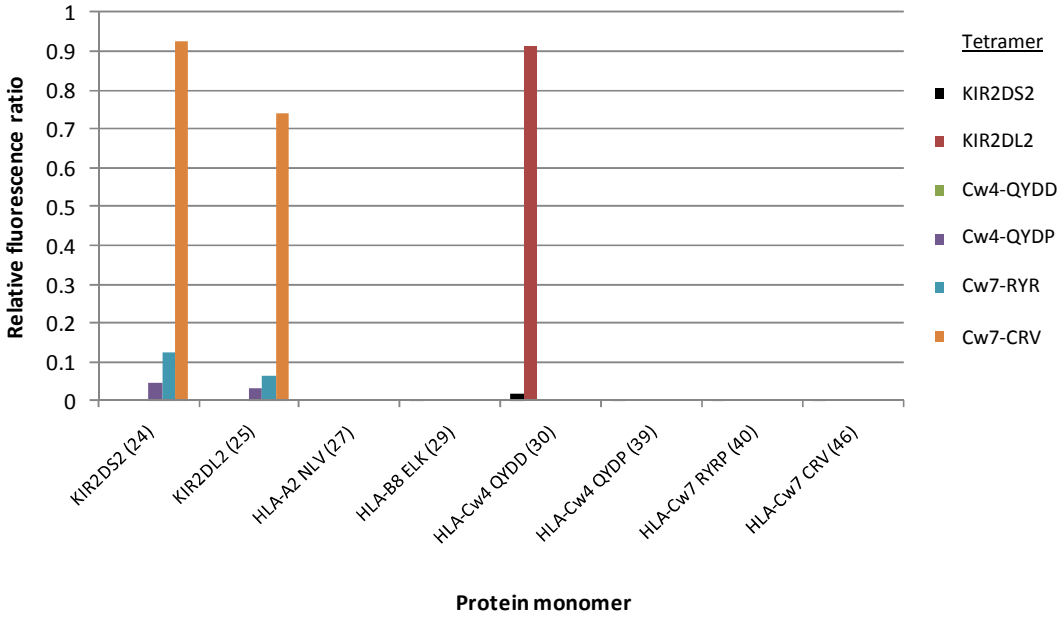


Figure 3-22: Luminex assay using Bio-Plex COOH microspheres. KIR2DS2 and KIR2DL2 monomers were coated onto beads and tested for recognition with HLA tetramers and vice versa.

Somewhat surprisingly KIR2DS2 and KIR2DL2 were only seen to bind HLA-Cw*0702 when the KIR protein was on the bead and an HLA tetramer used. This may be due to inefficient levels of HLA-Cw*0702 being coated onto the beads (data not shown). In contrast KIR2DL2 bound HLA-Cw*0401-QYDD when the HLA protein was on the bead and a KIR tetramer used.

3.4 *EX VIVO* RECOGNITION OF 2DKIRS USING HLA-C TETRAMERS

HLA-C tetramers had been generated and were used to bind KIRs at the cellular level. Although not as useful a reagent as a specific KIR2DS2 antibody, there are potential advantages to the use of these novel tetramers, including the fact that they are a natural ligand. PBMCs were stained with HLA-C tetramers in order to see if any cells were positively stained. The HLA-C tetramers did not stain lymphoid cells which were isolated directly *ex vivo.* However, tetramers did not stain cells which were cultured *in vitro* for a period of at least seven days. Figure 3-23 shows an example of staining by HLA-Cw*0401 tetramers in one donor. After culture the percentage of tetramer-positive cells had increased in several of the culture conditions. The addition of IL-2 to the culture made a profound difference to the percentage of lymphoid cells that were stained with the tetramer. Within the T cell subset the staining was more profound in the $CD8^+$ T cell subset where approximately 10% of cells showed positive staining, but there was also evidence of staining in the $CD4^+$ populations. The addition peptide alone also increased the proportion of tetramer-positive cells although to a rather lesser extent. The CW4-QYDD tetramer also stained more cells than the Cw4-QYDP reagent. HLA-Cw*0702 tetramers were not able to stain lymphoid cells in any of the donors tested. Collective data from 5 donors is shown in Figure 3-24.

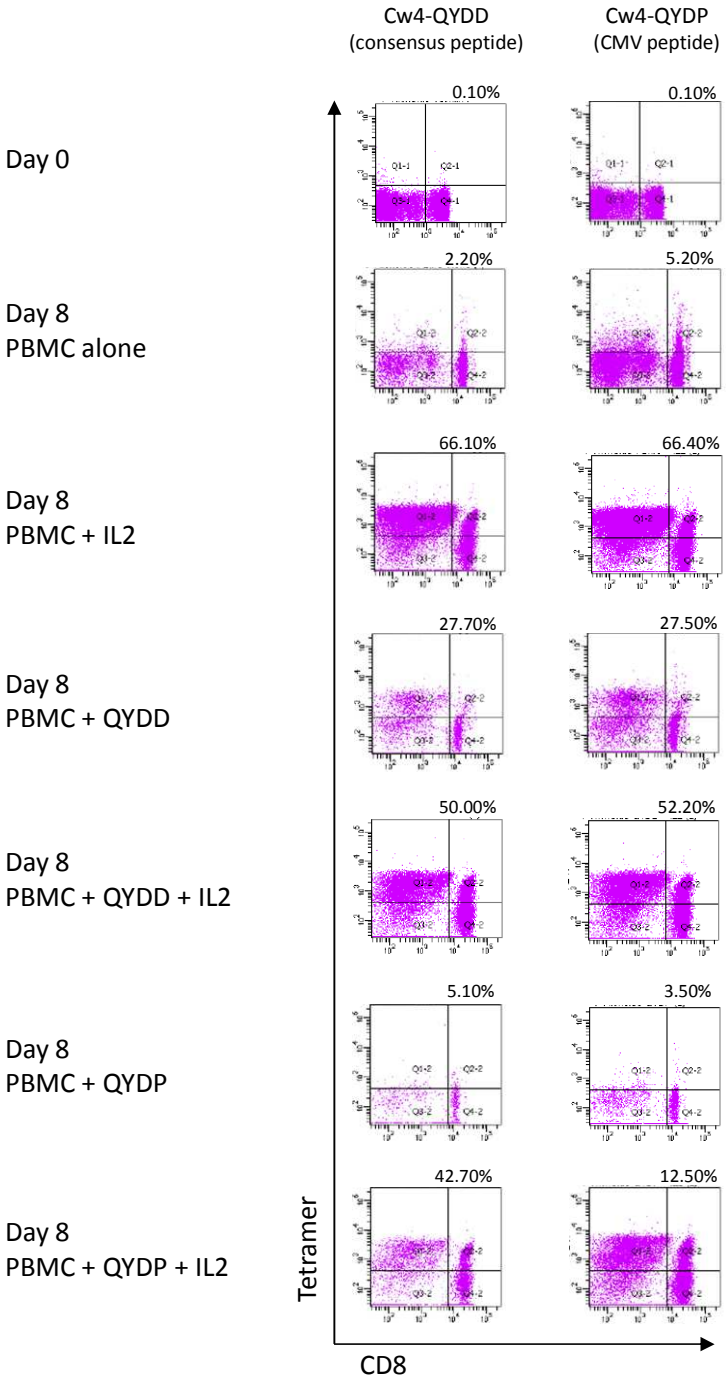


Figure 3-23: HLA-C tetramer staining of peptide lines. An example of HLA-C tetramer staining of PBMCs cultured for one week with or without peptide and IL-2. Percentages are proportion of lymphocytes that are tetramer-positive.

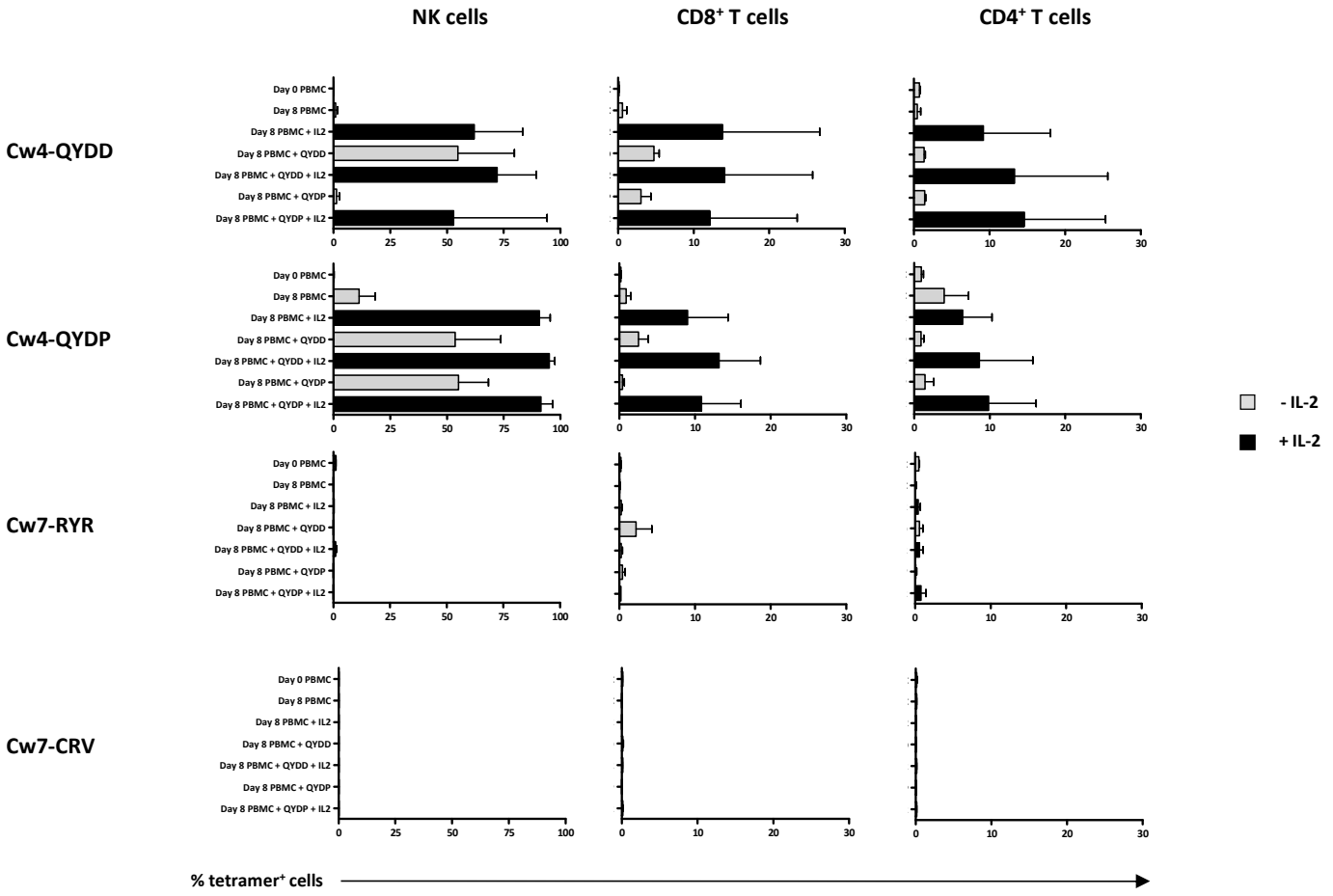


Figure 3-24: HLA-C tetramer staining of peptide lines. Collective HLA-C tetramer staining from peptide lines of 5 donors. Percentages graphed are the frequency of lymphocytes that are tetramer^{+}.

3.5 DISCUSSION

The activating receptor KIR2DS2 shares 99% homology with its inhibitory counterpart KIR2DL2 (extracellular domains), making the generation of specific reagents to differentiate between these proteins a difficult task. Recombinant KIR2DS2 and KIR2DL2 proteins were generated to attempt antibody production via phage display. However with only four amino acid changes between the two proteins these attempts have so far proved to be unsuccessful.

The aim of the work presented in this chapter was to investigate the interaction between the 2-domain KIRs (KIR2DS2 and KIR2DL2) and their HLA-C ligands. Recombinant KIR2DS2 and KIR2DL2 proteins were successfully generated, as were HLA-Cw*0702 and HLA-Cw*0401 which were chosen as examples of HLA-C group 1 and group 2 alleles. Refolding HLA-C, especially HLA-Cw*0702 was not straightforward. Standard conditions could not be used and even after optimisation SDS-PAGE analysis of peak fractions collected during FPLC revealed that the stoichiometry of the folded HLA molecule was not 1:1, β_2m : heavy chain (Figure 3-11b). Much less β2m than heavy chain was incorporated into the monomer. Despite this, monomers were all well biotinylated and recognised by W6/32 Ab.

To examine binding, and assess whether or not interactions were peptide dependent, HLA-C monomers were refolded with a panel of peptides (Table 2-6). BIAcore analysis of KIR2DS2 and KIR2DL2 was attempted against HLA-Cw*0401 and HLA-Cw*0702. However, in this assay the KIR proteins bound the ligands coated to the chip in a non-specific manner. This may have been caused by problems immobilising the ligand proteins to the sensor surface. We also wondered whether protein purity could have an effect and so KIR proteins were re-

expressed this time in Rosetta cells. However, the resulting protein, although appearing purer on an SDS-PAGE gel did not improve the BIAcore results.

The reasons for the failure to observe ligand binding by BIAcore are unclear as this is a very sensitive technique for studying protein interactions. It was considered possible that my recombinant protein had mutations compared to the native sequence but this was excluded by sequencing of the expression plasmid. Glycosylation-dependency of the KIR-HLA-C interaction is another possibility as proteins were generated in a prokaryotic expression system.

An alternative approach to examine 2DKIR interactions with HLA-C was therefore undertaken. A Luminex assay, based on a technique used routinely to measure HLA class I-specific antibodies in patients after allo-transplantation, was adapted to measure recombinant KIR2DS2 and KIR2DL2 protein interactions with HLA-coated beads. HLA class I was initially used to optimise the assay, but class II beads were also available and intended to be tested. No binding was observed between KIR2DS2 or KIR2DL2 proteins and the HLA-coated beads which raised further doubt over the integrity of the recombinant proteins that I had generated. At this stage it became important to show that the proteins had been correctly folded but I was able to confirm this with an ELISA assay incorporating the GL183 antibody. However, when the GL183-based ELISA was performed with 2DKIR proteins diluted in Luminex buffer this recognition was lost. This was unexpected as, although the exact composition of the commercially produced Luminex buffer is unknown, the only ingredient listed is 0.1% sodium azide. During this project another group published data using this same

method, although they only investigated the inhibitory 2DKIR proteins. Moesta *et al.* produced KIR2DL1-, KIR2DL2- and KIR2DL3-Fc fusion proteins as opposed to recombinant proteins and measured binding to the HLA class I coated beads (Moesta *et al.* 2008). They successfully showed that KIR2DL2 bound all C1-group allotypes but also several C2-group allotypes (Cw*0501 and Cw*0202), and 2 HLA-B allotypes (B*4601 and B*7301) that share polymorphisms with HLA-C. This novel observation confirmed the potential of the Luminex technology and has led to a reappraisal of the previous dogma of the 2DKIR binding pattern.

A second Luminex assay was designed in which the recombinant KIR and HLA-C protein monomers were coupled to spectrally distinct COOH beads. Interactions between these beads were then measured against tetrameric HLA-C or KIR proteins. With this assay the KIR2DS2 and KIR2DL2 proteins were shown to bind to HLA-Cw*0702. Recognition was seen with two different HLA-Cw*0702 monomers which had been refolded around the RYR and CRV peptides, although binding to RYR was much less marked. Interestingly KIR2DL2 was also observed to bind HLA-Cw*0401 QYDD, and the BIAcore experiments may back this up, although this needs readdressing. Although this interaction is in conflict with the 'KIR2DL2-C1' and 'KIR2DL1-C2' paradigm, evidence is now emerging that the binding specificity of KIR ligands is not as well defined as had previously been believed. The Parham group have shown that KIR2DL2 can recognise C2-group allotypes when polymorphisms occur at amino acids 16 (Pro→Arg) and 148 (Arg→Cys) (Dr. Paul Norman, personal communication). These particular amino acids reside opposite the binding site and when substituted the positive charge causes the structure to bend and the binding site opens.

Recombinant protein monomers were also tetramerised to further investigate the KIR-HLA interactions. KIR2DS2 and KIR2DL2 tetramers were used to stain PBMC. A small frequency of cells stained positive, but this was a much lower percentage than might be expected by the number of cells expressing HLA-C. It may be that KIR binding is peptide dependent and therefore only a fraction of HLA-C molecules on the cell surface will bind KIRs. HLA-C tetramers were also synthesised and used to stain PBMC. The frequency of cells detected initially was low, but in experiments carried out on cultured cells showed that the percentage of HLA-Cw4 tetramer-binding cells increased. We observed that staining was highest in cells cultured with IL-2 and there is indeed evidence in the literature that CD158a and CD158b are upregulated by IL-2 (Kogure *et al.* 1999). Cultured cells were tested with 2 Cw4 tetramers containing different peptides. We were interested to see whether binding was peptide dependent, and from the data there were different patterns of binding in some cultures. However, this data is not yet conclusive and a larger panel of peptides needs to be examined to reach a better understanding.

This chapter shows that much remains to be determined regarding the binding specificity of the KIR2D proteins. To some extent, the interpretation will depend on the specificity of the technique that is used. Ultimately it will be the molecular interaction at the cell surface that determines the functional significance of KIR protein binding but biophysical studies, such as those in this chapter, and reagents such as HLA-C tetramers, have the potential to generate important novel information.

Chapter 4

The expression of Killer Immunoglobulin-like Receptors on T cells and other lymphocyte subsets

As discussed in the introduction there has been little information so far relating to the pattern of KIR expression on cell types other than NK cells. There have been numerous studies which have highlighted disease associations with specific KIR alleles (described in section 1.1.2.7), but very little has been done to address what these receptors do and how they interact with their ligands at a cellular level. Before these questions can be answered a clearer picture of the expression pattern of these receptors on different cell types needs to be gained, and it needs to be determined whether expression is stable over time, or if there are factors that can change expression levels.

The role that KIR proteins perform on T cells is not fully understood, but signalling through KIRs on T cells may inhibit T cell receptor mediated activation and KIR expression has been suggested to be one mechanism of controlling T cell mediated immune responses (Phillips *et*

al. 1995). An aberrant pattern of KIR expression on T cells could thus be of importance in autoimmune as well as infectious disorders.

One setting where NKR expression has been examined is in the elderly population. It has been shown that there is increased frequency of some NKRs on T lymphocytes with age (Tarazona *et al.* 2000). It is not clear whether expression of all KIR antigens increases in this way and this information is essential to allow understanding of the role of KIR in disease as many of the conditions that KIRs have been associated with affect the elderly population.

In light of our group's observation that KIR2DS2 had a protective role against CMV reactivation in patients who had undergone a stem cell transplant, (Cook *et al.* 2005), I was also keen to investigate whether there is any relationship between CMV infection and KIR expression. Although CMV encodes many genes that can influence MHC class I expression, no specific KIR has yet been shown to play an important modulatory influence on the immune response to CMV (Carr *et al.* 2002). This may reflect a true absence of effect but may also be masked by the complexity of the polygenic interactions between CMV, MHC alleles, and the NK cell haplotype. The aim of the work presented in this chapter therefore was to determine the pattern of KIR expression on T cells and other lymphocyte subsets in healthy donors and to investigate the effect that age and CMV have on this.

4.1 KIR EXPRESSION ON LYMPHOCYTE SUBSETS

Previous studies examining KIR expression on lymphocytes have focussed mainly on the expression pattern on NK cells. A few small studies have also shown expression on subsets of $CD8^+$ and $CD4^+$ T lymphocytes (Mingari *et al.* 1996; Ugolini *et al.* 2000; Lanier 2005). Therefore in an initial series of experiments the expression of KIRs was characterised on NK cells as well as major T cell subsets ($CD8^+$, $CD4^+$ and γδ), non-invariant NKT (NKT-like) cells and B cells to give a more complete overview of KIR distribution on these cell types. Lymphocytes from 10 donors were isolated and stained for typical cell surface markers to characterise each subset as shown in Table 4-1 and co-stained for KIR expression.

Table 4-1: Cell surface markers used to identify lymphocyte subsets

Lymphocyte subset	Cell surface markers
NK cells	$CD3^-$ $CD56^+$
NKT-like cells	$CD3^+$ $CD56^+$
$CD8^+$ T cells	$CD3^+$ $CD8^+$
$CD4^+$ T cells	$CD3^+$ $CD4^+$
γδ T cells	$CD3^+$ γδ TCR
B cells	$CD19^+$

Using flow cytometry each lymphocyte subset was examined for KIR expression. The antibodies that we used usually detect several KIR proteins due to the high degree of homology between receptors. Specificities of the α-KIR Abs used in these experiements are summarised in Table 4-2.

Table 4-2: α-KIR antibodies and their specificities

Antigen	Ab clone	KIRs detected	Inhibitory	Activating	HLA ligand
CD158a	HP-3E4	KIR2DL1, KIR2DS1	✓	✓	HLA-C group 2
CD158b	GL183	KIR2DL2, KIR2DL3, KIR2DS2	✓	✓	HLA-C group 1
CD158e1	DX9	KIR3DL1	✓		HLA-Bw4
CD158i	FES172	KIR2DS4		✓	?HLA-Cw4 [1]

[1] Studies have indicated that KIR2DS4 may have a restricted affinity for HLA-C ligands interacting specifically and with low affinity with HLA-Cw4 rather than HLA-Cw6 (Katz *et al.* 2001). More recently a non-class I MHC ligand for KIR2DS4 has been shown, on melanoma cell lines (Katz *et al.* 2004).

In initial experiments antibodies specific to CD158a, CD158b, CD158e1 and CD158i were pooled to give total frequency of KIR expression on the lymphocytes. Figure 4-1 shows an example of staining from 1 donor, representative of all 10 donors stained, with data from all 10 donors summarised in Table 4-3.

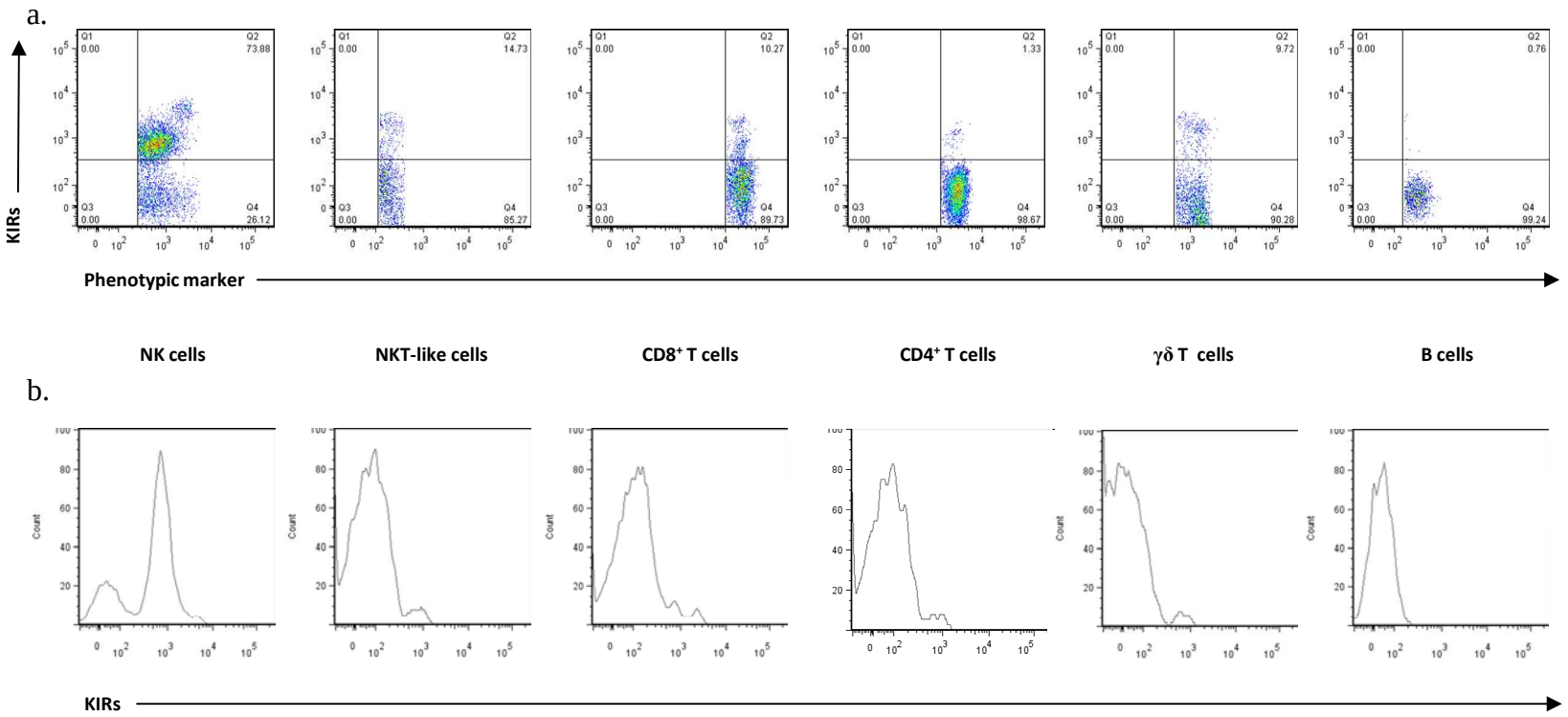


Figure 4-1: KIR expression on lymphocyte subsets. PBMC from healthy donors were stained for KIR molecules and other phenotypic cell surface markers. KIR expression was examined on NK cells, NKT-like cells, $CD8^+$ and $CD4^+$ T cells, -γδ T cells and B cells. (a) representative plots and (b) histograms showing KIR expression on each lymphocyte subset is shown.

KIRs are predominantly expressed on NK cells, although NKT-like cells and -γδ T cells were also found to express KIR at frequencies of ~15% and ~10% respectively. KIRs were also expressed on αβ T cells with approximately 10% of $CD8^+$ T cells and 1.5% of $CD4^+$ T cells staining positively with the KIR Ab mix. Finally no or very few B cells were found to express KIR.

Table 4-3: KIR expression on lymphocyte subsets

Donor	NK cells	NKT-like cells	$CD8^+$ T cells	$CD4^+$ T cells	γδ T cells	B cells
1	81.25%	13.80%	12.54%	1.57%	11.42%	0.00%
2	79.14%	15.26%	11.23%	0.95%	8.52%	0.15%
3	90.84%	12.79%	9.32%	1.27%	7.98%	0.01%
4	73.88%	14.73%	10.27%	1.33%	9.72%	0.76%
5	69.36%	13.25%	14.19%	1.97%	10.28%	0.04%
6	74.67%	12.07%	10.74%	1.69%	12.88%	0.09%
7	91.54%	14.98%	11.62%	2.06%	9.29%	0.24%
8	83.84%	15.06%	10.33%	1.84%	8.76%	0.09%
9	78.96%	11.97%	9.84%	1.02%	10.05%	0.17%
10	75.27%	16.51%	10.05%	1.30%	9.47%	0.37%
Median	**79.05%**	**14.27%**	**10.54%**	**1.45%**	**9.60%**	**0.12%**

T cells are important in the control of CMV replication and a number of studies are suggesting that KIR proteins may play important roles in the CMV-specific immune response. KIR2DS2 expression has a role in suppression of CMV reactivation (Cook *et al.* 2004; Cook *et al.* 2005), and increased expression of KIR has been seen on $CD4^+$ $CD28^-$ cells in patients with rheumatoid arthritis. It is now appreciated that this T cell subset is almost exclusively CMV-specific (Namekawa *et al.* 2000; Yen *et al.* 2001). Interestingly, Ly49 is a functional

ortholog of KIR in mice and has been shown to act as a murine ligand for CMV (Adam *et al.* 2006). A better understanding of KIR expression by T cells in healthy donors is needed. I therefore sought to examine the KIR phenotype of $CD4^+$ and $CD8^+$ T cells more closely and went on to determine the influence of CMV seropositivity on this profile. NK cells were included in experiments as a comparison. Blood samples were collected from 100 donors, and lymphocyte subsets were stained with α-KIR Abs to measure the frequency of cells expressing KIRs. The percentage of total KIR^+ cells in the subsets is shown for each donor in Figure 4-2. KIRs were expressed on significantly higher numbers of NK cells (median of 42%) than T cells, and within the T cell subset, significantly more $CD8^+$ T cells expressed KIRs than $CD4^+$ T cells (median values of 15.5% and 6.7% respectively). These findings are in agreement with current literature (van Bergen *et al.* 2004).

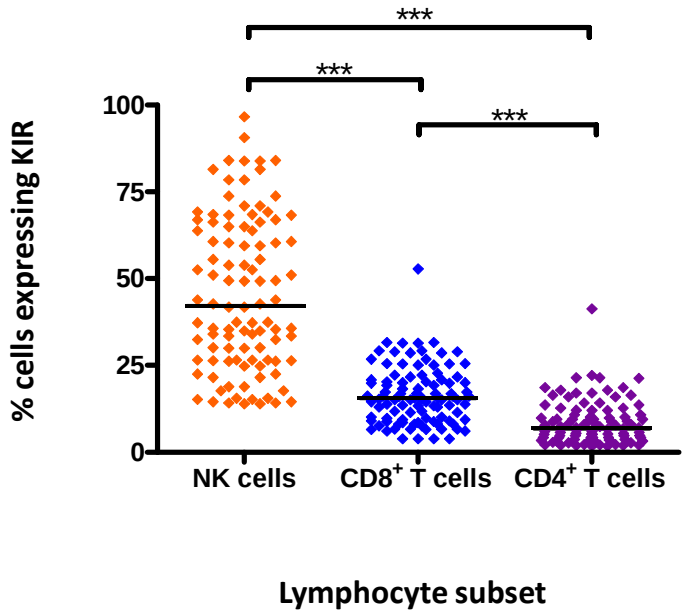


Figure 4-2: Total KIR expression on NK cells and T cells. PBMC from healthy donors were stained with Abs specific for KIRs and other phenotypic cell surface markers. Percentage of cells expressing KIRs was measured within NK cells, $CD8^+$ and $CD4^+$ T cells. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. Plotted values are the percentage of KIR^+ cells within each lymphocyte subset. A Dunn's Multiple Comparison test was carried out to determine whether the median values were significantly different.
*** = p <0.001

To define which KIRs were expressed on T cells and NK cells, an 11 colour Ab staining panel was developed. This included all four antibodies specific for KIRs (CD158a, CD158b, CD158e1 and CD158i), phenotyping antibodies to identify the lymphocyte subsets and a 'dump channel' to exclude dead cells, monocytes and B cells. The same 100 donor panel was analysed with this panel and individual KIR expression was measured on lymphocyte subsets. Figure 4-3a shows example staining from one donor, and Figure 4-3b shows the collective data from all 100 donors. A similar pattern is observed as for the combined KIR staining shown in Figure 4-2. For each KIR, expression is more frequent on NK cells, followed by $CD8^+$ T cells and then $CD4^+$ T cells.

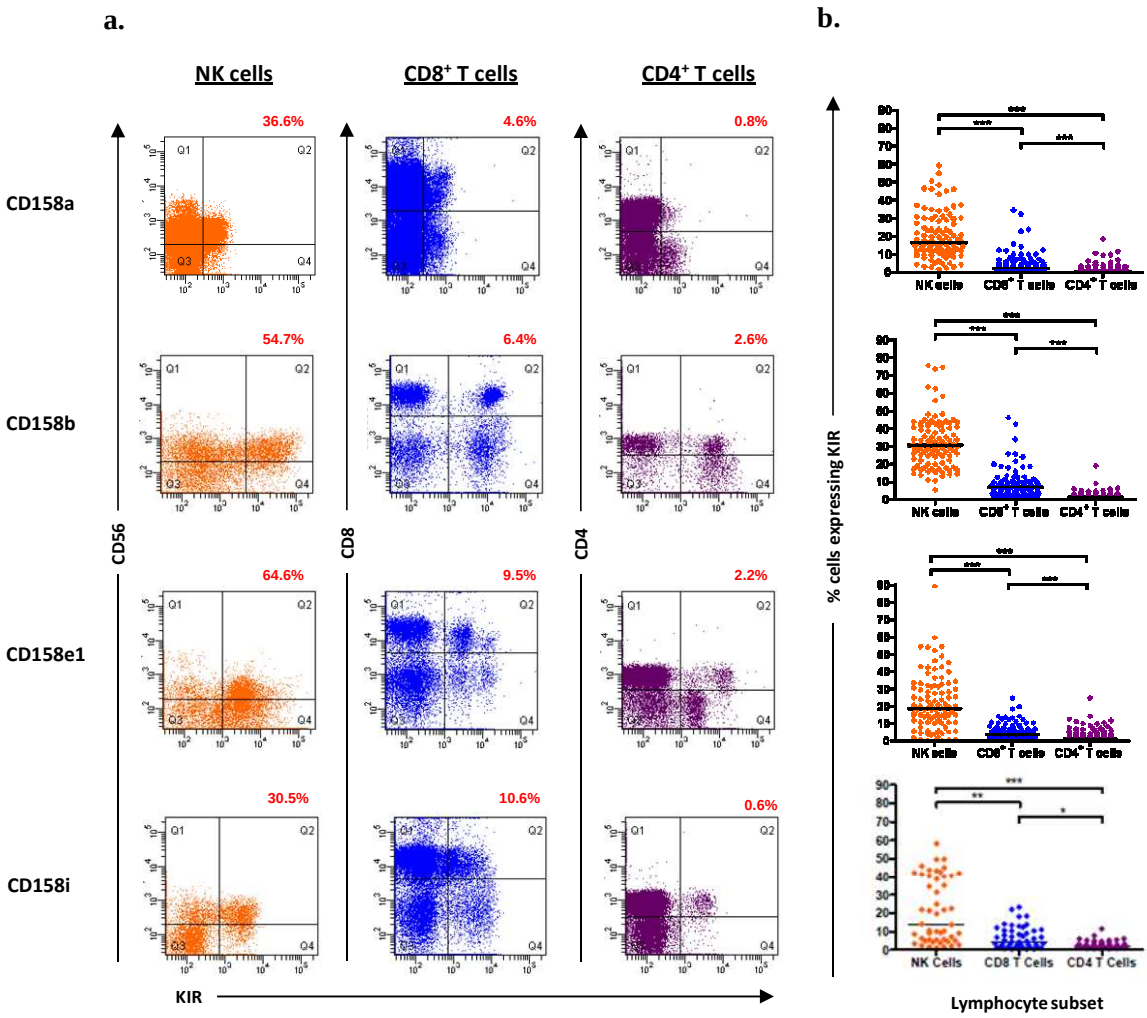


Figure 4-3: Individual KIR expression on NK and T cells. KIR expression was measured on NK, $CD8^+$ and $CD4^+$ T cells. (a) Representative staining data from one donor. The percentage of each lymphocyte subset expressing that KIR receptor (quadrant Q2) is shown. NK and T cells were stained in different 11-colour Ab panels and two different secondary Abs were used to detect CD158b. (b) Collective data from 100 donors. Comparisons between the groups were analysed using a Dunn's Multiple Comparison test.
*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

Multivariate analyses were also performed to ensure differences seen were not influenced by other factors such as CMV serostaus and age. ANOVA yielded a clear difference in KIR expression between NK, $CD8^+$ T and $CD4^+$ T cells (p <0.001), which was subsequently confirmed by univariate analyses for total KIR and each KIR separately (all p <0.001). KIR^+ cells were always more frequent within the NK subset than either $CD8^+$ or $CD4^+$ T cell subset (all p <0.001). A larger proportion of $CD8^+$ T cells were KIR^+ than $CD4^+$ T cells; the largest difference was seen for total KIR, CD158b, and CD158e1 expression (all p <0.005) whereas somewhat smaller differences were observed for CD158a and CD158i (all p <0.05). These effects remained unaltered after adjustment for CMV status and age.

When lymphocytes were stained with the α-CD158e1 Ab (DX9), some donors showed two clear lymphoid populations which stained with different intensities. This has been attributed to the antibody binding differentially to polymorphic KIR3DL1 molecules (Gardiner *et al.* 2001). The bright population stains 'KIR3DL1*015-like' molecules due to stronger binding, and the dim population binds 'KIR3DL1*005-like' allotypes that have one or two amino acid substitutions out of four possible at residues 182, 283, 320, and 373. These changes are enough to result in a lower binding affinity. KIR3DL1*004 does not stain with DX9 Ab and has substitutions at all four positions (Gardiner *et al.* 2001). However when donors stained positively for both sets of molecules they were not uniformly expressed across the different cell subsets. Figure 4-4 illustrates the pattern of CD158e1 staining. Figure 4-4a shows the percentage of each lymphocyte subset staining positive with the DX9 Ab. Example FACS plots from two donors are shown in Figure 4-4b. It can be seen that NK cells mostly have one positively-stained population, whereas $CD3^+$ T cells have two. Collective data from all donors is illustrated in Figure 4-4c. NK cells were seen to almost exclusively express the

poor-binding allotypes (95% : 5%), whereas expression on T cells was more balanced (60% : 40%). Upon closer inspection $CD8^+$ T cells appeared to mainly express those poor-binding allotypes (70%), although expression is not as homogenous as on NK cells. $CD4^+$ T cells appeared to have a much more heterogeneous repertoire with almost equal levels of poor-binding allotypes to strong-binding (55% : 45%).

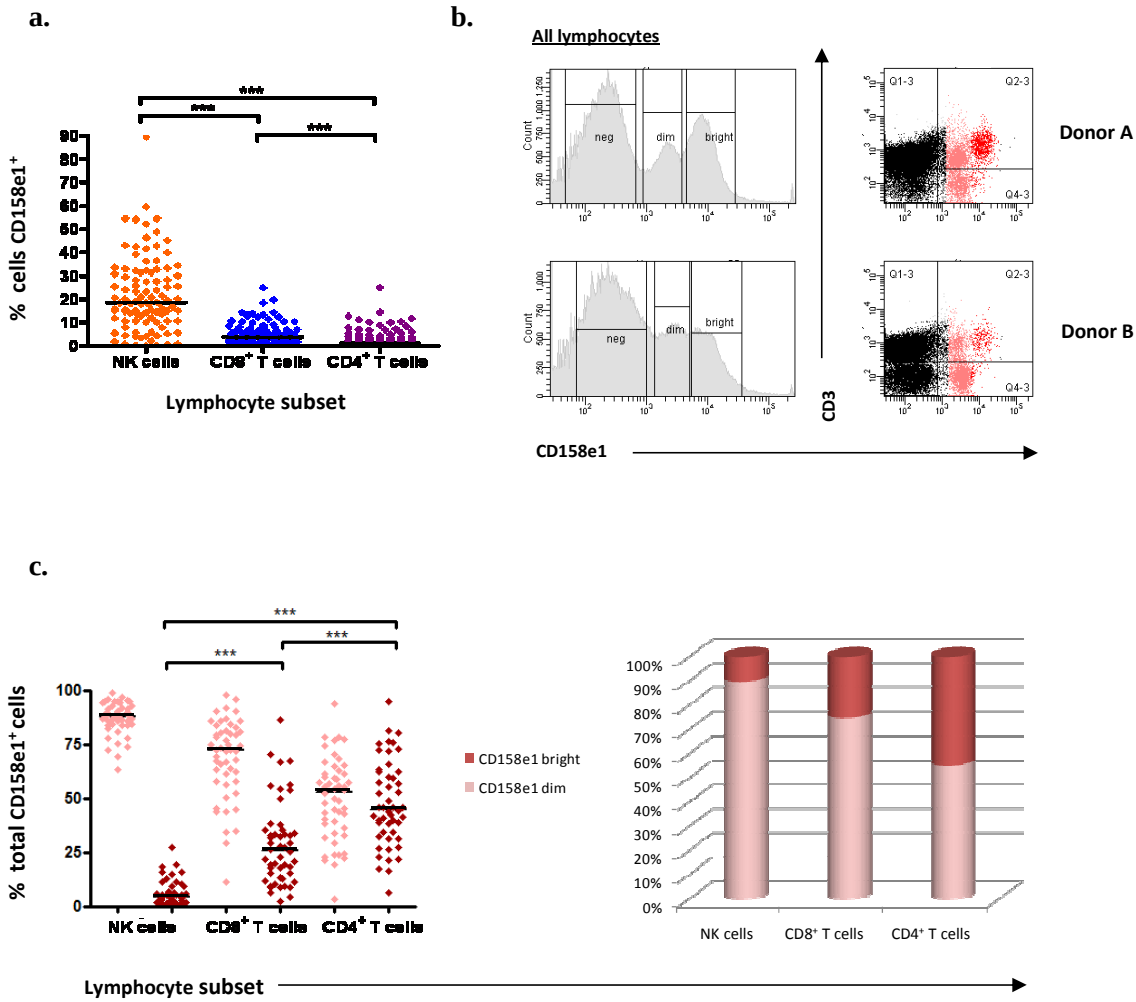


Figure 4-4: Differing CD158e1 allotype expression on NK and T cells. **(**a) Total CD158e expression on NK and T Cells. (b) CD158e positivity was divided into 2 populations – those KIR3DL1 alleles that were strong binding (bright) and those that were poor binding (dim). Plots are gated on all lymphocytes. (c) Frequencies of bright and dim-staining cells are shown as a percentage of total CD158e1^{+} cells within each cell type. One-way ANOVA analysis was carried out to test for significance. *** = $p < 0.001$

It has been shown that functionally, these subtypes of KIR3DL1 differ in inhibition signal strength (Carr *et al.* 2005). This along with data from this study is summarised in Table 4-4.

Table 4-4: Characteristics of KIR3DL1 subtypes

KIR3DL1 allotypes	DX9 Ab binding	Inhibition signal strength	Expression	Proportion of CD158e1$^+$ NK cells	Proportion of CD158e1$^+$ CD8$^+$ T cells	Proportion of CD158e1$^+$ CD4$^+$ T cells
*001, 002, 008, 009, 015, 020	Bright	High	Cell surface	5%	30%	45%
*005, 006, 007	Dim	Low	Cell surface	95%	70%	55%
*004	Negative	Null	Sequestered within cell	-	-	-

4.2 KIR EXPRESSION ON T CELL MEMORY SUBSETS

Few studies have examined the pattern of KIR expression on individual subsets of memory T cells. In 2001, Anfossi *et al.* showed that KIR^+ T cells exhibited a memory effector phenotype with no expression of CCR7 and only low levels of CD27 and CD28 (Anfossi *et al.* 2001). It has also been suggested that the expression of KIR on T cells is more restricted than the expression of other NKRs with, again, preferential expression on the $CD28^-$ subset (Abedin *et al.* 2005; Vallejo 2006; 2007).

To perform a more detailed analysis of how memory subset profile influenced the frequency of KIR expression my 11 colour antibody panel was employed to examine KIR expression on $CD8^+$ and $CD4^+$ T cell memory subsets. In order to characterise T cell memory subsets I used the markers of CCR7 and CD45RA (Table 4-5).

Table 4-5: Cell surface markers used to classify T cell memory subsets

T cell memory subset	Cell surface markers
Naïve	$CCR7^+$ $CD45RA^+$
T_{CM}	$CCR7^+$ $CD45RA^-$
T_{EM}	$CCR7^-$ $CD45RA^-$
T_{EMRA}	$CCR7^-$ $CD45RA^+$

PBMCs from 50 donors were studied by FACS analysis. Dead cells, monocytes and B cells were excluded from analysis but a selection gate was set around $CD3^+$ T cells. $CD8^+$ and $CD4^+$ T cell memory subsets were further gated as described in Table 4-3, and the frequency of KIR expressing cells recorded for each of these subsets. Figure 4-5 shows the frequency of total KIR expression observed for each of the T cell subsets.

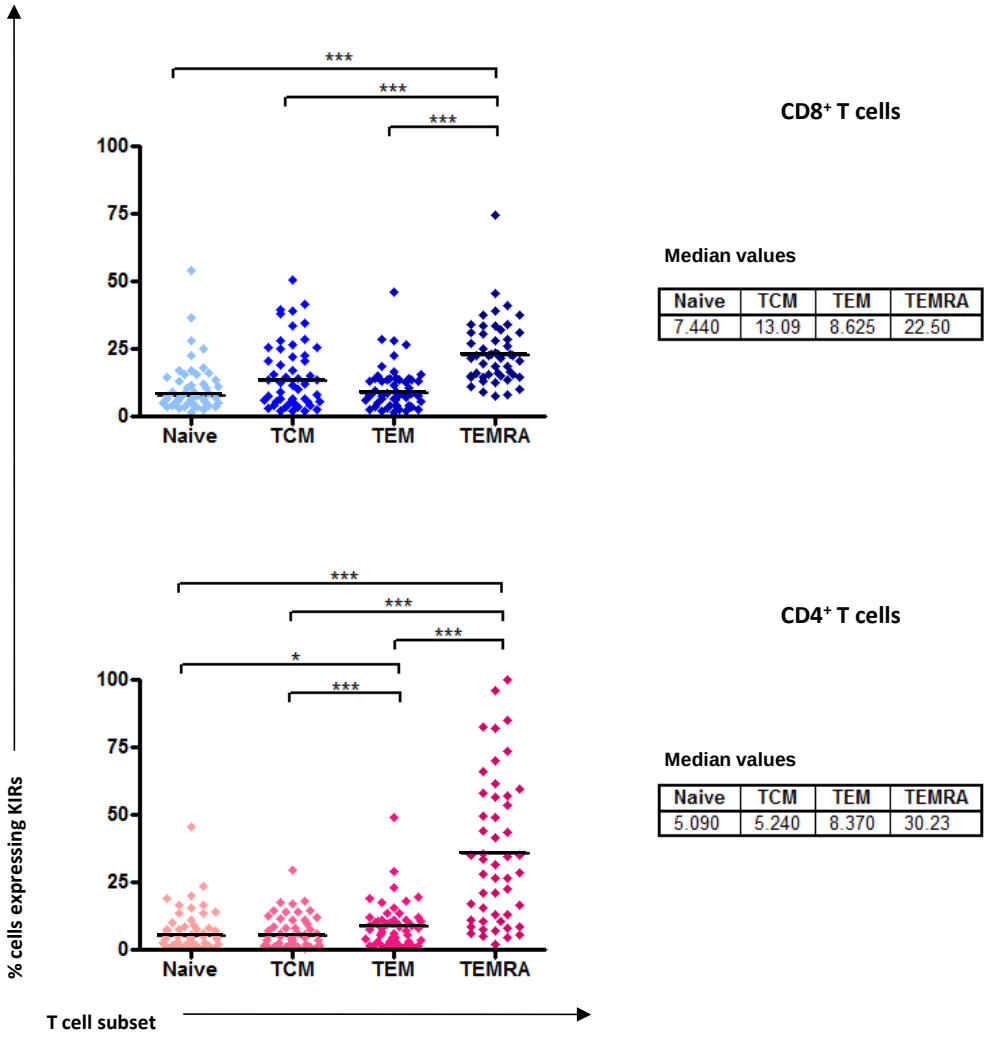


Figure 4-5: T cell differentiation leads to an increase in total KIR positivity. KIR expression was measured on $CD8^+$ and $CD4^+$ T cell memory subsets. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. Plotted values are the percentage of KIR^+ cells within each subset. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

In the $CD8^+$ compartment, effector memory 'revertant' $CD45RA^+$ T cells (T_{EMRA}) were seen to have a 3-fold increase in the frequency of KIR expression when compared to naïve T cells, while in the $CD4^+$ compartment a 6-fold increase in frequency was observed when compared to the naïve population. KIR expression by the other $CD8^+$ memory subsets is varied, with central memory T cells (T_{CM}) more frequently expressing KIR when compared to effector memory cells (T_{EM}) (median expression of 13% vs. 8.6%). However, in the $CD4^+$ T cell subsets an increase in the frequency of KIR^+ cells was observed as cells move along the 'classical differentiation' pathway from N → T_{CM} → T_{EM}. As discussed above, the highest concentration of KIR expression is seen on T_{EMRA} cells.

As well as total KIR expression, I also examined expression of the four individual KIR proteins on the different T cell memory subsets was also measured. As can be seen in figure 4-6, the pattern of expression of the four individual KIRs on $CD8^+$ T cells was similar to that of the total KIR. The frequency of KIR expression increases with the transition from naïve cells to T_{CM}, decreases in the T_{EM} subset before increasing greatly within the T_{EMRA} population.

Figure 4-7 shows KIR expression analysis on $CD4^+$ T cells. Again, the pattern seen with total KIR expression remains the same when looking at each of the KIR antigens individually. Frequency of KIR expression increases gradually as naïve cells undergo transition to T_{CM} and then T_{EM}. There is then a huge increase in the frequency of KIR^+ cells within the T_{EMRA} population. However, in contrast to the total frequency of KIR expression, CD158i appears to be downregulated on T_{CM} relative to the naïve population.

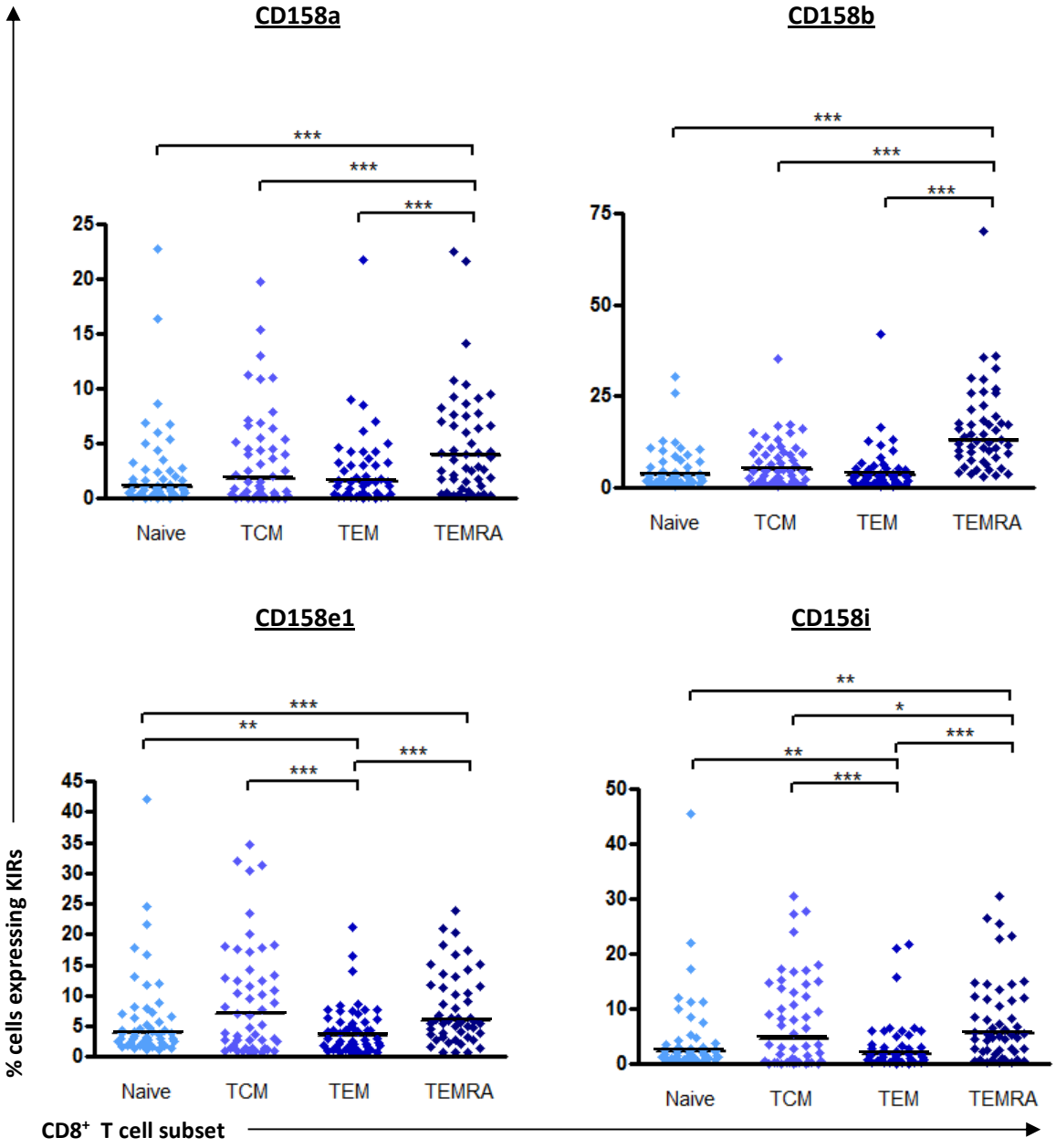


Figure 4-6: CD8$^+$ T cell differentiation is associated with an increase in frequency of all individual KIRs. Individual KIR expression was measured on CD8$^+$ T cell memory subsets. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. Plotted values are the percentage of KIR$^+$ cells within each subset. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different.
*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

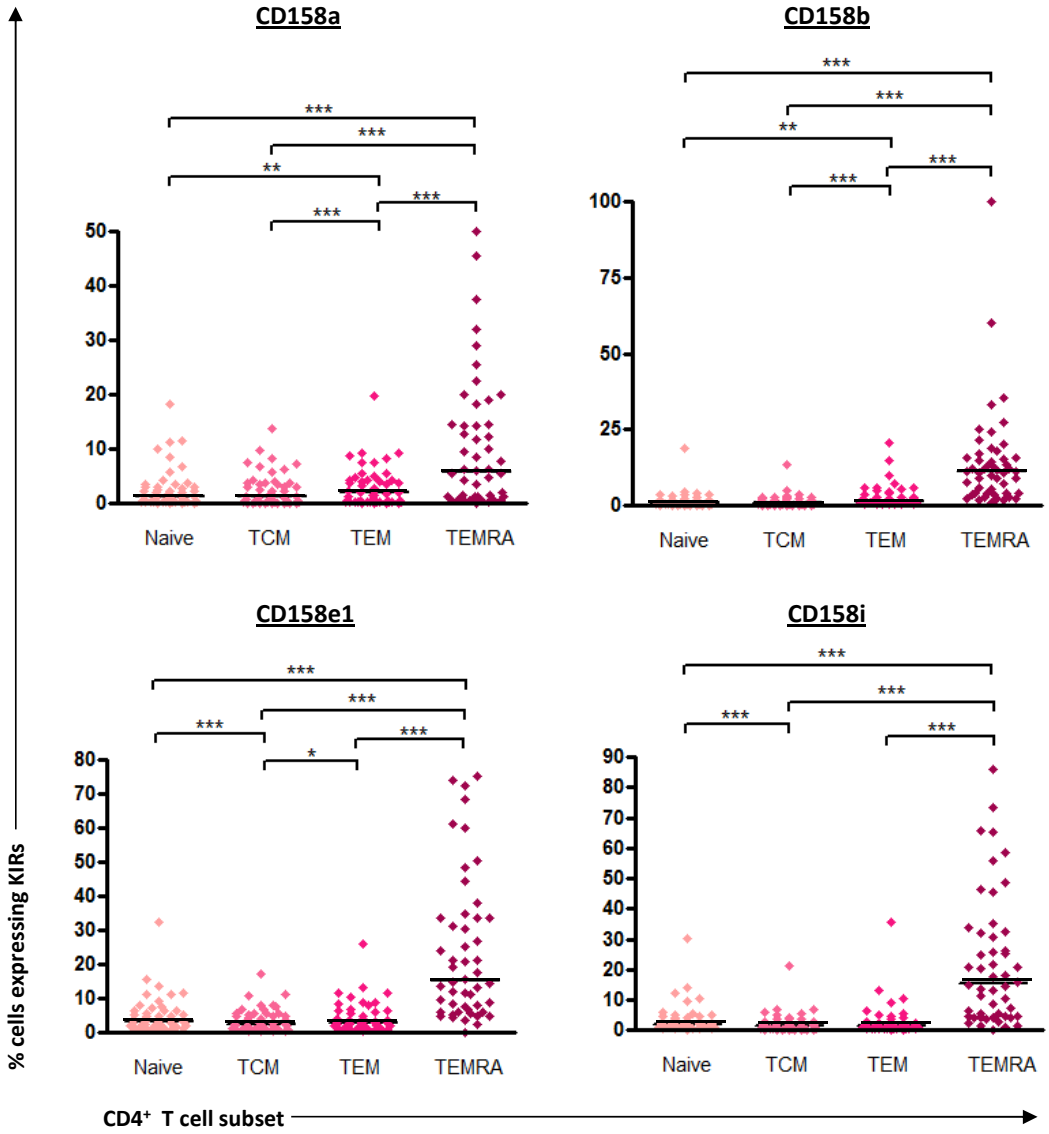


Figure 4-7: CD4$^+$ T cell differentiation is associated with an increase in frequency of most individual KIRs. Individual KIR expression was measured on CD4$^+$ T cell memory subsets. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. Plotted values are the percentage of KIR$^+$ cells within each subset. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different.
*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

4.3 KIR CO-EXPRESSION ON T CELL MEMORY SUBSETS

The ability to determine the expression of the four KIR determinants simultaneously enabled analysis of the co-expression of these receptors. This type of study has not previously been reported and so KIR^+ cells were examined for expression of single or multiple types of KIRs. The pattern of expression was also examined during differentiation to see if it remained stable.

The initial analysis was performed on $CD8^+$ T cells. KIR^+ cells were divided into four groups – those that stained positively for 1, 2, 3, or all 4 KIR antibodies respectively, and the percentages of each T cell memory subset that fell into each group were determined. Figure 4-8a shows the results of this analysis with the proportion of KIR^+ cells staining with 1, 2, 3 or 4 KIR Abs (Figure 4-8b). $CD8^+$ T_{EM} and T_{EMRA} populations were found to have a more focussed KIR repertoire than naïve and T_{CM}. Median values of 89 and 88% of KIR^+ T_{EM} and T_{EMRA} cells respectively stain positive with 1 or 2 antibodies, whereas 75 and 72% of naïve and T_{CM} do.

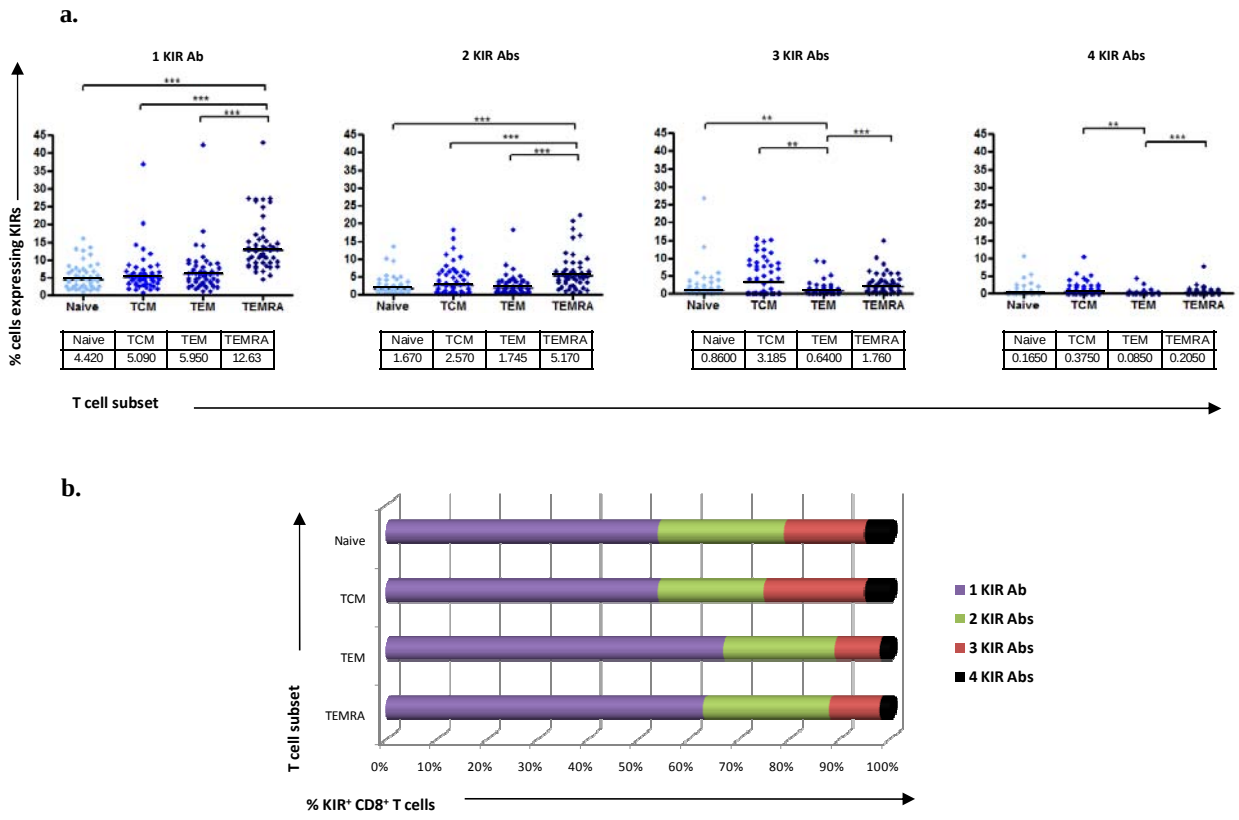


Figure 4-8: CD8$^+$ T_{EM} and T_{EMRA} populations have a more focussed KIR repertoire than naïve and T_{CM}. KIR co-expression was determined on CD8$^+$ T cell memory subsets. Cells were grouped according to the number of KIR Abs they stained positive for. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. (a) Plotted values are the percentage of KIR$^+$ cells within each T cell subset. (b) Plotted values show the proportion of KIR$^+$ cells that fall into each group. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

KIR co-expression was also measured at on $CD4^+$ T cell subsets (Figure 4-9). When looking at the proportion of KIR^+ cells it can be seen that T_{CM} and T_{EM} populations appear to have a more focussed KIR repertoire than naïve and T_{EMRA}. Of KIR^+ cells in both T_{CM} and T_{EM} populations 70% stain for 1 antibody and 90% for 1 or 2 antibodies. This is in contrast to naïve and T_{EMRA} where 51 and 33% of KIR^+ cells stain only for 1 KIR antibody, and 79 and 68% 1 or 2 antibodies. It can be seen that the $CD4^+$ T_{EMRA} population express a particularly diverse KIR repertoire with 10% of cells expressing KIR proteins detected by all 4 KIR Abs.

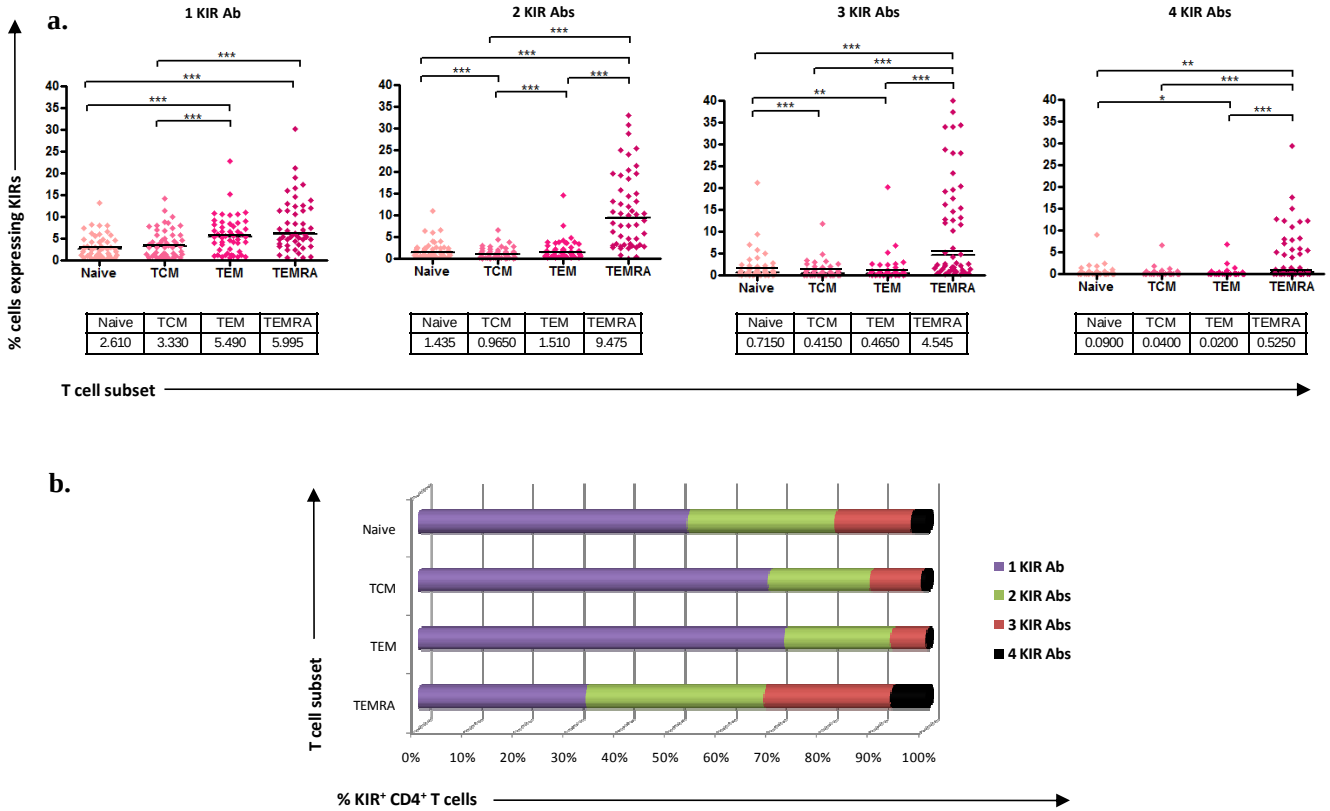


Figure 4-9: CD4$^+$ T_{CM} and T_{EM} populations have a more focussed KIR repertoire than naïve and T_{EMRA}. KIR co-expression was measured on CD4$^+$ T cell memory subsets. Cells were grouped according to the number of KIR Abs they stained positive for. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. (a) Plotted values are the percentage of KIR$^+$ cells within each T cell subset. (b) Plotted values show the proportion of KIR$^+$ cells that fall into each group. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

4.4 EFFECT OF AGE ON KIR EXPRESSION

The decline in immune function with age is a well documented phenomenon and appears to occur regardless of geographical and ethnic background. Those over the age of 65 have been shown to have increased morbidity and mortality in association with infectious disease, low response to vaccinations, and a higher incidence of malignancies and autoimmune disorders (Castle 2000; Ramos-Casals *et al.* 2003; Denduluri *et al.* 2004; Simonsen *et al.* 2005). The age-related decline in thymic function causes extensive remodelling of the T cell system (Sauce *et al.* 2009). Whilst TCR repertoire contraction is characteristic of the ageing immune system, there is increasing evidence that clonal T cells of elderly persons may express a variety of receptors normally found on NK cells (Tarazona *et al.* 2000).

In light of the effects observed with the expression of other NKR expression on lymphoid cells with ageing, KIR expression was examined within our donor cohort and plotted against age. PBMC samples from CMV seronegative donors only were used in this analysis due to an association seen between CMV seropositivity and KIR expression (see section 4.5). Both total KIR positivity and individual KIR expression were examined on lymphoid subsets in 25 donors. Figure 4-10 shows that total KIR expression increases on NK and $CD8^+$ T cells in association with age. $CD4^+$ T cells also showed a trend towards increased frequency of KIR expression although this did not reach statistical significance (p=0.0630). This is probably due to the lesser number of data points when only looking at CMV seronegative subjects, and this effect is augmented by the fact that most elderly donors (over 60 years of age) are CMV seropositive, meaning the regression analysis could only be applied over a narrow age range.

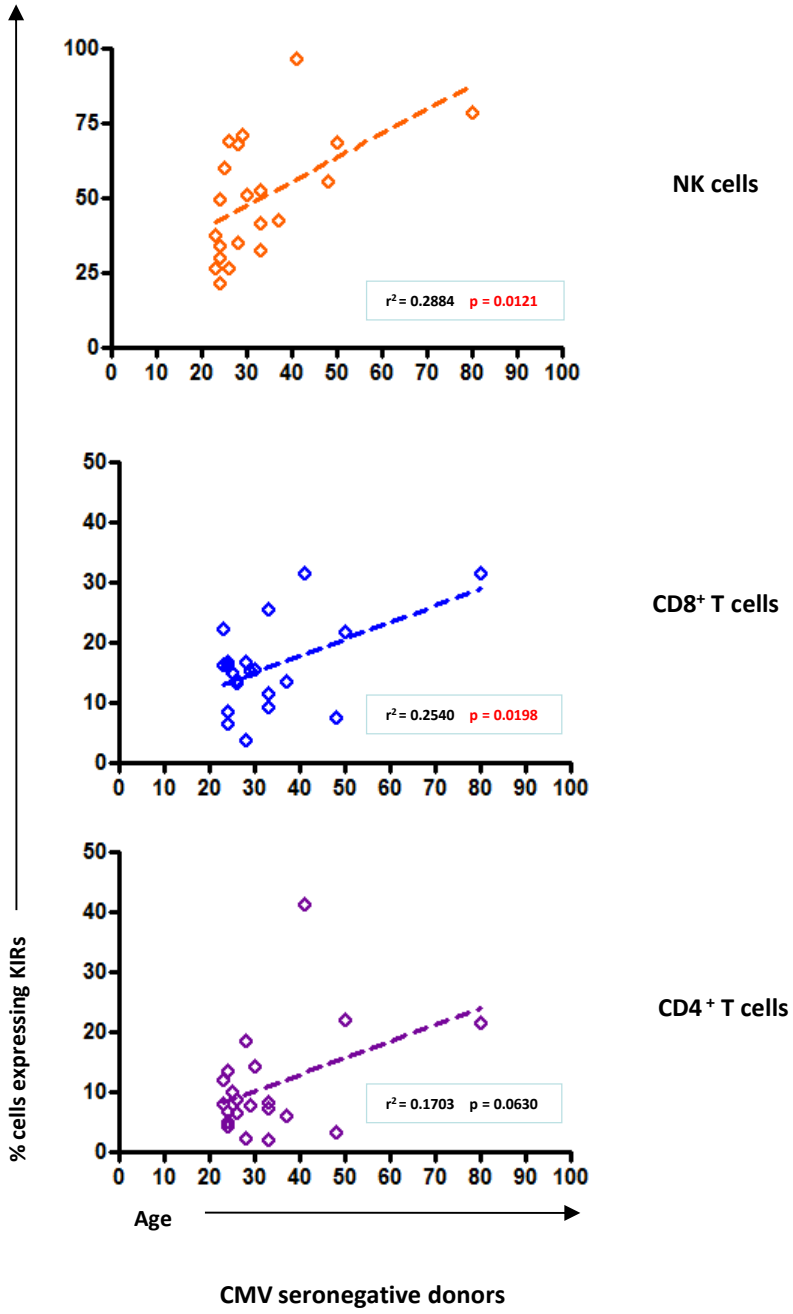


Figure 4-10: Frequency of combined KIR expression increases on NK and CD8$^+$ T cell subsets with age. The frequency of KIR$^+$ cells in each lymphocyte subset was measured using samples derived from healthy donors who were CMV seronegative. The plots above show these frequencies in relation to the donor age. Linear regression analysis was applied to each data set to test significance.

CMV seronegative donors were then analysed for the frequency of individual KIR antigen expression. Most of the trends observed when pooling all KIR Abs held true for the individual antigens (Figure 4-11). Three out of 4 antigens (CD158a, CD158b and CD158i) showed a significant increase with age on NK cells, and there were also 3 out of 4 (CD158a, CD158b and CD158e1) statistically significant correlations for $CD8^+$ T cells. No age effect on expression of individual KIR proteins was witnessed on $CD4^+$ T cells.

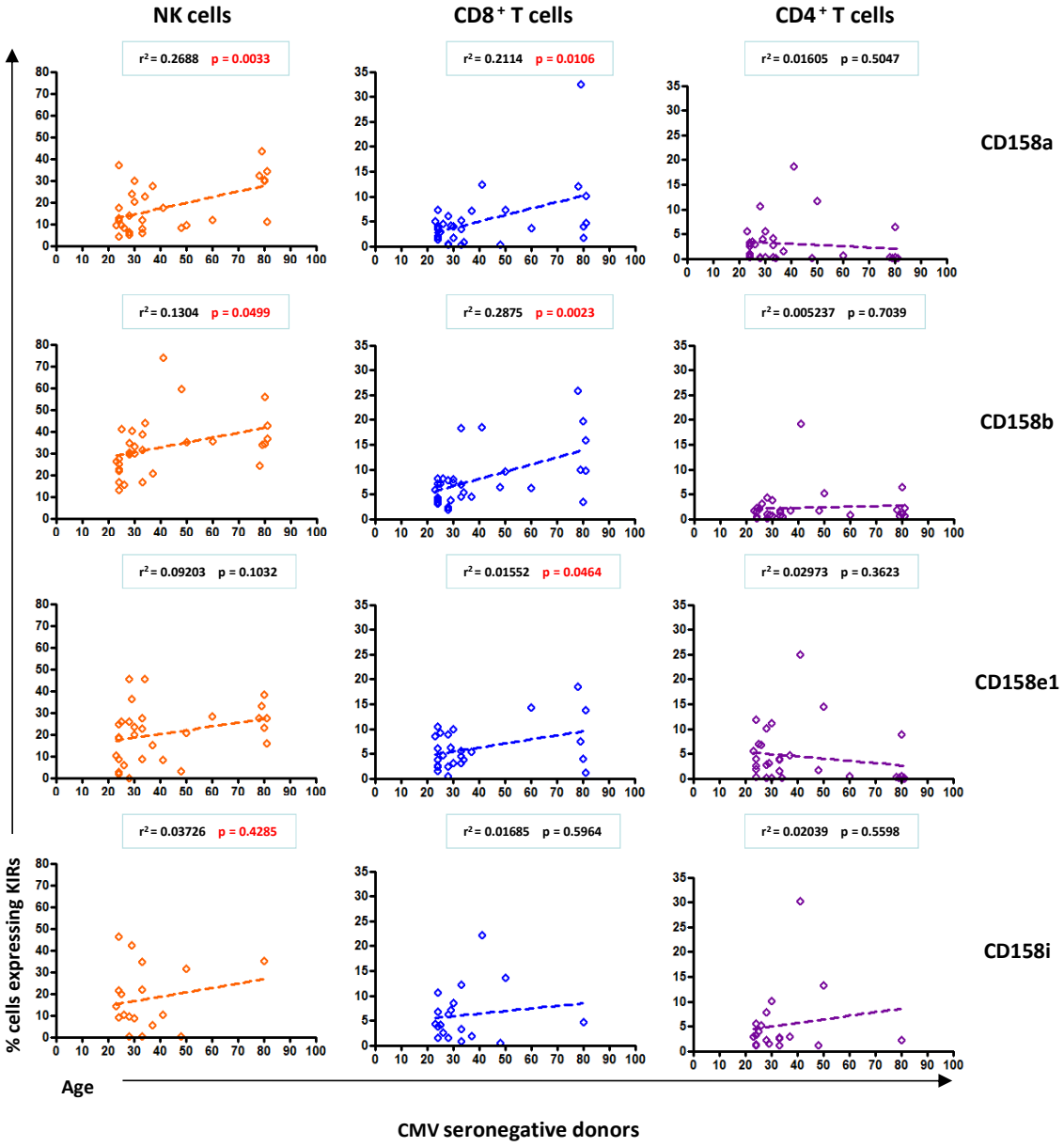


Figure 4-11: Frequency of KIR antigen expression increases on NK and CD8$^+$ T cells with age. The same seronegative donors were examined and each KIR antigen analysed individually. The percentage of each subset expressing the relevant KIR was plotted against age and linear regression analysis applied to test significance of trends.

4.5 EFFECT OF CMV ON T CELL EXPRESSION OF KIRS

Apart from NK cells, expression of some NKRs is also found on the resting cytotoxic effector cells of CMV carriers (Gamadia *et al.* 2001). In agreement with this, cross sectional cohort studies have shown that CMV infection leaves an imprint in the NKR repertoire on T cells (Guma *et al.* 2004). Whether NKRs on T cells regulate recognition and subsequent elimination of CMV-infected cells is not clear. Huard and Karlsson postulated that repeated stimulation by antigen *in vivo* would upregulate NKR expression (Huard *et al.* 2000). We therefore analyzed expression of KIR antigens on T cells (and NK cells) of healthy individuals who are CMV seropositive exposed to virus and compared the findings with seronegative donors.

Figure 4-12 shows that CMV seropositive donors have a lower frequency of KIR expressing NK cells, $CD8^+$ T cells and $CD4^+$ T cells than CMV seronegative donors. This is true for total KIR expression, and for most individual KIR antigens (apart from CD158a on NK cells and CD158b on $CD8^+$ T cells). The most striking effect is seen with CD158e1 expression, especially on NK cells (median expression of 20.8% compared with 0.03%). $CD4^+$ T cells displayed marked differences in expression of total KIR, CD158a and CD158i where the decrease in median expression is between 2-4-fold for each. Somewhat surprisingly however, not all differences proved to be statistically significant. This may be partly due to the large variation in KIR expression between individuals, and another factor to consider may be age, as the median ages in the 2 groups of donors does differ. To address the latter point further statistical analysis was undertaken.

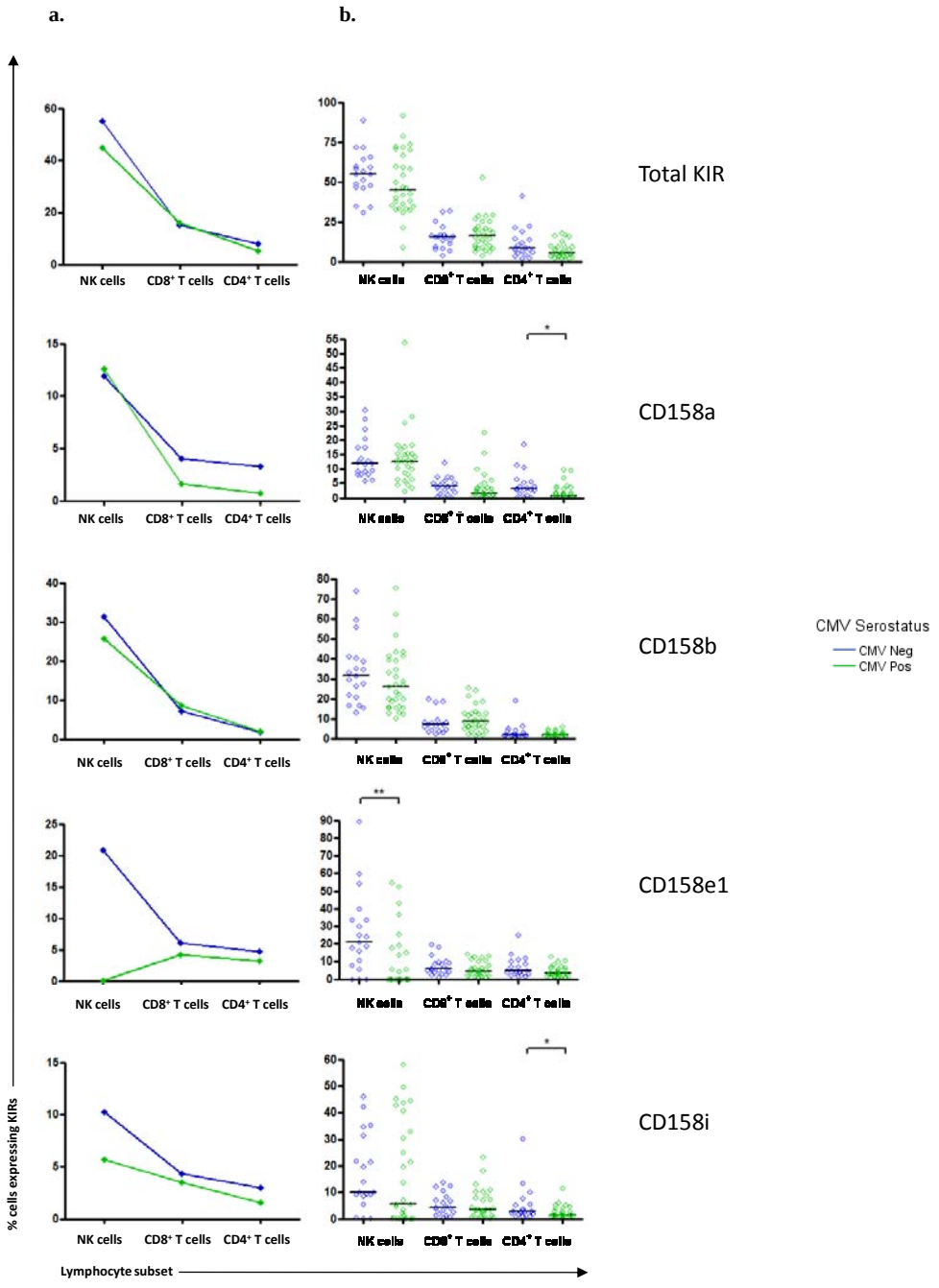


Figure 4-12: CMV infection results in lower levels of KIR expression. As described previously, total and individual KIR expression was measured on lymphocyte subsets of both CMV seronegative and seropositive donors and the frequency of expression compared. (a) Median frequencies of KIR^+ cells within each subset. (b) Comparisons between the groups were analysed using a Mann-Whitney, two-tailed t test. ** = $p < 0.01$, * = $p < 0.05$

ANOVA was utilized to further determine the association between CMV serostatus and KIR expression. A positive serostatus was associated with lower total KIR expression on $CD4^+$ T cells (p <0.05) and also reduced expression of CD158a (p <0.01), CD158e1 (p = 0.05), and CD158i (p <0.05). In addition NK cells exhibited a lower expression of CD158e1 (p <0.05). After adjustment for age, differences were still significant for CD158a and CD158i expression on $CD4^+$ T cells (p <0.05) and CD158e1 on NK cells (p <0.05).

In the previous section (4.4) the effect of age on frequency of KIR expression was only conducted on samples from CMV seronegative donors. This same analysis was conducted on CMV seropositive donors and compared to the previous results. From Figure 4-13 it can be seen clearly that the trends look completely different. Although there are few donors in the CMV seronegative cohort, statistical analysis showed that the regression analyses were significantly different implying that, when accounting for age, CMV has an effect on frequency of KIR expression. More specifically the trend lines in the CMV seropositive data suggest there is a weak negative correlation – i.e. the frequency of KIR-expressing cells is reduced with age when combined with CMV. Although these trends are not significant in themselves, they are the reverse to the effect seen in the CMV seronegative cohort, supporting the data that CMV decreases the frequency of KIR expression.

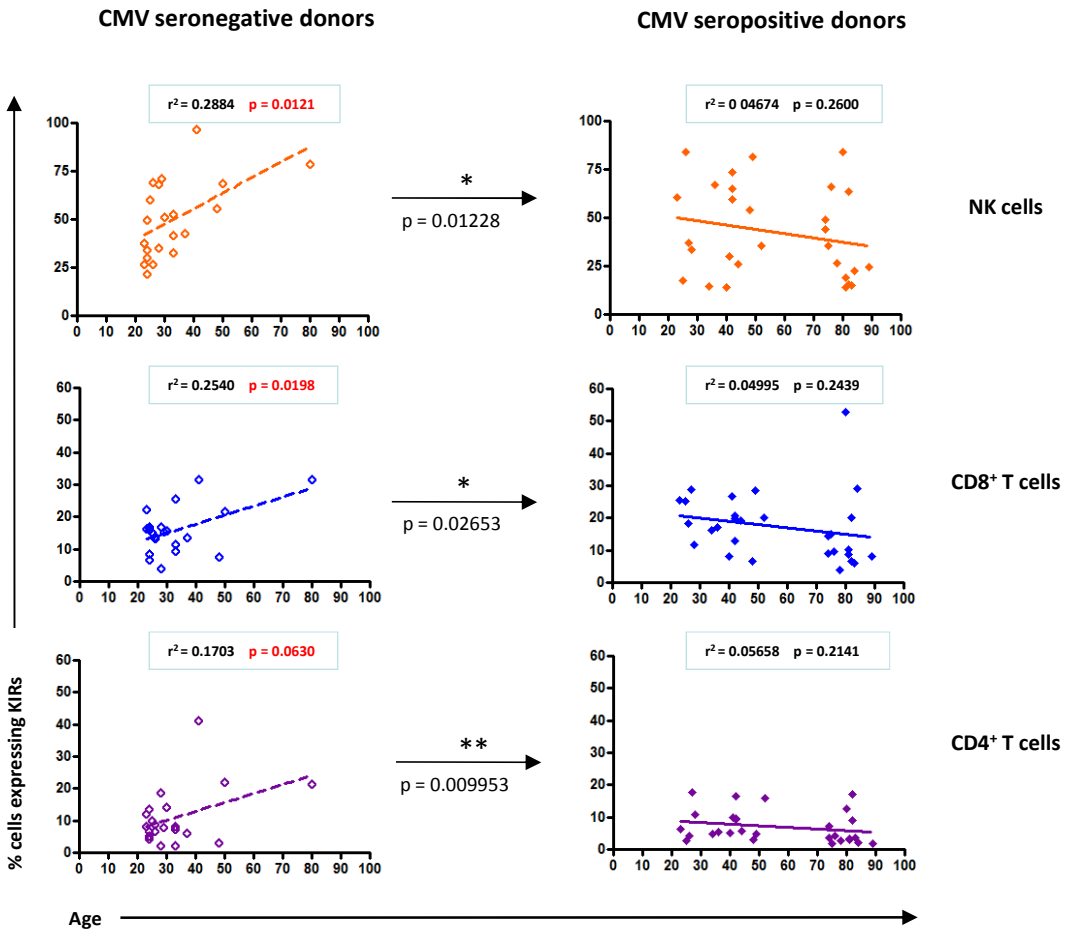


Figure 4-13: Effect of CMV on total KIR expression on lymphocyte subsets. Lymphocyte samples from healthy donors were segregated according to CMV serostatus and the frequency of KIR^+ cells in each lymphocyte subset recorded. The plots above show these frequencies in relation to the donor age. A linear regression analysis was applied to each data set. Significances of each trend were tested as well as the significance of the difference between groups.
** = $p < 0.01$, * = $p < 0.05$

Linear regression was used to test if the effects of ageing were moderated by serostatus. There indeed appeared to be a robust age-by-serostatus interaction for frequency of total KIR expression on NK cells, $CD8^+$ T cells, and $CD4^+$ T cells - all $p < 0.05$.

The same analysis was performed to investigate individual KIR antigen expression. Figures 4-14, 4-15 and 4-16 show the differences for each KIR antigen on NK cells, $CD8^+$ T cells and $CD4^+$ T cells, respectively. CD158a and CD158e1 KIR expression was observed to be significantly different on $CD8^+$ T cells between seronegative and seropositive donors. Multivariate analysis showed the pattern observed for total KIR was replicated with CD158b expression (NK cells $p < 0.01$; $CD8^+$ T cells $p < 0.05$; $CD4^+$ T cells $p < 0.005$). A significant age-by-serostatus interaction effect was also observed for CD158e1 on $CD8^+$ T cells ($p < 0.05$).

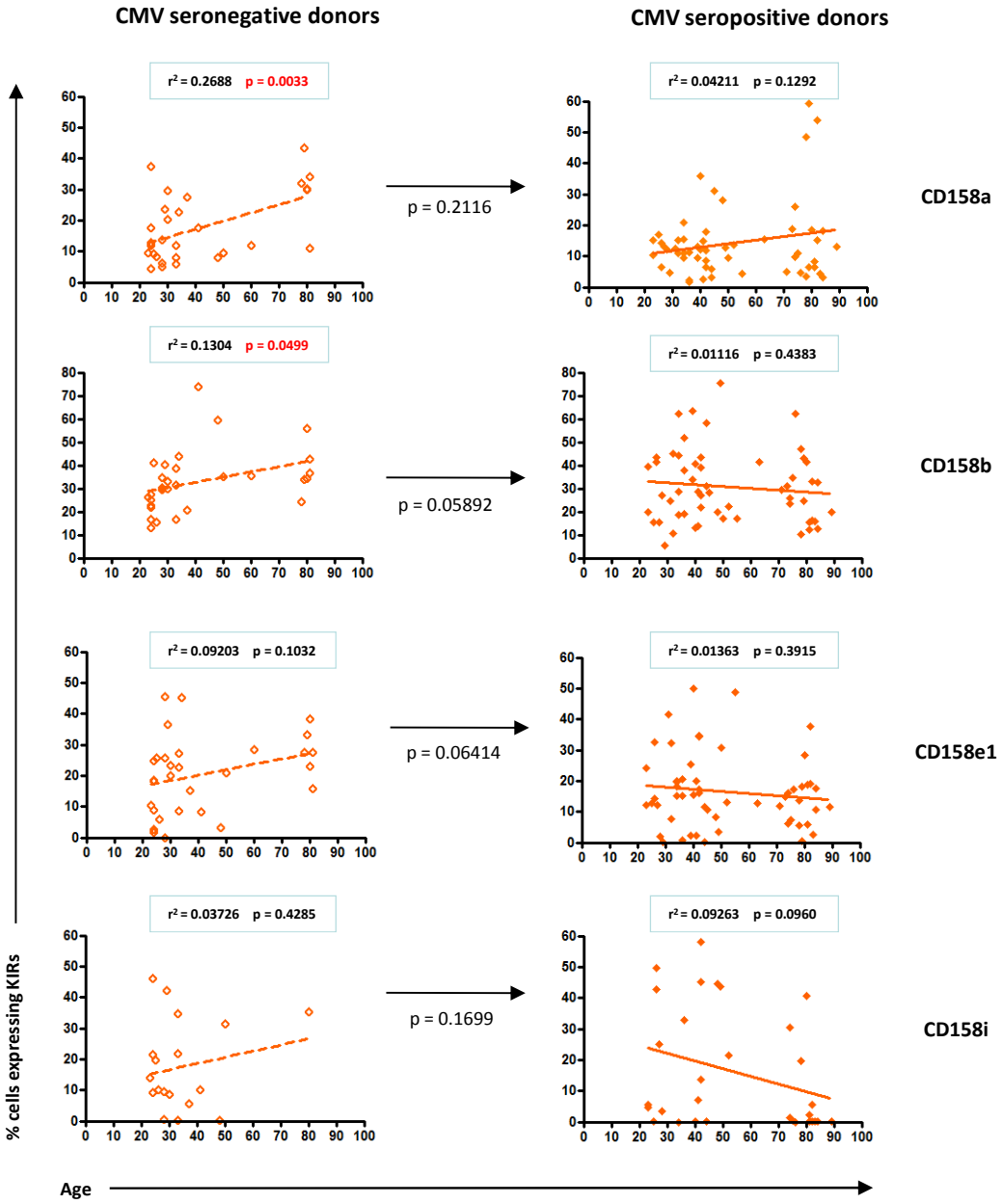


Figure 4-14: Effect of age and CMV on individual KIR expression on NK cells. Frequency of individual KIR expression was measured on NK cells of both CMV seronegative and seropositive donors. A linear regression analysis was applied to each data set. The significance of each trend was tested as well as the significance of the difference between groups.

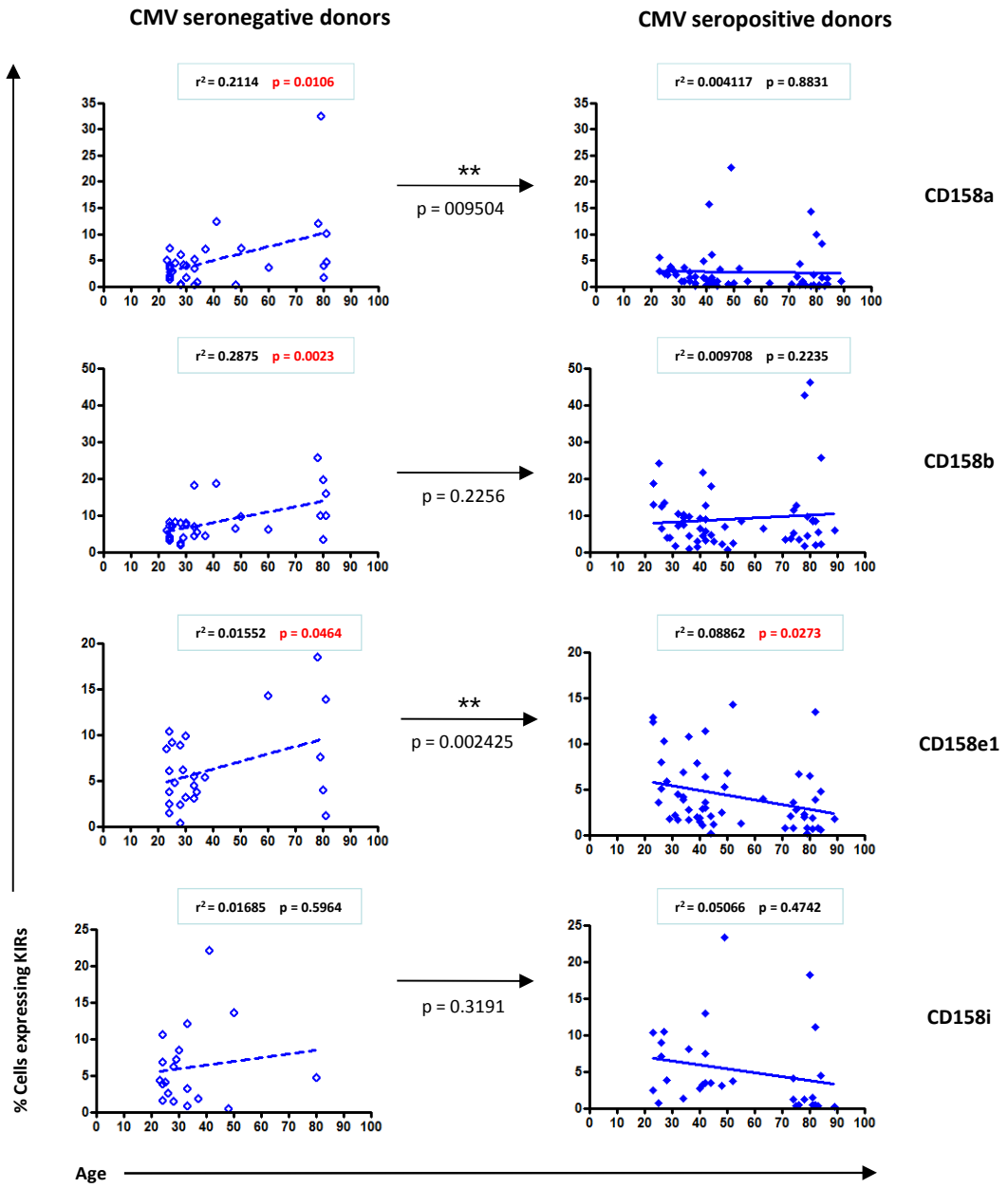


Figure 4-15: Effect of age and CMV on individual KIR expression on CD8$^+$ T cells. Frequency of individual KIR expression was measured on CD8$^+$ T cells of both CMV seronegative and seropositive donors. A linear regression analysis was applied to each data set. The significance of each trend was tested as well as the significance of the difference between groups. ** = $p < 0.01$, * = $p < 0.05$

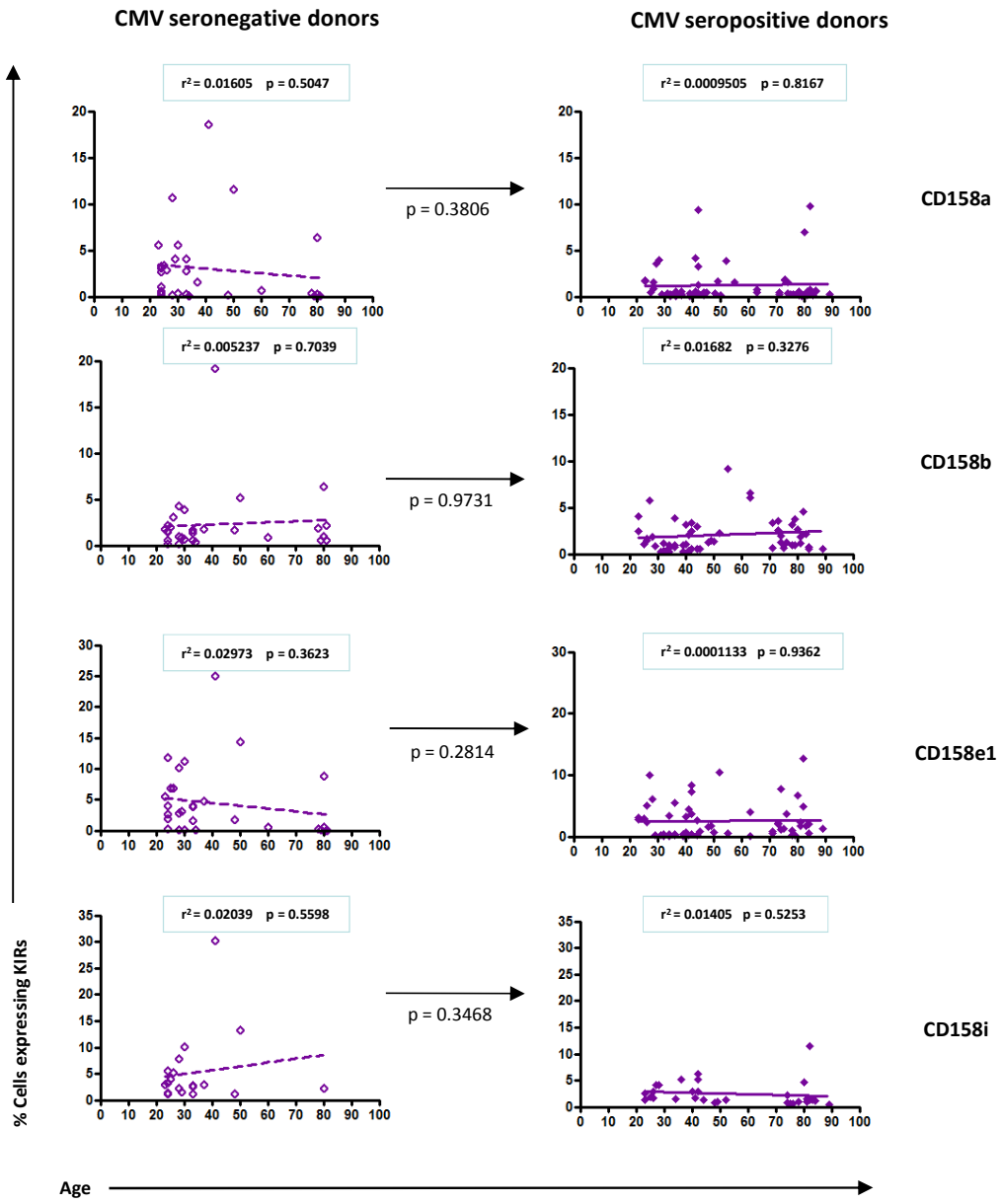
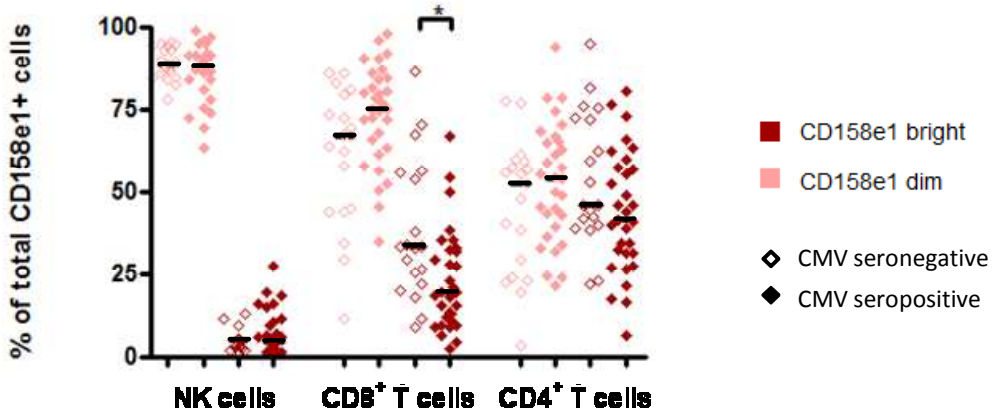


Figure 4-16: Effect of age and CMV on individual KIR expression on CD4$^+$ T cells. Frequency of individual KIR expression was measured on CD4$^+$ T cells of both CMV seronegative and seropositive donors. A linear regression analysis was applied to each data set. The significance of each trend was tested as well as the significance of the difference between groups.

In brief, individual KIR antigen expression was not significantly different between cohorts on NK cells. Both CD158b and CD158e1 nearly reached significance with the probability that trends were significantly different being 0.058 and 0.064 respectively (Figure 4-14). It appears that it is the cumulative expression that is affected (Figure 4-13, top panel). However on $CD8^+$ T cells CD158a and CD158e1 expression were significantly different (p = 0.009 and p = 0.002 respectively) between the two groups (Figure 4-15). None of the individual antigens appeared to have significantly different expression levels on CD4+ T cells when analysed according to age and CMV serostatus (Figure 4-16). Again, it is only the cumulative expression of all KIR antigens that is significantly reduced in CMV seropositive donors (Figure 4-13, bottom panel).

After determining that CMV did indeed affect KIR expression levels the previous data was reanalysed segregating donors according to CMV serostatus. In view of the observation that CMV decreased frequency of CD158e1-expressing cells, this was investigated further, and in reference to the differential staining pattern observed in Figure 4-4, the effect of CMV on specific CD158e1 allotypes being expressed was examined. Figure 4-17 shows the same data from Figure 4-4, but the donors have been separated according to CMV serostatus. Even though CMV serostatus has been shown to affect the frequency of cells expressing CD158e1, it appears to have no affect on the ratio of dim : bright allotypes on NK cells or $CD4^+$ T cells. However when analysing $CD8^+$ T cells, it can be seen that CMV seropositive donors have a higher percentage of dim staining allotypes, and less bright staining.

a.



b.

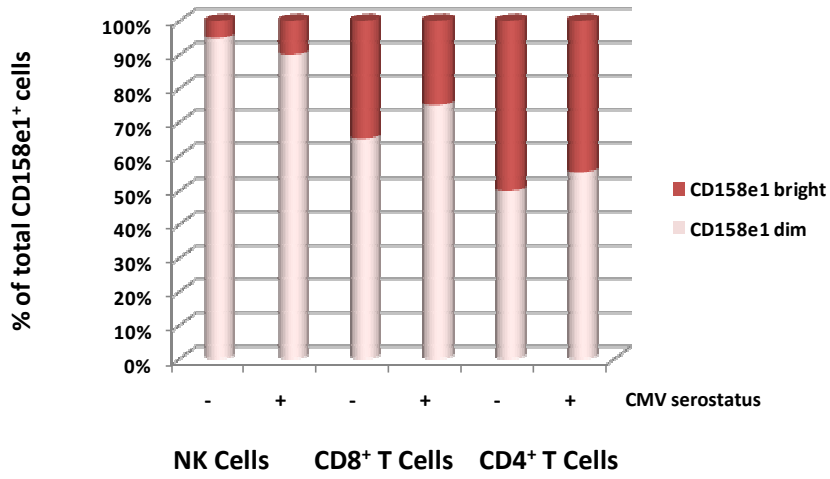
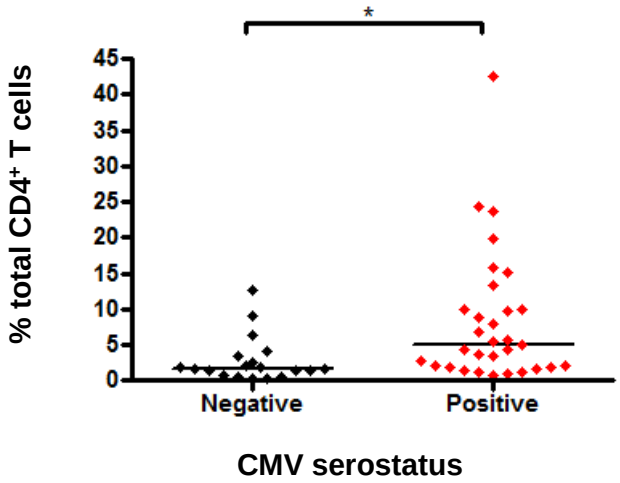


Figure 4-17: Effect of CMV on CD158e1 allotype expression on NK and T cells. CD158e1 expression frequency data was segregated according to CMV serostatus and reanalysed. Nonparametric, paired analysis (Wilcoxon signed rank test) was used to test for significant changes in allelic ratio. * = p <0.05

Section 4.2 had investigated KIR expression on memory T cell subsets. The effect of CMV on the pattern of KIR expression on T cells at different stages of differentiation was next investigated. A particular point to address here is the presence of $CD4^+$ T_{EMRA} cells. In the literature it is generally considered that the T_{EMRA} subset is lacking from $CD4^+$ T cells, however we have observed a very small subset of $CD4^+$ T_{EMRA} cells, and this is in agreement with findings of Gupta *et al.* and Sallusto *et al.* (Sallusto *et al.* 2001; Gupta 2005) although the latter author did not discuss this small population in their results. What has been alluded to is that viral infections induce a $CD4^+$ $CD45RA^+$ population (Hooper *et al.* 1999; van Leeuwen *et al.* 2004; Weinberger *et al.* 2007). For this reason we wanted to look whether there was a difference in KIR expression during T cell differentiation in CMV seronegative and seropositive donors.

The frequency of $CD4^+$ T_{EMRA} cells was measured in CMV seronegative and seropositive donors. The result, which can be seen in Figure 4-18, was that CMV seropositive donors had a larger population of $CD4^+$ T_{EMRA} cells, around a 3-fold increase versus CMV seronegative donors (4.9% and 1.6% respectively).



Negative	Positive
1.640	4.920

Figure 4-18: $CD4^+$ T_{EMRA} cells are more common in CMV seropositive donors compared to healthy donors. PBMC were stained, and following 'dump channel' exclusion, $CD3^+$ $CD4^+$ T cells were analysed. T_{EMRA} cells were identified by gating on $CCR7^-$ $CD45RA^+$ cells. Data was plotted as the percentage of total $CD4^+$ cells that are defined as T_{EMRA}. A Mann-Whitney test was used to check significance. * = p <0.05

This finding is in agreement with current literature. There have been several studies showing that antigen-experienced T cells such as virus-specific T cells revert back to expressing CD45RA (Harari *et al.* 2004).

When comparing the frequency of KIR expression on T cell memory differentiation subsets in CMV seronegative and seropositive donors a different pattern of expression is observed (Figure 4-19). The frequency of KIR^+ cells is highest in T_{CM} and T_{EMRA} populations, but remains fairly low in naïve and T_{EMRA}. In CMV seropositive donors, KIR^+ cells are increased in the T_{EMRA} population only. This pattern of KIR expression being maintained on a steady proportion of cells throughout early stages of differentiation and then showing a marked increase in the T_{EMRA} population mirrors the KIR expression pattern in the $CD4^+$ subset.

When considering the $CD4^+$ T cell population it can be seen that the overall pattern of KIR expression is similar in CMV negative and CMV seropositive donors when comparing naïve, T_{CM} and T_{EM} populations. However, the increased frequency of KIR expression on T_{EMRA} cells is much more pronounced on the CMV seronegative cohort. It is also worth noting that naïve and T_{CM} populations as well as the T_{EMRA} more frequently express KIRs in the seronegative donors when compared to the equivalent populations in CMV seropositive donors.

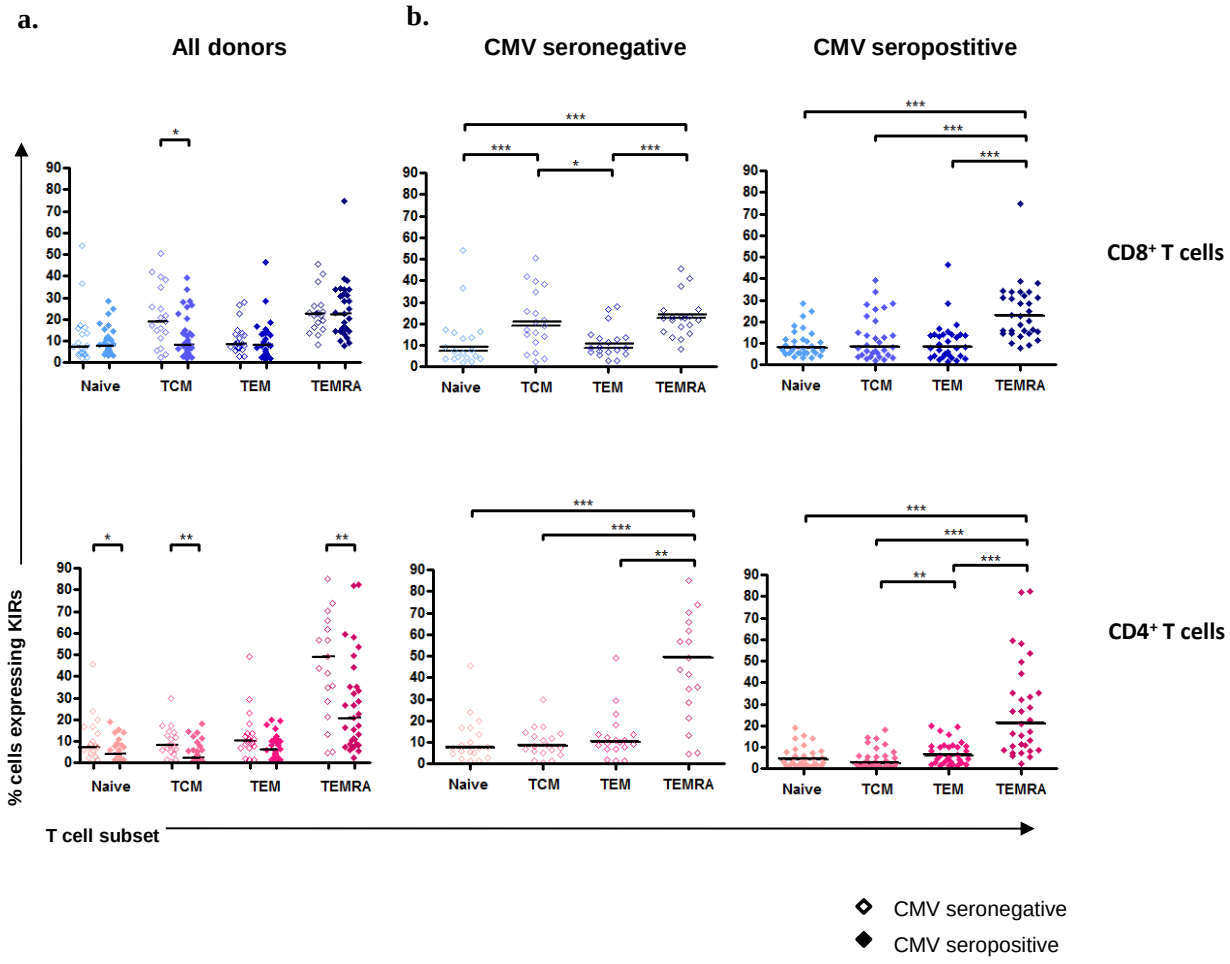


Figure 4-19: T cell differentiation is associated with an increase in total KIR positivity in both CMV seronegative and seropositive donors. Donors were segregated according to CMV serostatus and the frequency of KIR expression measured on $CD8^+$ and $CD4^+$ T cell memory subsets in both cohorts. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. Plotted values are the percentage of KIR^+ cells within each subset. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

This analysis was repeated looking at each individual KIR antigen. Similar results were seen as for total KIR expression on $CD8^+$ lymphocytes (Figure 4-20). CMV seronegative donors more frequently expressed KIR than CMV seropositive donors. This was especially pronounced in the T_{CM} population. The frequency of KIR^+ cells is increased in T_{EMRA} populations of both cohorts of donors. Interestingly CD158b was found to be more frequently expressed on T_{EMRA} cells in CMV seropositive donors when compared to the equivalent population from CMV seronegative donors.

When looking at the frequency of individual KIR expression on $CD4^+$ T cells (Figure 4-21), the patterns resemble that seen when measuring total KIR expression – frequencies of KIR^+ cells gradually increase (although remain low) until cells become highly differentiated. In all cases the T_{EMRA} population more frequently expresses KIR molecules. This is true for both CMV seronegative and seropositive donors. However, KIRs are less frequently expressed by $CD4^+$ T cells in CMV seropositive donors. This is true for all 4 antigens tested on each memory subset.

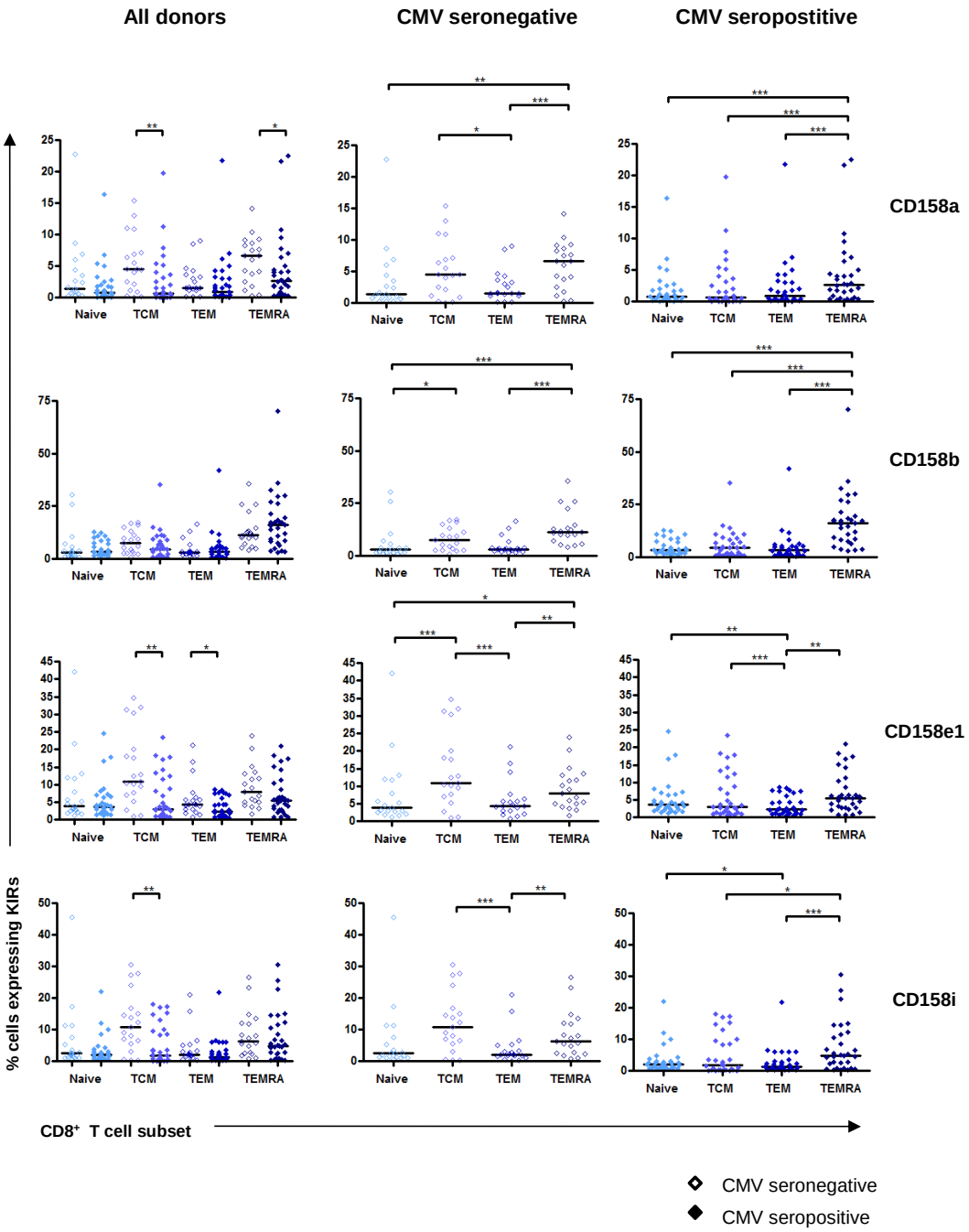


Figure 4-20: CD8+ T cell differentiation is associated with an increase in frequency of all individual KIRs in both CMV seronegative and seropositive donors. Individual KIR expression was measured on $CD8^+$ T cell memory subsets in CMV seronegative and seropositive donors. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. Plotted values are the percentage of KIR^+ cells within each subset. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

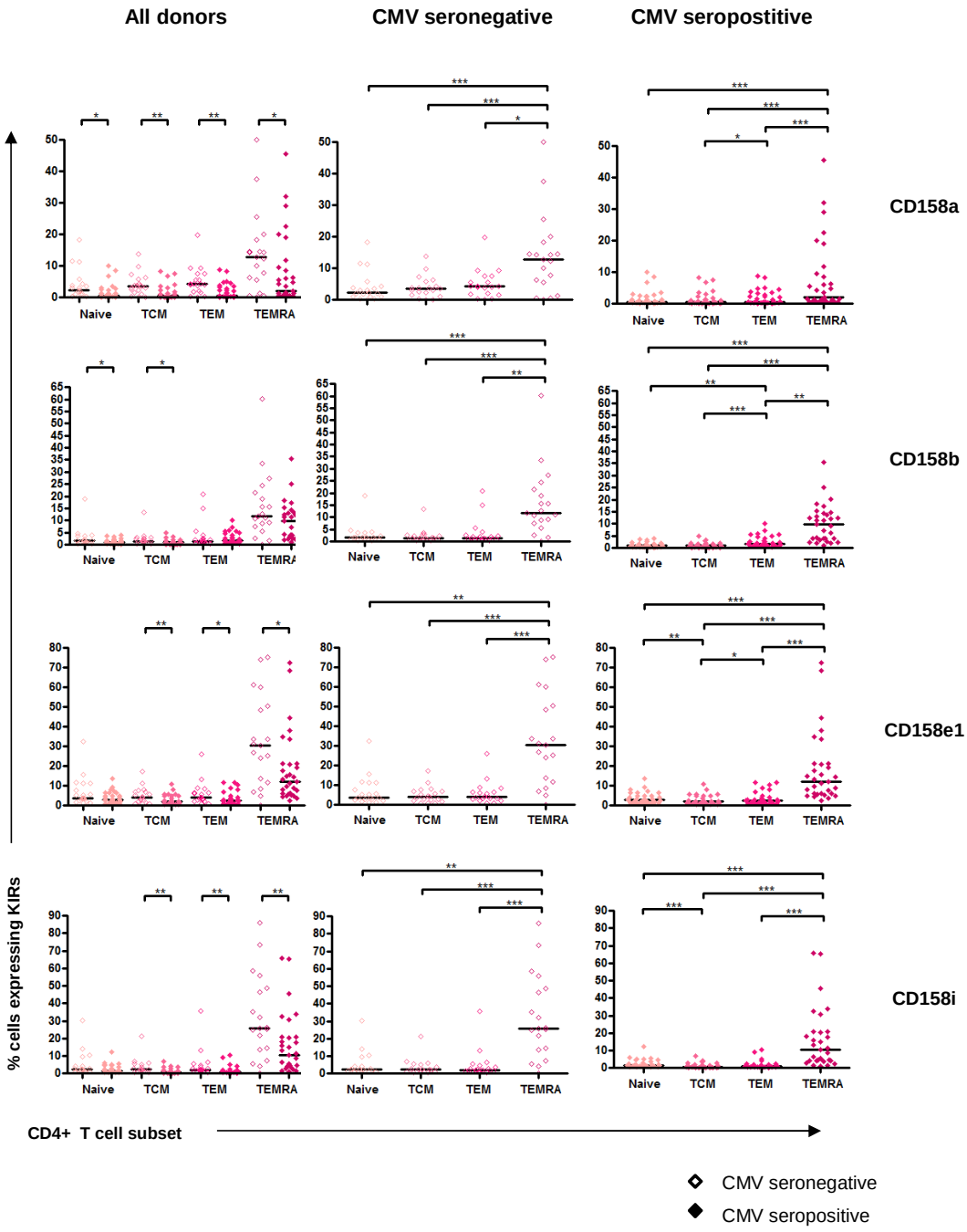


Figure 4-21: CD4$^+$ T cell differentiation is associated with an increase in frequency of all individual KIRs in both CMV seronegative and seropositive donors. Individual KIR expression was measured on CD4$^+$ T cell memory subsets in CMV seronegative and seropositive donors. Dead cells, monocytes and B lymphocytes were excluded using a 'dump channel'. Plotted values are the percentage of KIR$^+$ cells within each subset. A Dunn's Multiple Comparison test was carried out to determine whether the median values are significantly different. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

Finally, KIR co-expression on T cell subsets was compared between CMV seronegative and seropositive donors. Figures 4-22 and 4-23 show the results for $CD8^+$ and $CD4^+$ T cells, respectively. It can be seen clearly that CMV seropositive donors have a more limited KIR repertoire than seronegative donors. This was true for both $CD8^+$ and $CD4^+$ T cells, and within each memory subset. CMV seropositive donors can be seen to have a higher percentage of KIR^+ cells staining for 1 Ab and 1 or 2 Abs than seronegative donors.

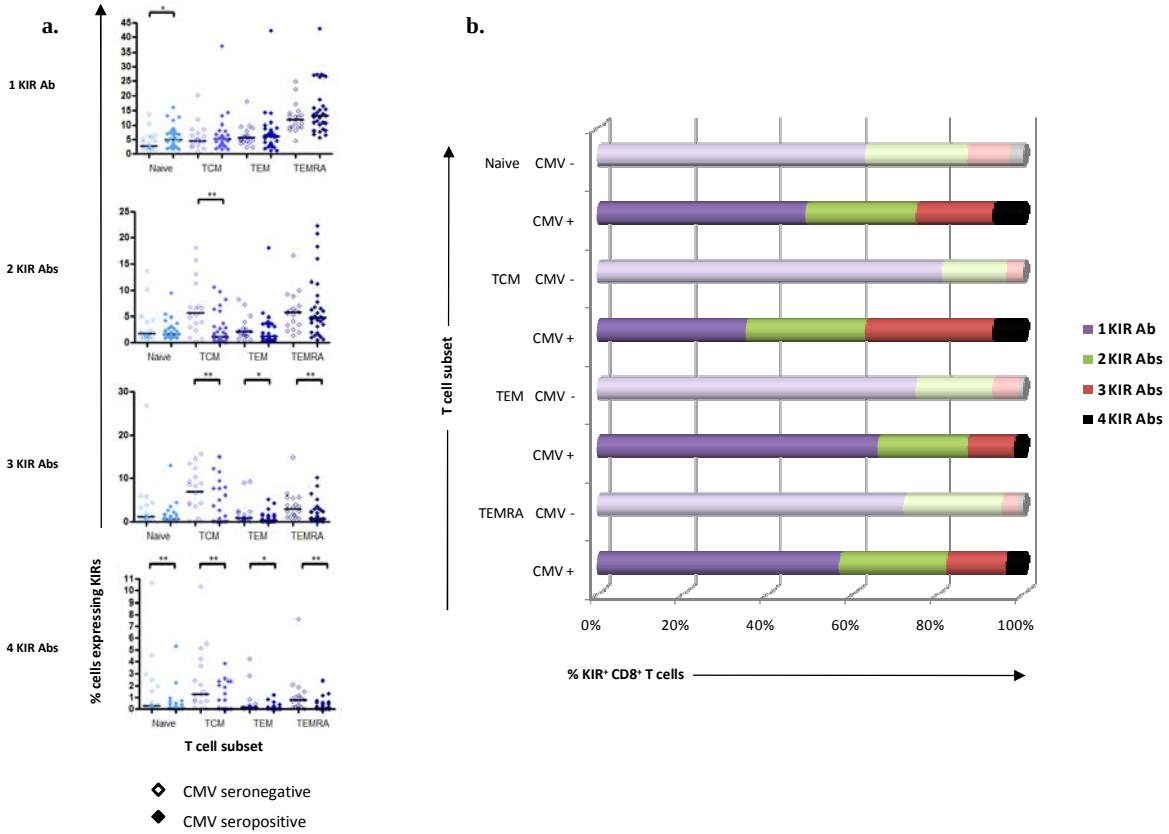


Figure 4-22: CMV seropositive donors have a more focussed CD8$^+$ T cell repertoire than seronegative donors. KIR co-expression was measured on CD8$^+$ T cell memory subsets. Cells were grouped according to the number of KIR Abs they stained positive for and results segregated into CMV$^+$ and CMV$^-$. (a) Plotted values are the percentage of KIR$^+$ cells within each T cell subset. One-way ANOVA analysis was carried out to test for significant differences between cohorts. (b) Plotted values show the proportion of KIR$^+$ cells that fall into each group.
*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

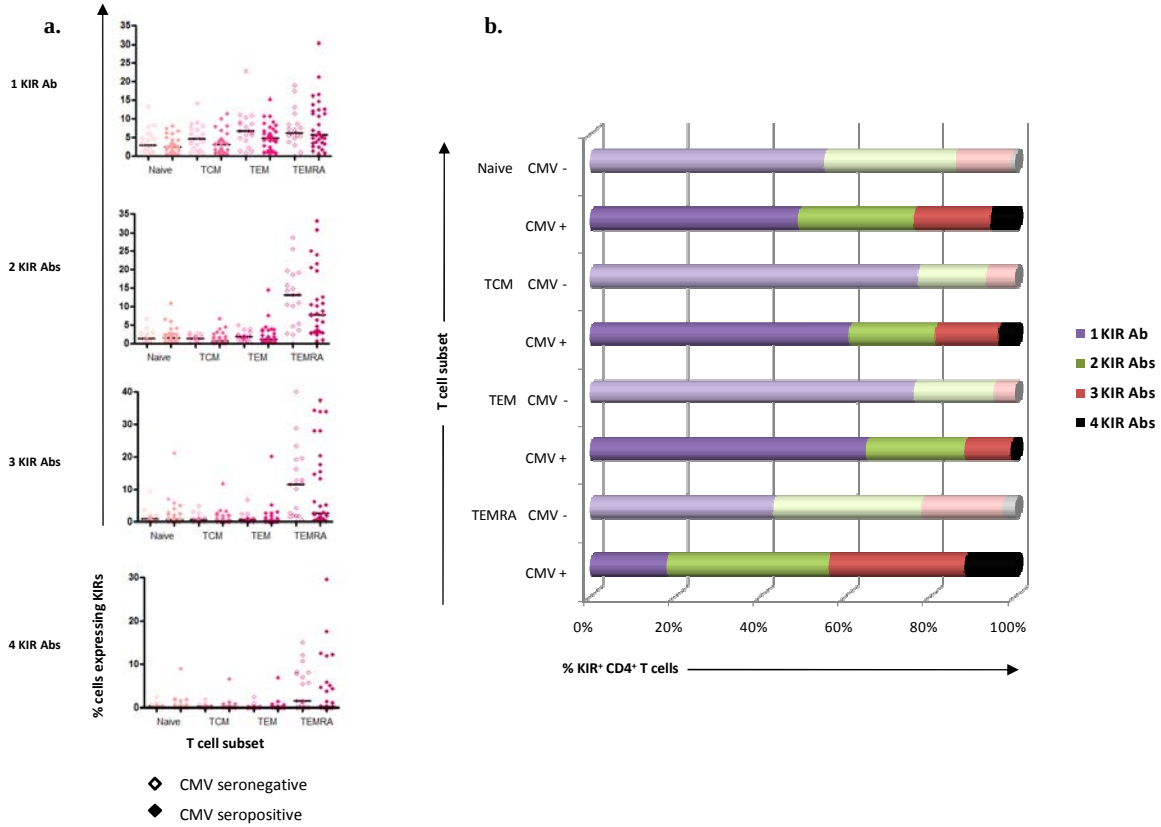


Figure 4-23: CMV seropositive donors have a more focussed CD4$^+$ T cell repertoire than seronegative donors. KIR co-expression was measured on CD4$^+$ T cell memory subsets. Cells were grouped according to the number of KIR Abs they stained positive for and results segregated into CMV$^+$ and CMV$^-$. (a) Plotted values are the percentage of KIR$^+$ cells within each T cell subset. One-way ANOVA analysis was carried out to test for significant differences between cohorts. (b) Plotted values show the proportion of KIR$^+$ cells that fall into each group.
*** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

4.6 DISCUSSION

Polychromatic flow cytometry is proving to be a powerful tool for examining changes in immune parameters. This study is the most comprehensive work looking at KIR expression on T cells to date. It has begun to answer some questions, but at the same time has initiated many more. All of the analyses performed compared the frequency of KIR^+ cells in each T cell subset, and it could be argued that recording absolute number of cells would be informative and possibly more robust as sizes of the memory T cell compartments differ greatly.

Firstly, KIR expression on the main lymphocyte subsets was examined and NK cells, NKT-like cells, $CD8^+$ T cells, γδ T cells and $CD4^+$ T cells were found to express KIR (in order of highest to lowest frequency). Few if any B cells were found to express KIR. This study then focussed on NK and T cells, and looked at total and individual KIR expression on these cells. All KIRs were expressed on these lymphocyte subsets.

One interesting point to note is the frequency of $CD4^+$ T cells expressing KIR within this cohort of donors. Significantly higher proportions of $CD4^+$ T cells expressed KIRs relative to that reported previously (median of 6.7% compared with reports of typically 1%. The latter figure is what is seen from each antibody independently in this study). One explanation could be the combination of antibodies in the KIR 'antibody cocktail' used in the present study. No previous studies have included α-CD158i Ab (which has a median expression of 2.2%).

α-CD158e1 Ab was observed to stain cells at two different intensities, and it was previously reported that these correlated to two subgroups of KIR3DL1 allotypes (Gardiner *et al.* 2001). Interestingly these allotypes were not expressed at the same ratio on each cell type. NK cells almost exclusively express the dim binding allotypes, whereas a much larger proportion of the $CD158e1^{+}$ T cells expressed the bright staining allotypes (CD4 > CD8). These allotypes are also functionally different - those that bind the DX9 Ab with high affinity and result in a bright staining population have also been shown to bind their HLA-Bw4 ligand with higher affinity and result in a stronger inhibitory signal being sent to the cell (Carr *et al.* 2005). One explanation for the observed differences in expression levels could be that this controls the balance of signals that the cell receives. NK cells express high frequencies of KIRs and it is therefore plausible that they do not require such a high affinity for their ligand. T cells however express much lower levels of KIRs and having a larger proportion of these being high affinity may be advantageous to fine tune the overall signalling to the cell. Another interesting point is that there is a third subgroup of 3DL1 alleles – 3DL1*004. This allele does not stain with DX9 Ab and does not function – it is sequestered in the cell. 3DL1*004 is present at a frequency of ~20% in the Caucasian population and thus this allele may have been the target for natural selection. One possibility is that it performs its function within the cell, in contrast to other KIR3DL1 allotypes. Alternatively, 3DL1*004 could represent an inactivated form of a KIR3DL1 allotype that was expressed at the cell surface and became the target for subversion by a viral ligand (Arase *et al.* 2002). By this means, the virus could have inhibited the antiviral NK cell response. In this circumstance, there would be competitive advantage to a variant allele whose protein was sequestered inside the cell and unable to interact with a viral protein expressed on the surface of infected cells.

KIR expression on T cells varies according to the stage of differentiation. Levels are highest in the most differentiated T_{EMRA} population, but all memory subsets do express KIR. This finding goes against published studies by Abedin *et al.* and Vallejo that suggest KIRs are only expressed on senescent or pre-senescent memory T cells (Abedin *et al.* 2005; Vallejo 2006). This difference in these results may be explained by several possibilities. Neither CMV serostatus or age of donors is stated in these studies. Also not all four KIR antibodies used in this study were used in previous studies e.g Arlettaz *et al* used only GL183 Ab (Arlettaz *et al.* 2004). In this study the pattern of expression is the same for total KIR and individual KIR antigens, but differs between $CD8^+$ and $CD4^+$ T cells. As well as the frequency of cells expressing KIRs within each subset, co-expression of different KIR molecules was also investigated. Different memory subsets have varying degrees of receptor diversity. This may possibly be related to the KIR frequency in some cases – as the number of KIRs increases the repertoire contracts and becomes more focussed. This may be a mechanism of achieving a balance of signals.

The KIR repertoire may contract as $CD8^+$ T cells differentiate to compensate for the increase in the frequency of cells expressing KIR or vice versa. KIR expression appears to be extremely complex and there are a lot of mechanisms in place to carefully maintain the balance of signalling. This may be another of these mechanisms. Control of KIR expression appears to be heavily regulated with each lymphocyte subset being controlled independently. Many different factors are believed to control expression – in fact Trowsdale *et al.* have speculated that KIR expression is likely to involve up to 15 different transcription promoter sequences which are located within 500 bp and 5' to the initiation codon (Trowsdale *et al.* 2001). Other mechanisms controlling KIR levels have been suggested, including hormonal

and cytokine regulation of expression (Ponte *et al.* 1999; Mingari *et al.* 2000). Despite the many potential factors contributing to altered expression, levels of KIR appear stable year to year (Shilling *et al.* 2002).

Because the frequency of KIR expression on $CD4^+$ T cells is low, it is plausible that naïve cells express as diverse a range as possible to maximise potential interactions. As T cells differentiate the repertoire contracts and a steady balance is maintained. The T_{EMRA} population are most diverse. As T_{EMRA} cells are rare, again it could be down to the balance between frequency of expression and diversity. Another possibility could be that there is a viral link – as $CD4^+$ T_{EMRA} cells are increased with CMV infection it could be postulated that CMV is driving the upregulation of multiple KIRs on these cells.

Finally the effect of CMV on KIR expression was examined. It was found that CMV seropositive donors have lower numbers of KIR^+ cells in NK cell, $CD8^+$ and $CD4^+$ T cell populations. It also seems that the KIR repertoire in these donors is more focussed. This could be an indication that CMV is shaping the KIR repertoire (in the same way it does other NK receptors (Guma *et al.* 2004). An interesting example of this can be seen when looking at CD158e1 expression. This KIR receptor is downregulated in CMV seropositive donors (median value of 20.8% in seronegative donors vs. 0.03% in seropositive donors on NK cells). Furthermore in CMV seropositive donors, the proportion of $CD8^+$ T cells expressing bright-staining allotypes is decreased. CMV appears to be dampening down the inhibitory KIR signal, this time not in number of receptors being expressed, but in their binding affinity.

It may be plausible that CMV is targeting this receptor in some way to evade the immune system. It would be interesting to investigate this further and determine CD158e1 allotype expression on CMV-specific T cells.

Like many other NKRs, age was found to affect the frequency of KIR-expressing cells in that an increased number of NK and $CD8^+$ T cells were found to express these receptors. This effect was only seen in the CMV seronegative cohort as the opposing action of CMV masked it in the seropositive donors. Not only is the frequency of KIR^+ T cells reduced in CMV seropositive donors, but the receptor diversity is also more limited. KIR^+ T cells in CMV seropositive donors were found to include a higher proportion of cells expressing only 1 or 2 KIR types when compared to CMV seronegative donors. One possibility is that they are not required. CMV may drive KIR expression and it is possible that CMV could select for a specific KIR phenotype. This would be a potential explanation for the high percentage of KIR^+ cells only staining with 1 KIR antibody. Another explanation could be that this is an immune response 'counter measure' to CMV and in fact the change in KIR expression is not itself CMV-driven, but an effect of preferential selection of those cells doing the job.

The following figures pictorially show a summary of the effect of T cell differentiation and CMV on KIR expression (both frequencies, and co-expression). Figures 4-24 and 4-25 illustrate $CD8^+$ T cells and $CD4^+$ T cells, respectively. The size of each pie chart represents the frequency of KIR^+ cells in each memory subset, and the segments within each pie chart illustrate the KIR diversity of those KIR^+ cells. Changes in the $CD8^+$ T cell population appear to be more subtle on both counts. In contrast the changes within the $CD4^+$ T cell

compartment are startling. It is clear that as KIR^+ cells within the $CD4^+$ T cell population increase the repertoire gets more diverse. This seems to go against the idea of fine tuning a constant balance. Is this a last chance attempt to engage with a ligand and send a needed signal to the cell? Highly differentiated cells may need more KIRs to regulate effector function. If we could be sure it were inhibitory KIRs that were being measured it could also be postulated that KIRs are protecting the cells from exhaustion and conserving telomeres.

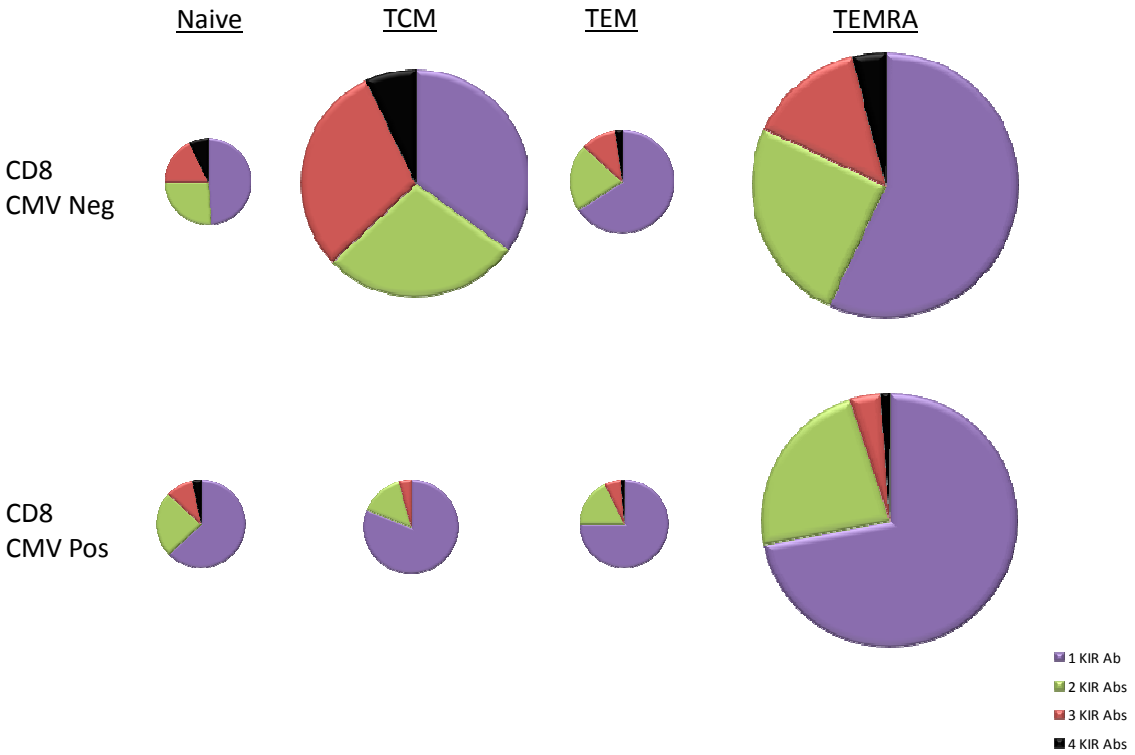


Figure 4-24: Summary of the effect of CMV and T cell differentiation on KIR expression on CD8$^+$ T cells. Size of the pie charts represents the frequency of KIR-expressing cells within that subset. The area within the pie chart represents KIR co-expression data.

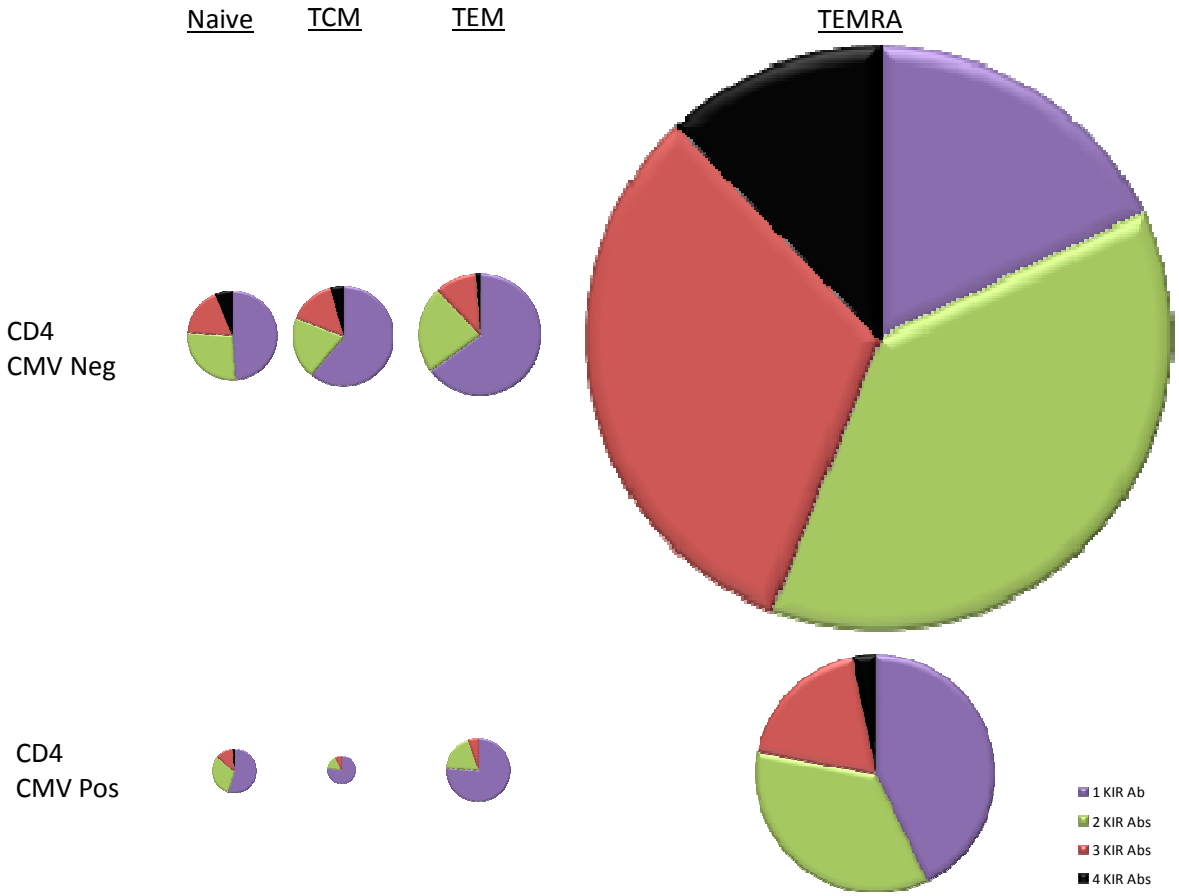


Figure 4-25: Summary of the effect of CMV and T cell differentiation on KIR expression on $CD4^+$ T cells. Size of the pie charts represents the frequency of KIR-expressing cells within that subset. The area within the pie chart represents KIR co-expression data.

When presenting the summary above, the frequency of KIR^+ $CD4^+$ cells in the CMV negative cohort was striking. As this is a very small population within these donors I decided to also present this data taking the population size of each subset into account. Figure 4-26 therefore shows the adjusted data according to population size of each memory subset. This gives a clearer picture of the actual number of KIR molecules that are present. It can be seen that CMV seropositive donors have lower numbers of KIR^+ T cells in naïve, T_{CM} and T_{EM} populations of $CD8^+$ and $CD4^+$ T cells. When looking at the T_{EMRA} populations it can be seen that there is no difference in the number of KIR^+ cells between CMV seropositive and CMV seronegative donors. This again reiterates the theory that a balance of KIR signalling needs to be maintained.

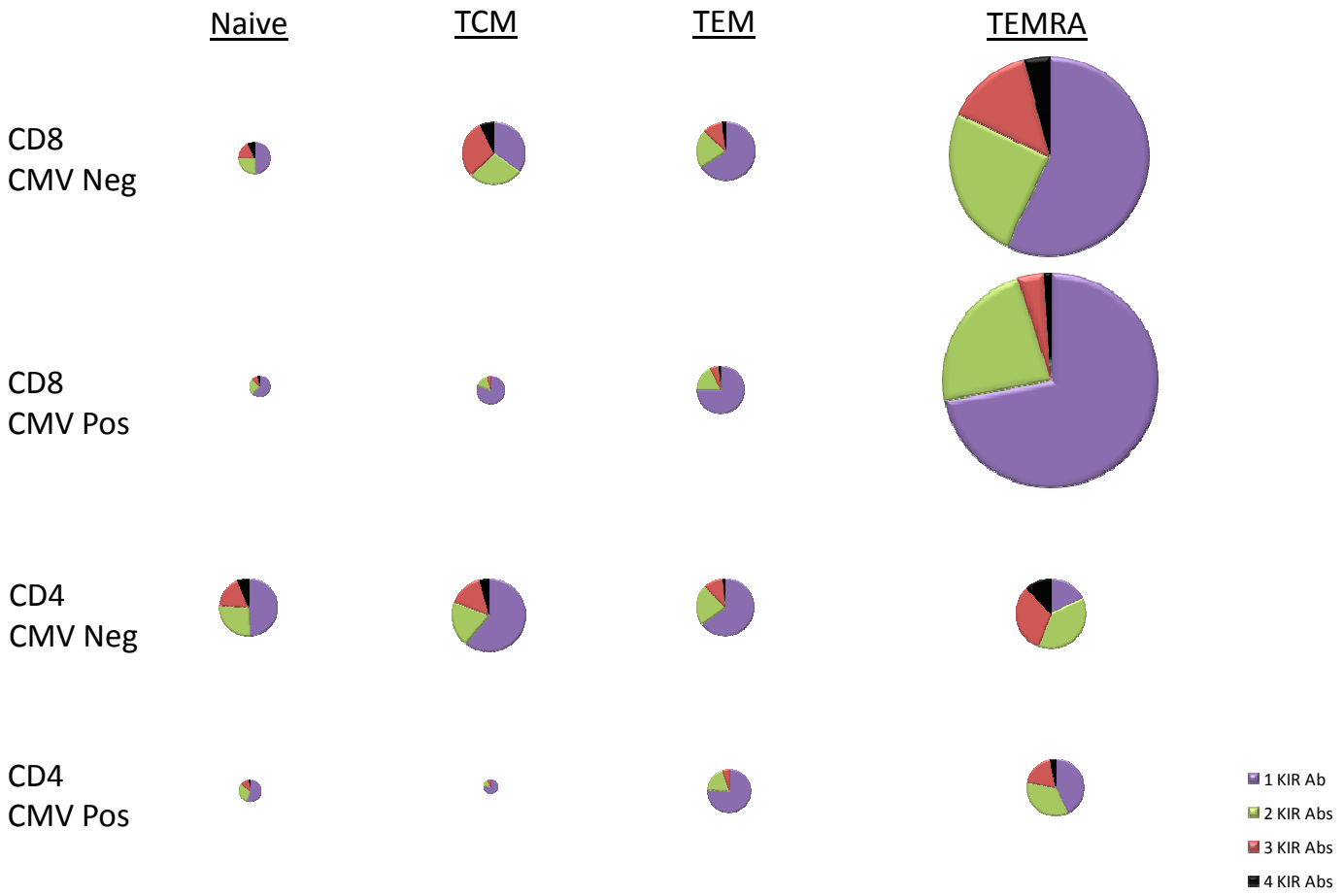


Figure 4-26: Summary of Figures 4-24 and 4-25 adjusted for population size of T cell memory subset. Size of the pie charts represents the frequency of KIR-expressing cells within that subset. Charts have been scaled in size according to the number of cells in each T cell subset. The area within the pie chart represents KIR co-expression data.

Chapter 5

Investigating the function of KIRs on T cells

The previous chapter summarised a fairly comprehensive study of KIR expression on lymphocytes in healthy donors. Of particular interest was the finding that CMV seropositve donors have lower levels of KIR expression on NK and T cells. This went against our initial hypothesis and so KIR expression in CMV seropositive donors was further investigated. Although overall KIR expression was reduced in the total T cell pool of these donors, it was unknown what the KIR repertoire would be on the CMV-specific T cell population.

Existing data analysing the correlation between KIR expression and CMV-specificity is scarce. In 2001, van Lier's group demonstrated that CMV-specific $CD8^+$ T cells were predominantly KIR negative. Furthermore, within the CMV-specific population there was a lower frequency of KIR^+ cells than within the total $CD8^+$ T cell pool (Gamadia *et al.* 2001). However, this data was limited to T cell responses against a single tetramer containing a pp65 derived peptide (NLVPMVATV) in only 7 donors. A second study in 2004 examined renal transplant patients that were immunosuppressed and developed CMV infection. This showed

representative data from 2, 32 and 37 weeks post-transplant illustrating again that there is a lower frequency of KIR-expressing cells in the tetramer positive $CD8^+$ T cells than within the total $CD8^+$ T cell pool (Anfossi *et al.* 2004). Again this study looked at a limited cohort of donors and used the same single tetramer to detect CMV-specific $CD8^+$ T cells. Thus the pattern, and functional significance, of KIR expression on CMV-specific T cells remains largely unresolved.

Therefore the aim of the work in this chapter was to investigate KIR expression on CMV-specific T cells in more detail within our healthy cohort of donors. Firstly, KIR expression was characterised on CMV-specific T cells and then attempts were made to determine the functional significance of this. Several groups have used rheumatoid arthritis (RA) patients to isolate $CD4^+$ $CD28^-$ T cells, a highly oligoclonal, largely CMV-specific, subset of T cells that is expanded this disease (Namekawa *et al.* 2000; Snyder *et al.* 2003; Snyder *et al.* 2004; van Bergen *et al.* 2009). CMV-specific $CD4^+$ T cells are $CD28^-$ and I therefore chose to attempt to clone these cells and investigate KIR function in this setting.

5.1 KIR EXPRESSION ON CMV-SPECIFIC T CELLS

From the original cohort of donors, 31 were CMV seropositive. To add to current work both $CD8^+$ and $CD4^+$ T cells were included in this study. Initial experiments compared total KIR expression on CMV-specific $CD8^+$ and $CD4^+$ T cells with KIR expression on the total $CD4^+$ and $CD8^+$ pool. To identify $CD8^+$ and $CD4^+$ CMV-specific T cells, PBMCs were stimulated with a combined pool of peptide mixes made up of a selection of peptides from the CMV proteins IE-1, pp65, pp50, gB and gH (see Table 2-2). This stimulus was chosen to better represent the whole CMV genome, rather than sole responses to a single pp65 peptide, and also cover responses to a range of HLA-types. The use of a peptide pool was also preferable to use of CMV lysate as all PBMC samples had been frozen to keep results consistent. We find that the T cell response is markedly dampened when lysate is used on frozen cells due to the lack of APCs to process it, but the response to peptide remains intact.

PBMC from the CMV seropositive donors were stimulated for 6 hours with the CMV peptide mix in the presence of Brefeldin A. This had previously been shown in our lab to be the optimal incubation period (Dr. Laura Crompton, personal communication). Following stimulation, cells were stained for surface markers, and after fixing and permeabilisation intracellular cytokine was stained using an IFNγ-specific mAb to identify peptide-reactive cells. The gating strategy for analysis is shown in Figure 5-1. Firstly, lymphocytes were identified by plotting cell size against granularity, and then dead cells, monocytes and B cells excluded from the analysis using the 'dump channel'. $CD8^+$ and $CD4^+$ T cells were gated on and then cells were divided according to IFNγ production. Those cells producing IFNγ in response to peptide stimulus were classed as CMV-specific and KIR expression was compared on this population and the control IFNγ-negative subset.

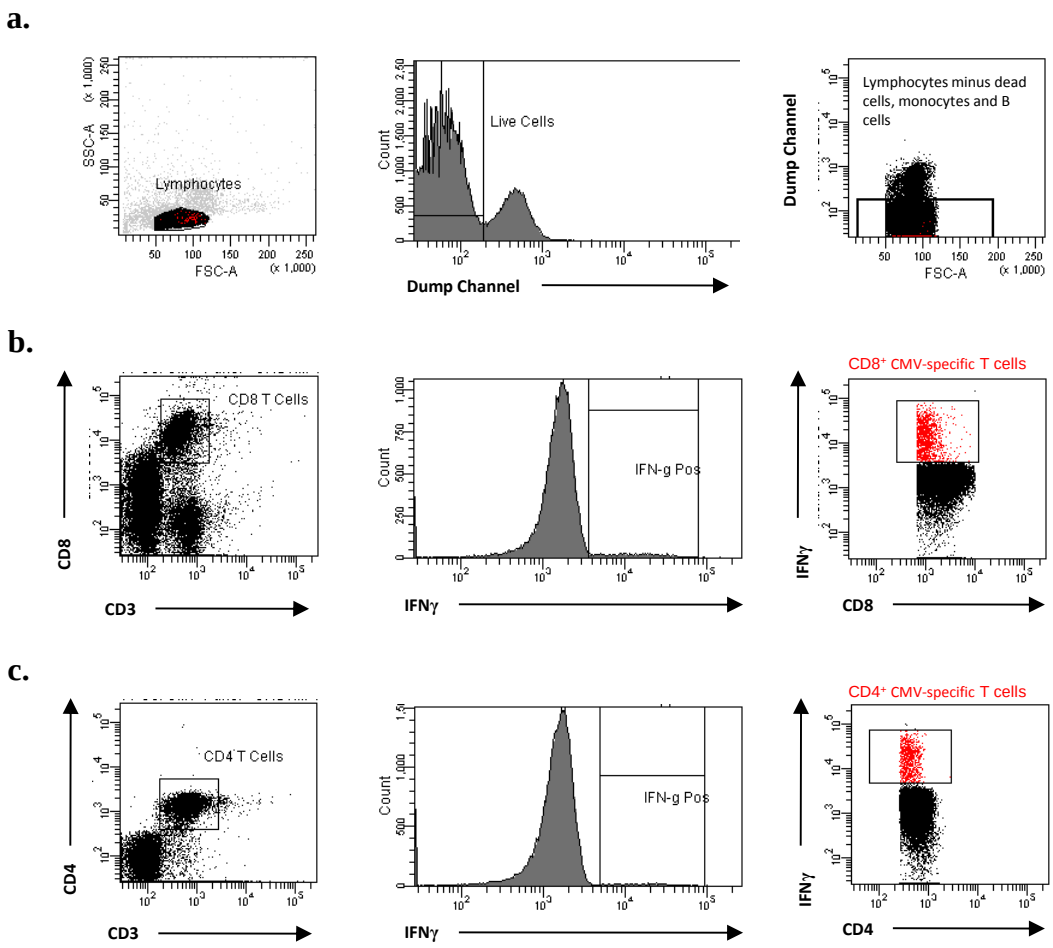


Figure 5-1: Gating strategy to identify CMV-specific $CD8^+$ and $CD4^+$ T cells. PBMC from CMV seropositive donors were stained for KIRs and additional phenotypic cell surface markers. (a) Lymphocytes were gated on and dead cells, monocytes and B lymphocytes were excluded using a "dump channel". T cells were gated on (b) $CD3^+CD8^+$ and (c) $CD3^+CD4^+$) and CMV-specific T cells selected based on the production of IFNγ in response to a CMV peptide mix.

Data from initial experiments is summarised in Figure 5-2 below. It can be seen that there is a significantly higher frequency of KIR^+ cells within the CMV-specific T cell compartment (both $CD8^+$ and $CD4^+$) when compared to the total T cell pool (p = < 0.001).

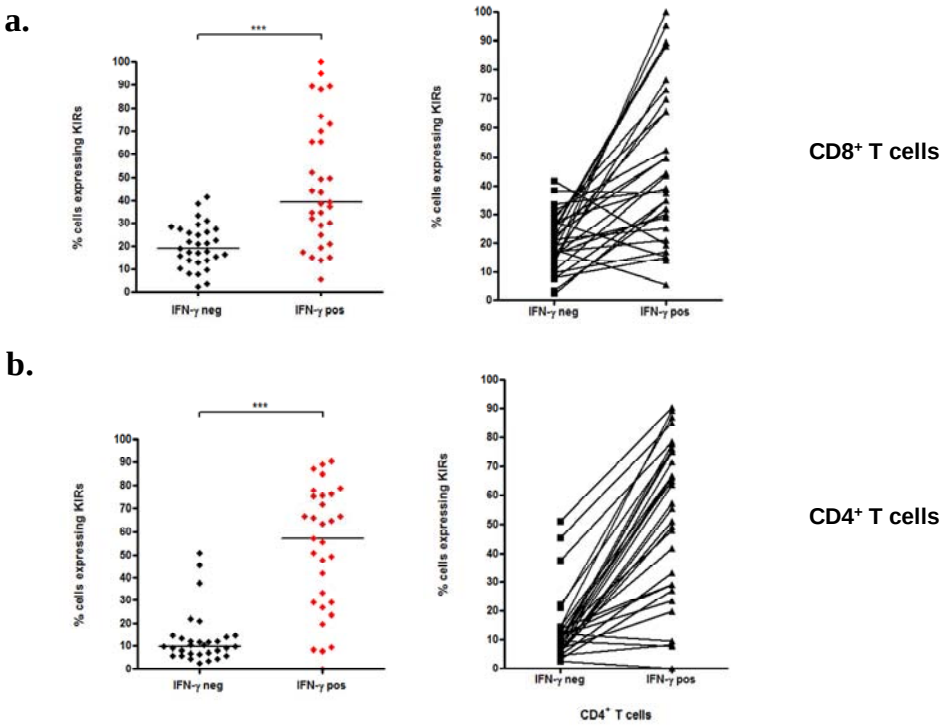


Figure 5-2: KIR expression is higher on CMV-specific T cells than on their IFNγ-negative counterparts. Total KIR expression was compared on (a) $CD8^+$ and (b) $CD4^+$ CMV-specific T cells with the rest of the T cell pool. Left panels show frequency of KIR^+ cells in each population for all donors. Right panels show the frequency of KIR^+ cells in matched $IFN\gamma^-$ and $IFN\gamma^+$ subsets within the same donor. Nonparametric, paired analysis (Wilcoxon signed rank test) was used to test for significant differences. *** = p <0.001

In chapter 4 we had observed increased expression of KIRs in T_{EMRA} populations of both $CD8^+$ and $CD4^+$ T cells. As CMV-specific cells are typically highly differentiated it was important to assess if the apparent increase expression of KIRs on CMV-specific cells compared to non-CMV-specific populations was simply a consequence of their differentiation state. By looking back at the data in the previous chapter (Figure 4-5), the frequency of KIR^+ cells in the overall T_{EMRA} population could be compared with that of the CMV-specific subset shown in Figure 5-2. Within CMV seropositive donors, a median value of 20% of both $CD8^+$ and $CD4^+$ T_{EMRA} cells express KIRs. In contrast 40% and 60% of $CD8^+$ and $CD4^+$ CMV-specific T cells respectively express KIRs. These results proved to be significant (p = <0.001 using a Mann-Whitney test), meaning that KIR-expressing cells are more frequent in the CMV-specific subset, and importantly that this increase in frequency is not purely due to T cell differentiation (Figure 5-3).

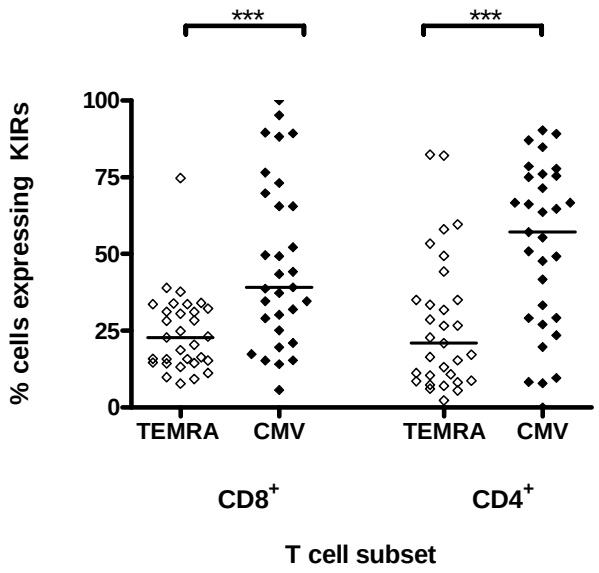


Figure 5-3: Increased KIR expression on CMV-specific T cells is not simply due to differentiation status. Median frequencies of KIR^+ T cells were compared between CMV-specific T cells and T_{EMRA} populations. Comparisons between the groups were analysed using a Mann-Whitney, two-tailed t test.
*** = $p < 0.001$

Following this result, we were interested to investigate which subpopulations of KIR proteins were increased on CMV-specific T cells. Thus, each of the 4 KIR proteins studied in the previous chapter was measured individually on CMV-specific T cells and, as before, compared to the overall T cell pool (Figure 5-4). Figure 5-4a shows that on $CD8^+$ CMV-specific T cells the expression of CD158e1 and CD158i was increased by approximately 4-fold compared to control cells. In contrast, expression of CD158b was significantly downregulated. CD158a expression was also reduced but the difference was not significant.

Some similarities were also observed when we analysed the $CD4^+$ data (Figure5-4b). CD158e1 and CD158i were again found to be upregulated on CMV-specific T cells, although in this setting the increase was 6-fold and 10-fold respectively. However, in contrast to $CD8^+$ CMV-specific T cells, CD158b was seen to be upregulated on $CD4^+$ CMV-specific T cells, with a median value 3-fold higher than that of the remaining $CD4^+$ T cell pool. No difference was seen in levels of CD158a expression. CD158e1 is an inhibitory molecule and CD158i is activating and so it appears that inhibitory and activating molecules are simultaneously upregulated on CMV-specific T cells. Due to the lack of specific antibodies it is unclear using HP-3E4 (CD158a) and GL183 (CD158b) Abs which specific KIR proteins are being measured.

Thus, not only is there differential expression of individual KIR proteins on CMV-specific T cells but there is also a different pattern of expression on $CD4^+$ and $CD8^+$ T cells. The most obvious difference is with CD158b expression which binds to the inhibitory KIR2DL2, KIR2DL3 and activating KIR2DS2 proteins. It is downregulated on $CD8^+$ CMV-specific T cells but modestly upregulated on the $CD4^+$ subset.

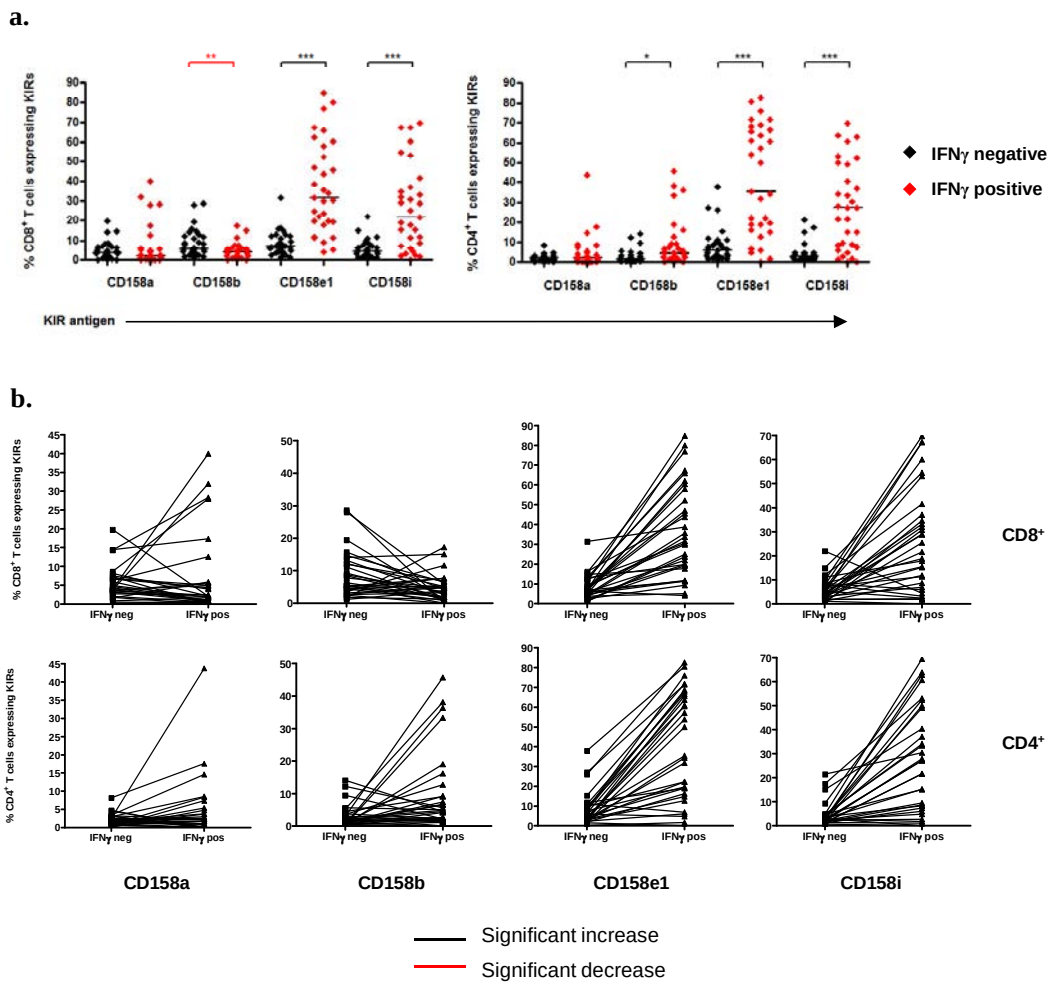


Figure 5-4: CD158e1 and CD158i are upregulated on both CMV-specific CD8$^+$ and CD4$^+$ T cells whilst CD158b is differentially regulated. (a) Median frequencies of individual KIR-expressing T cells were compared between CMV-specific T cells and the rest of the T cell pool. (b) Frequency of KIR$^+$ cells in matched IFNγ$^-$ and IFNγ$^+$ subsets within the same donor. Nonparametric, paired analysis (Wilcoxon signed rank test) was used to test for significant differences. *** = p <0.001, ** = p < 0.01, * = p <0.05

Finally KIR co-expression on CMV-specific T cells was compared with the KIR repertoire expressed on the rest of the T cell pool. As before KIR^+ cells were categorised into 4 groups according to the number of KIR-specific Abs to which they bound, and the results shown in Figure 5-5. $CD8^+$ and $CD4^+$ CMV-specific T cells have both a higher frequency of KIR-expressing cells, and a more diverse receptor repertoire than that of the total T cell pool (Figure 5-5b). Thus, a larger proportion of KIR^+ cells within the CMV-specific pool is made up from cells that stain positive for 2 or 3 KIR antibodies. Indeed 45% of KIR^+ $CD8^+$ CMV-specific T cells stain with more than one KIR antibody compared to only 30% of the KIR^+ cells within the rest of the T cell pool. Similar results were observed with $CD4^+$ CMV-specific T cells with median values of 40 and 28% respectively.

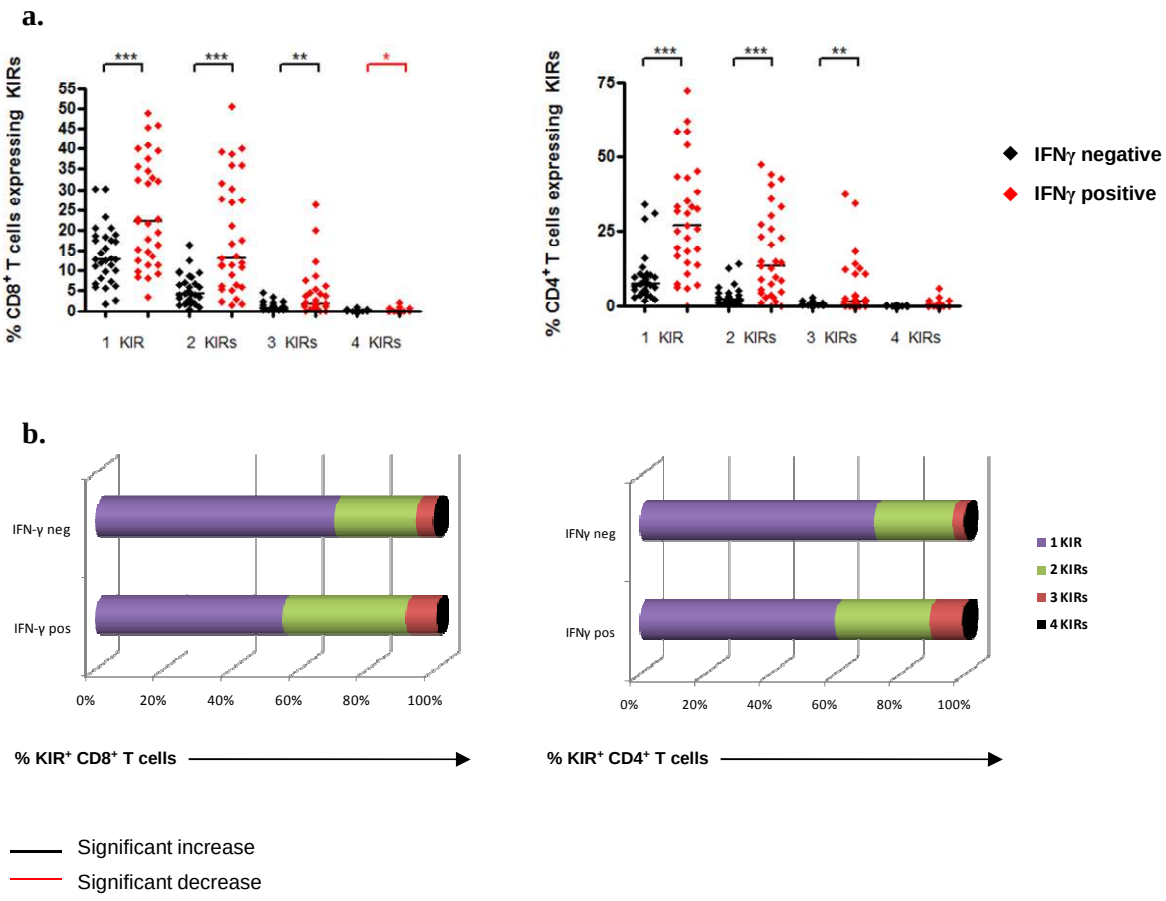


Figure 5-5: CMV-specific T cells have a more diverse KIR repertoire. Co-expression of KIRs was analysed on $CD8^+$ and $CD4^+$ CMV-specific T cells as well as the rest of the T cell pool. Cells were grouped according to the number of KIR antibodies they stained positive for. Dead cells, monocytes and B lymphocytes were excluded using a "dump channel". (a) Plotted values are the percentage of KIR^+ cells within each T cell subset. (b) Plotted values show the proportion of KIR^+ cells that fall into each group. A Dunn's Multiple Comparison test was carried out to determine whether the median values between the 2 populations are significantly different. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$

5.2 GENERATION OF KIR$^+$ CD4$^+$ CMV-SPECIFIC T CELL CLONES

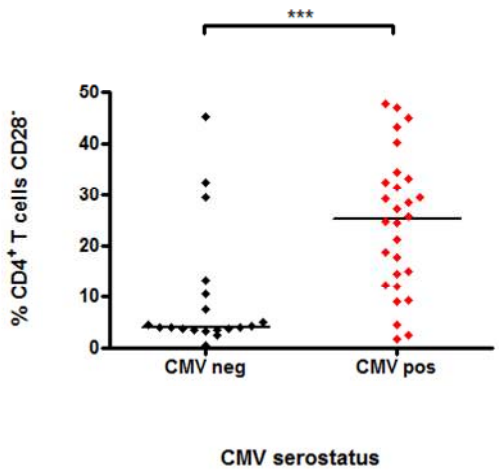
Previous reports to address KIR function have largely focussed on T cells derived from patients with RA. As described earlier these patients have an expansion of CD4$^+$ CD28$^-$ T cells which show high expression of KIR molecules. An expansion of CD4$^+$ CD28$^-$ T cells which express KIRs in clinical syndromes such as RA and acute coronary syndromes has been described (Yen *et al.* 2001). It is now clear that CMV infection results in an expansion of CD4$^+$ CD28$^-$ T cells (van Leeuwen *et al.* 2004), and CMV has also been shown to be the cause of the CD4$^+$ CD28$^-$ expansion in RA patients (Hooper *et al.* 1999). As such it may be plausible that the expansion of KIR$^+$ T cells in patients with RA is driven by CMV rather than the RA process itself.

Using the same cohort of donors as above, subsequent experiments analysed KIR expression on different subsets of CD4$^+$ T cells analysed directly *ex vivo*. Donors were separated according to CMV serostatus and within each group the frequency of CD4$^+$ T cells that were CD28$^-$ was determined. Consistent with earlier work showing CD4$^+$ CD28$^-$ T cells emerge as a consequence of CMV infection (van Leeuwen *et al.* 2004), Figure 5-6a shows that only around 4% of CD4$^+$ T cells are CD28$^-$ in the CMV seronegative group of donors, whereas this is increased to over 25% in the CMV seropositive group. As can be seen in Figure 5-6b, the CD4$^+$CD28$^-$ subset contains a significantly higher frequency of KIR-expressing cells in both groups of donors. Another interesting observation is that the CD28$^-$ subset in CMV seronegative donors has a higher frequency of KIR-expressing cells than the comparable subset in CMV seropositive donors. However, the absolute number of KIR$^+$ cells within the CD4$^+$ CD28- pool is lower in CMV seronegative donors due to the small overall CD4$^+$ CD28$^-$ pool in these donors (Figure 5-6c). Furthermore, when looking at the CD28$^+$ subset only it

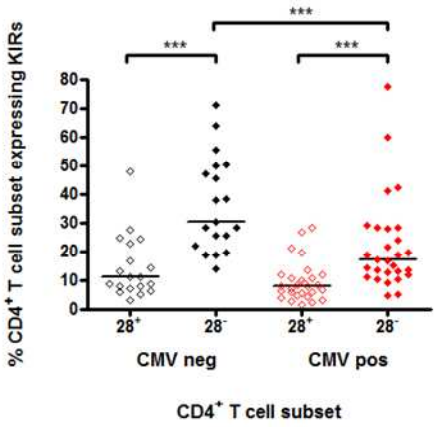
can be seen that CMV seronegative donors possess a higher number of KIR^+ $CD4^+$ cells. When examining the $CD28^-$ subset, CMV seronegative donors possess fewer KIR^+ cells than CMV seropositive donors.

A potentially interesting observation is that the total number of KIR^+ $CD4^+$ cells is equivalent between CMV seropositive and seronegative donors but they are differentially distributed within the $CD28^-$ and $CD28^+$ subsets. An explanation for this could be the need to maintain a balance of signals – as CMV seropositive donors have more $CD4^+$ $CD28^-$ cells not such a high proportion may need to express KIRs. This hypothesis would fit with the observation of the previous chapter – that CMV seropositive donors have a lower frequency of KIR-expressing cells.

a.



b.



c.

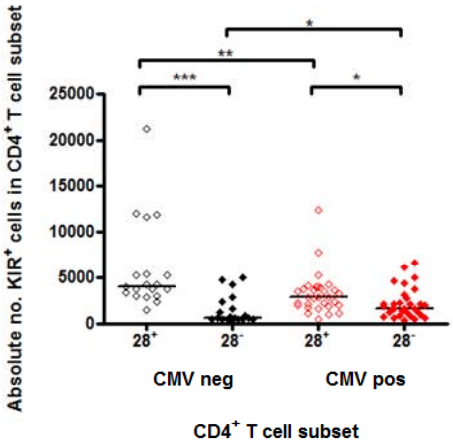
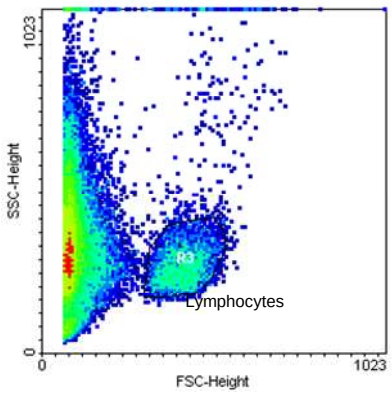


Figure 5-6: KIRs are more frequently expressed on the CD28$^-$ subset of CD4$^+$ T cells. **(**a) The percentage of CD4$^+$ T cells that were CD28$^-$ was compared between CMV seronegative and seropositive donors. (b) KIR expression was measured on each of these CD4$^+$ T cell subsets. (c) KIR expression on CD4$^+$ T cell subsets was adjusted according to population size. Nonparametric, paired analysis (Wilcoxon signed rank test) was used to test for significant differences between CD4+ T cell subsets within each group of donors. Comparisons between the groups were analysed using a Mann-Whitney, two-tailed t test.
*** = p <0.001, ** = p < 0.01, * = p <0.05

To study the function of KIRs on T cells I then went on to generate KIR^+ T cell clones. Based on previous findings that KIR^+ CD4+ T cells are more frequent in the CD28- population my first attempts at generating KIR^+ $CD4^+$ T cell clones were to FACsort $CD4^+$ $CD28^-$ cells. PBMCs were stained with CD4-specific and CD28-specific antibodies and sorted using a FACS Vantage cell sorter. Single $CD4^+$ $CD28^-$ cells were sorted into 96-well plates containing 200µl of cloning mix. An example of the gating is shown in Figure 5-7. Firstly the lymphocyte gate was set according to cell size and granularity (Figure 5-7a). Next, $CD4^+$ $CD28^-$ cells were selected by gating (Figure 5-7b) and the cell sorter isolated these cells into a 96-well plate at a density of 1 cell per well. Cells were left for 14 days and then screened for clone growth.

a.



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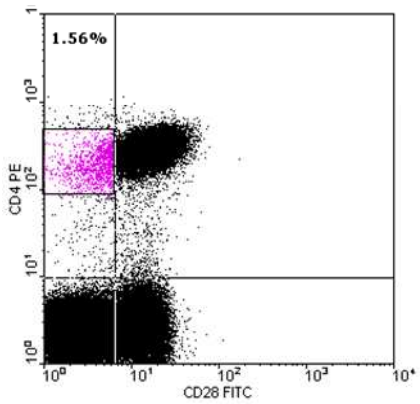

Figure 5-7: $CD4^+$ $CD28^-$ cells were selected via FACsorting. PBMC were stained with α-CD4-PE and α-CD28-FITC. (a) Lymphocytes were gated on based on cell size and granularity. (b) $CD4^+CD28^-$ cells were then sorted using a FACS Vantage cell sorter.

Initial attempts to isolate specific cells via this method were unsuccessful and yielded no proliferating clones. It was unclear whether this was due to technical problems with the FACS Vantage, or due to the impaired replicative potential of these T cells which tend to be terminally differentiated (Vallejo *et al.* 1999).

To overcome this, we concentrated solely on generating CMV-specific $CD4^+$ $CD28^-$ T cells (Fletcher *et al.* 2005). This approach was advantageous as I could use methods that are well-established within our group, and the isolated clones would be defined as CMV-specific. Thirty CMV seropositive laboratory donors were screened against a panel of four pp65-derived proteins (Table 2-2). PBMCs were stimulated with CMV peptides in the presence of Brefeldin A, an inhibitor of protein secretion from the Golgi apparatus, and the production of IFNγ by $CD4^+$ T cells was assessed by intracellular cytokine staining. PBMCs stimulated with CMV lysate generated from CMV-infected fibroblasts were used as a positive control for the assay. Unstimulated PBMCs or PBMCs stimulated with a 'mock' lysate, made from uninfected fibroblasts, were used as negative controls to confirm that any responses seen were specific for CMV-derived antigens within the lysate. As an additional control, PBMCs from healthy seronegative donors were stimulated with both mock and CMV lysate. Figure 5-8 shows typical responses to controls and CMV derived peptides in 2 CMV seropositive donors. Donor A has a strong response to peptide A1as well as the CMV lysate. Donor B showed responses against peptides A1 and A2. Both unstimulated samples and PBMCs treated with mock lysate produced no IFNγ.

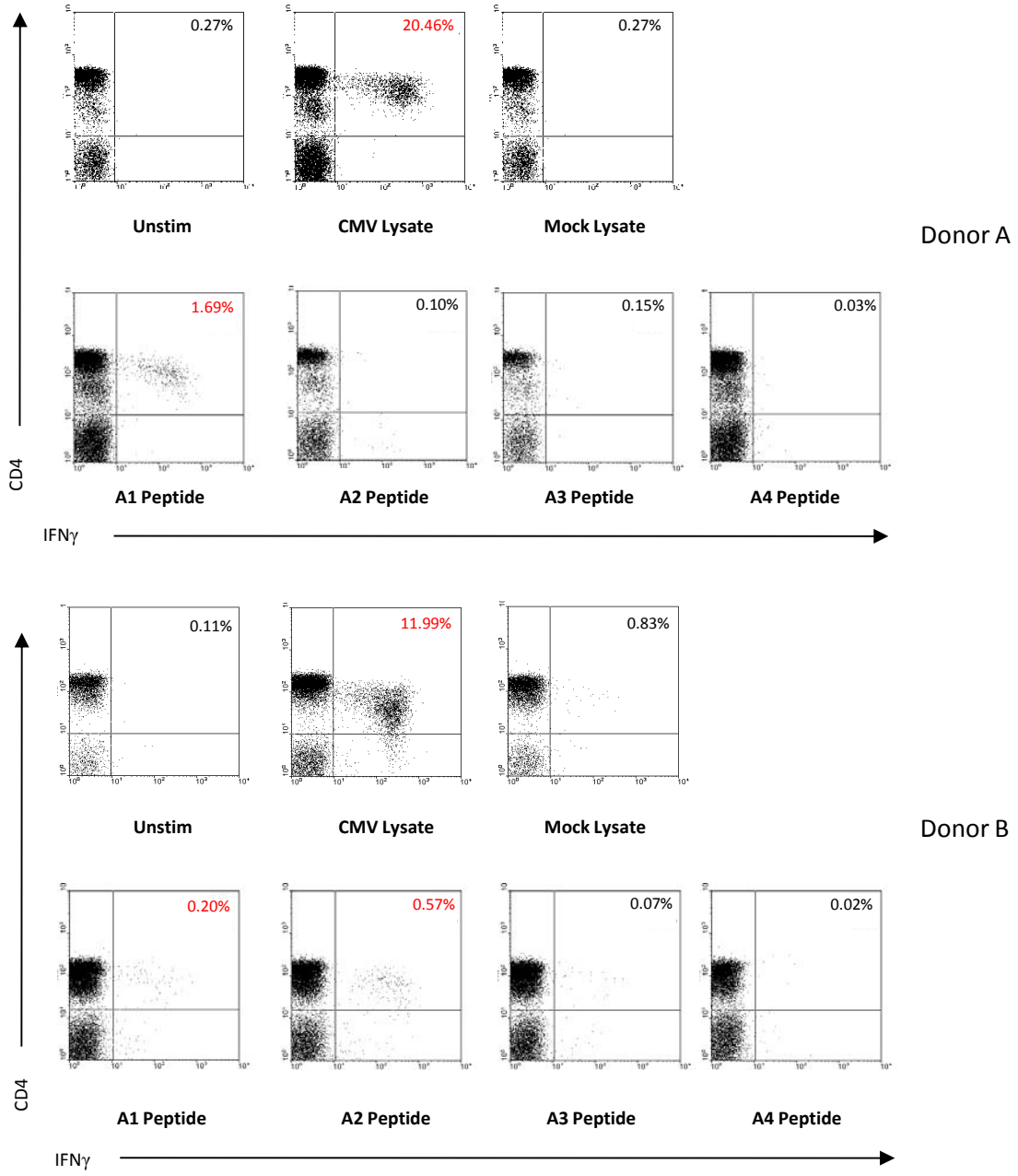

Figure 5-8: $CD4^+$ T cell responses to pp65-derived CMV peptides. PBMC were stimulated for 6 hours with CMV peptides. Brefeldin A was added to the samples 1 hour after infection. Cells were then stained for surface expression of CD3, CD4 and intracellular IFNγ. Percentages of total $CD4^+$ T cells which produced IFNγ following stimulation are indicated.

To increase the chance of cloning CMV-specific T cells from the background population, the cytokine capture assay was employed prior to limiting dilution. PBMCs from donors with peptide responses of over 0.5% were stimulated with CMV lysate. Cells that responded by secreting IFNγ were magnetically selected and cloned by limiting dilution. Figure 5-9 shows two examples of this procedure. The peptide-specific population is enriched from 1.96 % of the total population to 56.80% of the total population for donor A and from 0.18% to 46.50 % for donor B. Enriching for peptide-specific cells to such a large degree markedly increases the proportion of CMV-specific T cell clones that expand following the cloning process.

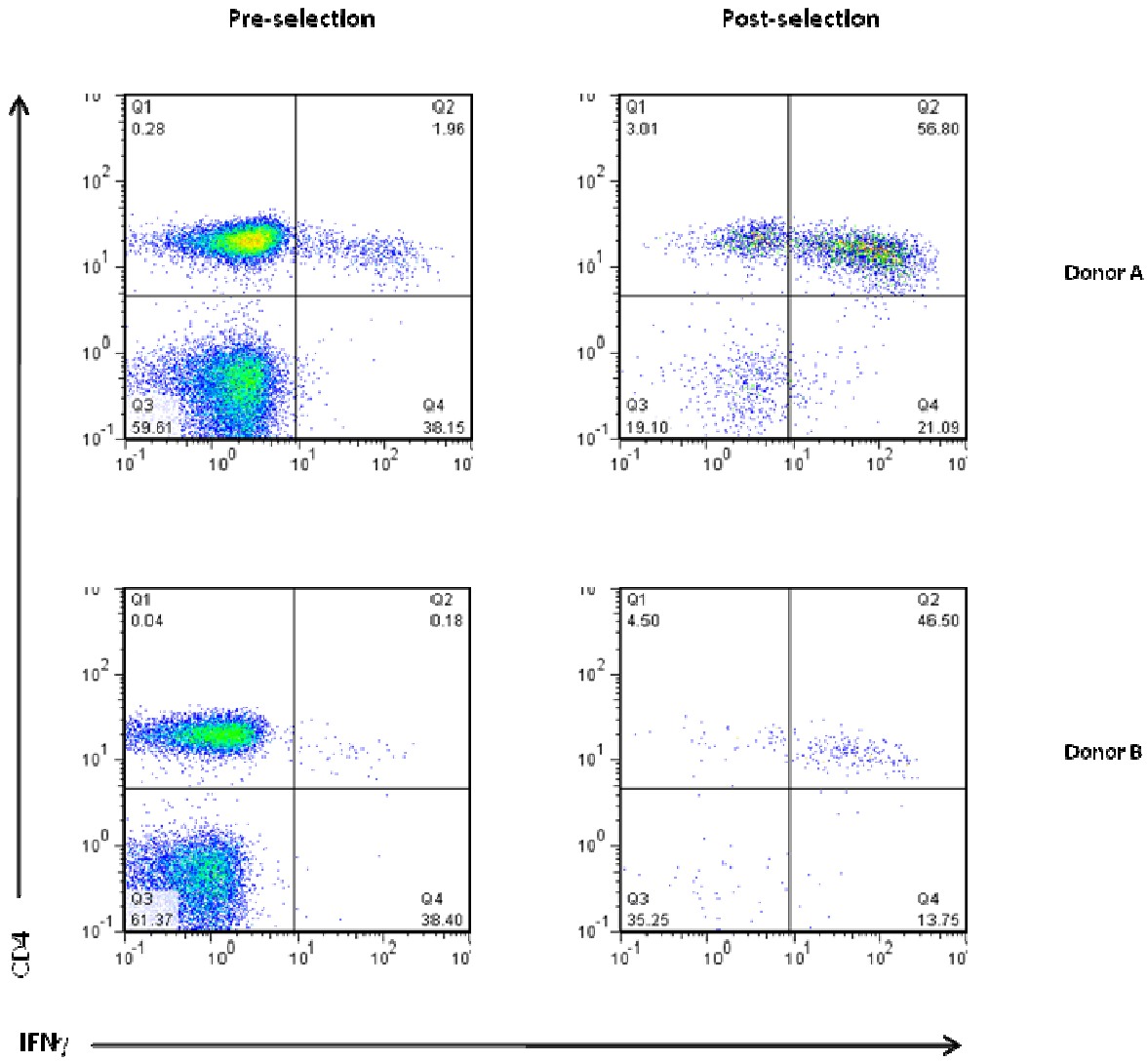

Figure 5-9: Enrichment of CMV-specific CD4$^+$ T cells using cytokine capture analysis. PBMCs from Donors A and B were stimulated with CMV peptides A1 or A2 respectively overnight and cells expressing IFNγ were isolated using the cytokine capture assay. Cells before and after enrichment were then stained for surface expression of CD4 and CD3 and assessed by flow cytometry. The numbers indicated are the percentage of CD4$^+$ IFNγ$^+$ cells in the total population analysed.

Unfortunately, these attempts to generate T cell clones were also unsuccessful despite use of the cytokine capture assay to enrich antigen-specific cells prior to limiting dilution cloning. As it was possible that $CD8^+$ T cells were outcompeting the $CD4^+$ T cells the final method was to deplete $CD8^+$ T cells from PBMC and set up a polyclonal T cell line (Khanna *et al.* 2000). In brief, PBMC samples taken from the same donors were $CD8^+$ T cell-depleted using α-CD8 Dynal beads. Remaining cells were cultured with the relevant peptide for 2 weeks to establish polyclonal cultures enriched in peptide-specific T cells. On day 14, cells from these peptide-reactivated cultures were used to set up limiting dilution cloning at both 0.3 and 3 cells per well. These cells were seeded in 96-well U bottomed plates along with 10^4 cells per well of autologous γ-irradiated LCL preloaded with 5μM relevant epitope peptide and 10^5 cells per well of pre-activated γ-irradiated allogeneic PBMC feeder cells. In a typical limiting dilution cloning experiment from a single peptide reactivation, 20 plates would be set up at 0.3 cells per well, and 10 plates at 3 cells per well. Three days following cloning, IL-2 (derived from MLA supernatant) was added to stimulate cell expansion.

Within approximately 2 – 5 weeks, growth of the microcultures could be observed. Typically up to 30 proliferating cultures could be identified on a cloning plate where cells had been seeded at 0.3 cells per well and as many as 90 proliferating cultures were identifiable on the cloning plates seeded at 3 cells per well. To enhance the probability that cultures had originated from a single cell, cultures at 0.3 cells per well were preferentially screened. Plates seeded at 3 cells per well were only screened where <30% of the wells had proliferated (Taswell *et al.* 1980; Munz *et al.* 2000).

In order to confirm the peptide-specificity of the $CD4^+$ T cell clones, individual clones were stimulated with irradiated HLA-matched LCLs which were pulsed with peptide. Secretion of IFNγ in response to simulation was used as a marker of recognition and quantified using an IFNγ ELISA. Unmanipulated LCLs and HLA-mismatched LCLs show that IFNγ secretion was due to recognition of CMV proteins and not EBV antigens. Figure 5-10 illustrates examples of 4 clones from 2 different donors. Three out of 4 of the clones shown here produced a high level of IFNγ only in response to LCLs pulsed with the pp65 derived peptide A1, indicating specific antigen recognition. Clone 44 however produced IFNγ in response to stimulation with LCL alone suggesting that the cells were not CMV-specific. This demonstrates that whilst using assays to select CMV-specific cells, inevitably clones which have other cross reactivities may also be cloned. Table 5-1 shows a summary of clones generated.

Table 5-1: Peptide-specific $CD4^+$ T cell clones generated

	Protein	Epitope sequence	Amino acid position	HLA restriction	No. of T cell clones generated
CMV	pp65 (UL183)	KYQEFFWDANDIYRI	509 – 523	HLA-DR1/3	177
	pp65 (UL183)	AGILARNLVPMVATV	489 – 503	HLA-DRB1*0701	5
EBV	EBNA3A	GPWVPEQWMFQGAPPSQGTD	780 – 799	HLA-DR1	44
	EBNA3C	SDDELPYIDPNMEPV	386 – 400	HLA-DQ5	36

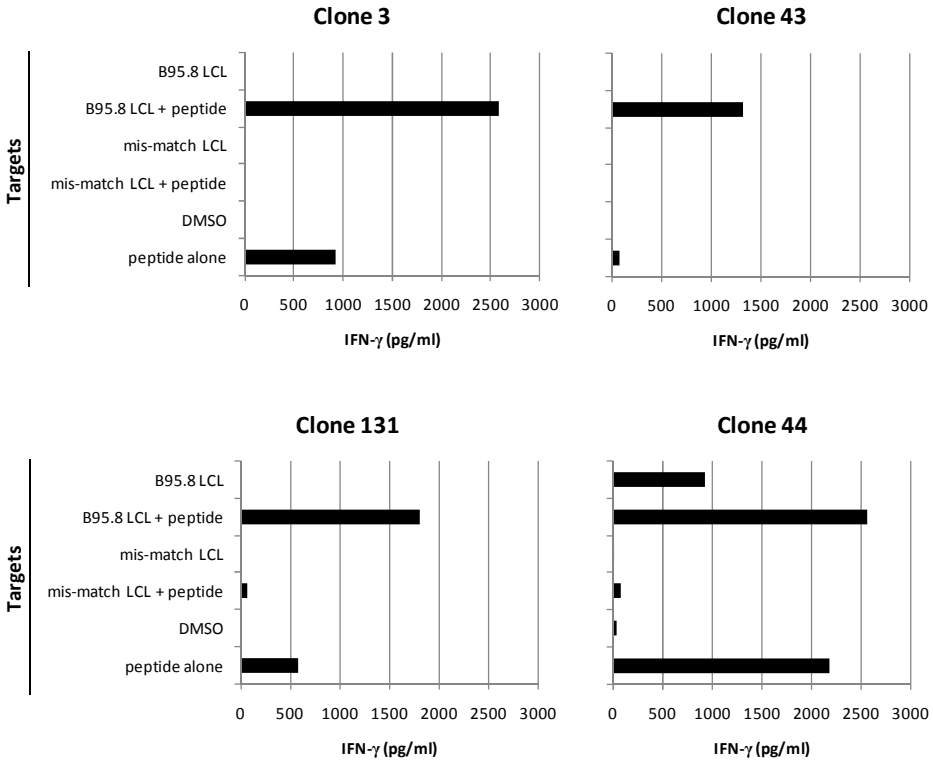

Figure 5-10: Screening of CD4$^+$ T cell clones for recognition of A1 peptide. APCs (LCLs) were pulsed with A1-peptide or DMSO as a negative control before being incubated overnight with T cell clones. Production of IFNγ was used as a marker of T cell recognition and IFNγ concentration of the supernatants was analysed by ELISA.

Given that the A1 peptide-specific $CD4^+$ T cell clones were generated via stimulation with a synthetic peptide, and it is known that this process can lead to peptide, but not antigen-specific, T cell clones (Khanna *et al.* 1995), it was important to show that they could recognise processed antigen and not just the peptide. To determine whether A1 peptide-specific $CD4^+$ T cells could recognise endogenously processed pp65 antigen, an MVA-pp65 construct in which the pp65 coding sequence was linked to a target sequence from the invariant chain (Ii) was employed. This provided a way of over-expressing pp65 within LCL target cells which was designed to be delivered directly into the class II processing pathway (Chaux *et al.* 1999).

T cell clones were stimulated with LCLs infected with MVA-pp65 or control MVA-pSC11. IFNγ was used as a marker of recognition and assessed by ELISA. Figure 5-11 shows typical examples of IFNγ production from 4 clones derived from 2 different donors following exposure to MVA-infected LCLs, in which recognition of LCLs infected with Ii targeted pp65 was clearly detected. This confirms that the established clones are capable of recognising processed antigen. No significant production of IFNγ was observed when clones were stimulated with unmanipulated LCL, mismatched LCL (with or without peptide) or control MVA (MVA-pSC11). This confirms the response was not due to recognition of EBV or vaccinia antigens.

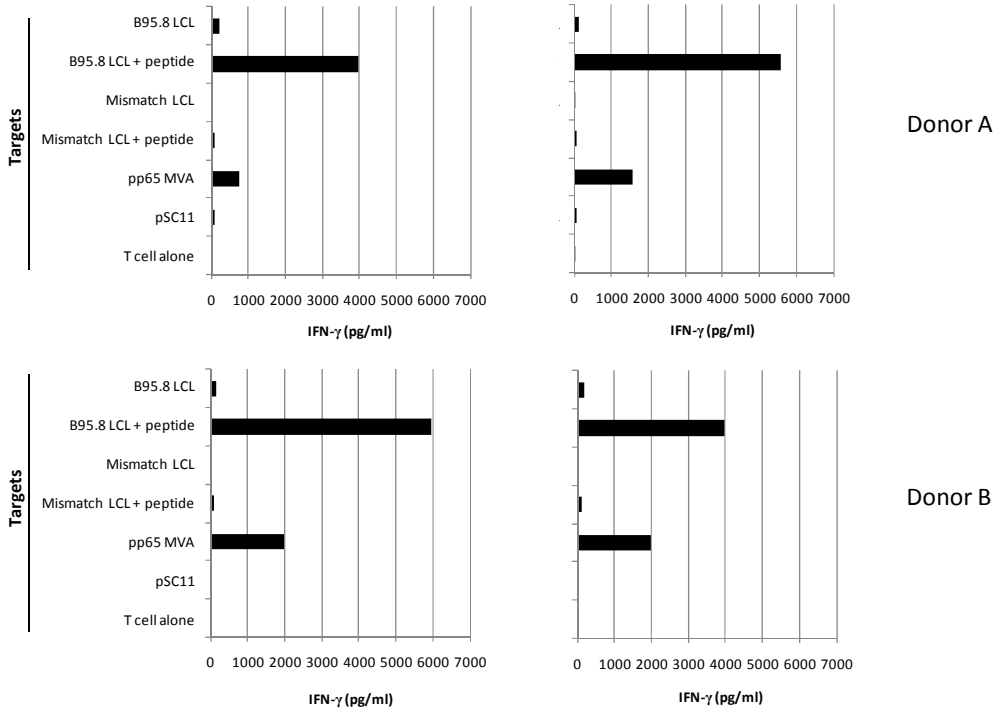

Figure 5-11: Capacity of A1 peptide-specific clones to recognise peptide derived from processed pp65 protein. APCs (LCLs) were either infected with MVA expressing CMV derived pp65 or an empty vector sequence (pSC11), pulsed with peptide, or left unmanipulated prior to overnight incubation with $CD4^+$ A1 peptide-specific T cell clones. Production of IFNγ was used as a marker of T cell recognition, and the IFNγ concentration of the supernatants was analysed by ELISA. Representative examples of antigen recognition by 4 clones derived from 2 different donors are shown.

An important function of antiviral T cells is the ability to lyse virally infected cells. Classically, this is a function of $CD8^+$ CTLs but more recently cytotoxic $CD4^+$ T cells have been reported (Appay *et al.* 2002; Zaunders *et al.* 2004; Long *et al.* 2005; Casazza *et al.* 2006). To determine whether these A1 peptide-specific $CD4^+$ T cell clones displayed cytolytic activity, chromium release assays were used. This method measures the release of the radioactive isotope Cr^{51} from Cr^{51}-labelled APC 'target' cells that have been pulsed with peptide prior to incubation with T cell clone 'effector' cells. Release of Cr^{51} into the supernatant is used as a measure of target cell lysis and thus the cytolytic ability of the effector T cells.

All clones tested were able to kill A1 peptide-loaded LCL, with killing apparent within 5 hours after incubation. Figure 5-12 shows representative examples of cytolytic activity of 4 clones from 2 different donors. Minimal chromium was detected in the supernatants following incubation with matched targets pulsed with A3 peptide or mismatched targets pulsed with A1 peptide indicating that killing was A1 peptide-specific.

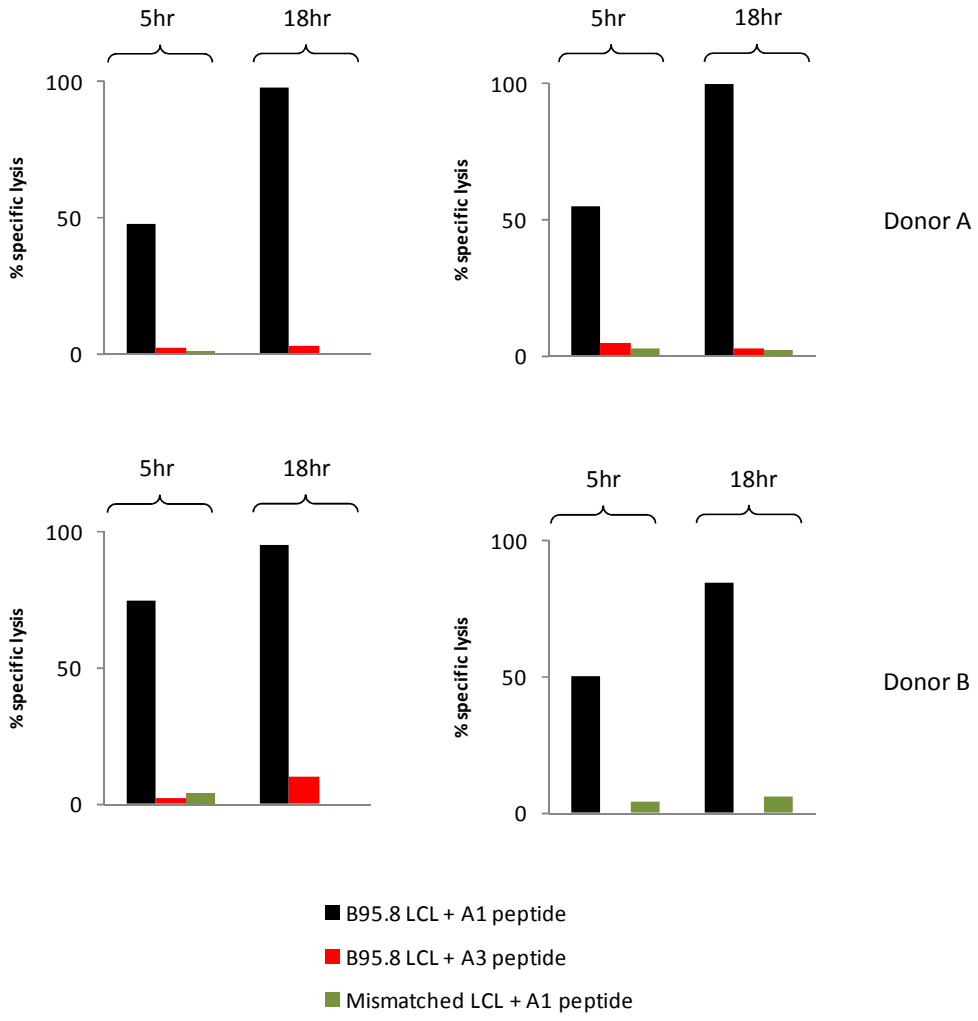

Figure 5-12: Killing of LCL targets by A1 peptide-specific CD4$^+$ T cell clones. 5 and 18 hour chromium release assays were conducted using autologous target cells pulsed with A1 and A3 peptides and A1 peptide-pulsed HLA-Class II mismatched targets. Representative examples for 4 clones derived from 2 donors are shown. Results are expressed as the average percent lysis of target cells from triplicate assays at effector : target ratio 2.5 : 1.

To further characterise the CMV-specific $CD4^+$ T cell clones, cells were stained for surface expression of memory markers to characterise their phenotype. Markers included CD4, CD8, CD45RA, CD45RO, CD28 and CD27. An example of staining from one donor is shown in Figure 5-13, which is representative of all clones stained. As shown, all cells exhibited an effector memory phenotype ($CD45RA^-$, $CD45RO^+$, $CD28^-$ and $CD27^-$) similar to that which has been described for $CD4^+$ T cells that arise during chronic viral infection (Amyes *et al.* 2003).

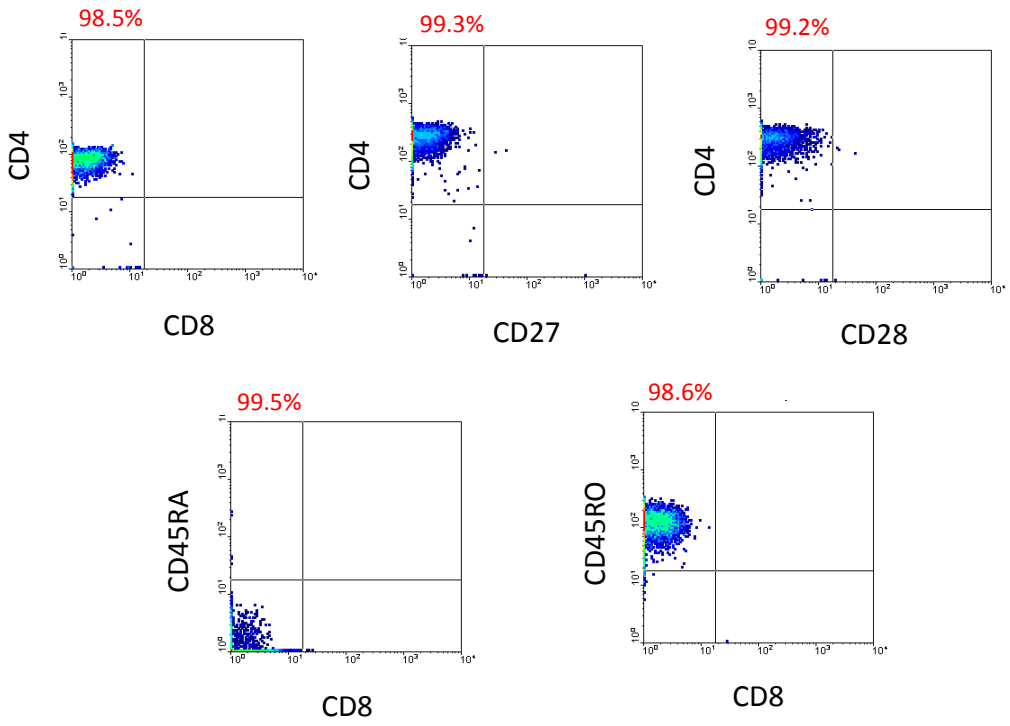

Figure 5-13: Cell surface phenotype analysis of A1-specific T cell clones. T cell clones were washed and stained for surface expression of CD3, CD4, and CD8 along with phenotype markers CD45RA, CD45RO, CD28 and CD27.

5.3 KIR EXPRESSION AND FUNCTION ON CD4$^+$ CMV-SPECIFIC T CELL CLONES

Having successfully generated antigen-specific CD4$^+$ T cell clones, and established that they were functional, we were interested to investigate their KIR expression. T cell clones were stained with CD158a-, CD158b- and CD158e1-specific antibodies. As illustrated in Figure 5-14 showing examples of staining from 2 A1 peptide-specific clones, KIRs were not constitutively expressed. Indeed, typically less than 5% of a T cell clone would be KIR$^+$.

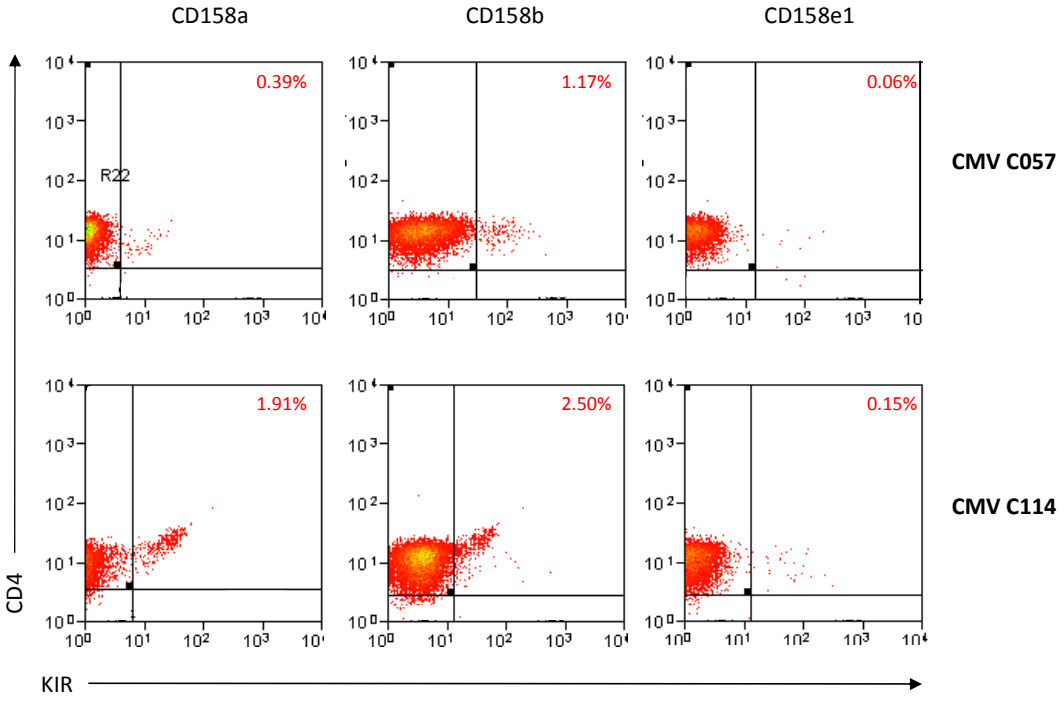

Figure 5-14: KIRs are not constitutively expressed on CD4$^+$ T cell clones. A1 peptide-specific CD4$^+$ T cell clones were stained with CD158a, CD158b and CD158e1 antibodies to establish KIR expression.

No more than 0.2% of cells expressing CD158e1 in any of the clones. There may be a number of reasons for this observation, including the possibility of donor selection. The gene frequency of KIR3DL1 in the population is 88%. Although this is high, the donors we cloned from were not KIR genotyped and so it is not certain that KIR3DL1 protein would be encoded for.

Interestingly, whilst I was culturing the clones I had the opportunity to document the expression of KIR proteins during the culture period. During these experiments I noticed that the frequency of cells expressing CD158a and CD158b increased during culture. In order to investigate this phenomenon further I then went on to assess if the antigenic-specificity of the ones was a determinant in this response. A panel of 15 clones were studied of which 5 were specific for the CMV A1 peptide, 5 recognised the EBV GPW peptide and an additional 5 had unknown specificity. Clones were examined for KIR expression at several points over a 12-week period. Interestingly, the frequency of cells which expressed both $CD158a^+$ and $CD158b^+$ cells increased significantly in all subsets. Table 5-2 shows a summary of the median values.

Table 5-2: KIR expression on CD4$^+$ T cell clones

Clone specificity	CD158a expression Day 0	CD158a expression Day 60	CD158b expression Day 0	CD158b expression Day 60
CMV – pp65 (A1 peptide)	1.45%	52.40%	1.17%	28.21%
EBV – EBNA3A (GPW peptide)	0.04%	30.25%	0.15%	25.41%
Non-specific	0.00%	22.14%	0.09%	18.95%

Percentages shown are median values. 5 of each clone type were tested.

The frequency of KIR$^+$ cells increased over time irrespective of the antigenic specificity of the cells and this was true for both CD158a as well (although to a lesser extent) as CD158b. The increase in KIR expression was greatest in the CMV-specific populations although this did not reach statistical significance. An example of staining is shown in Figure 5-15.

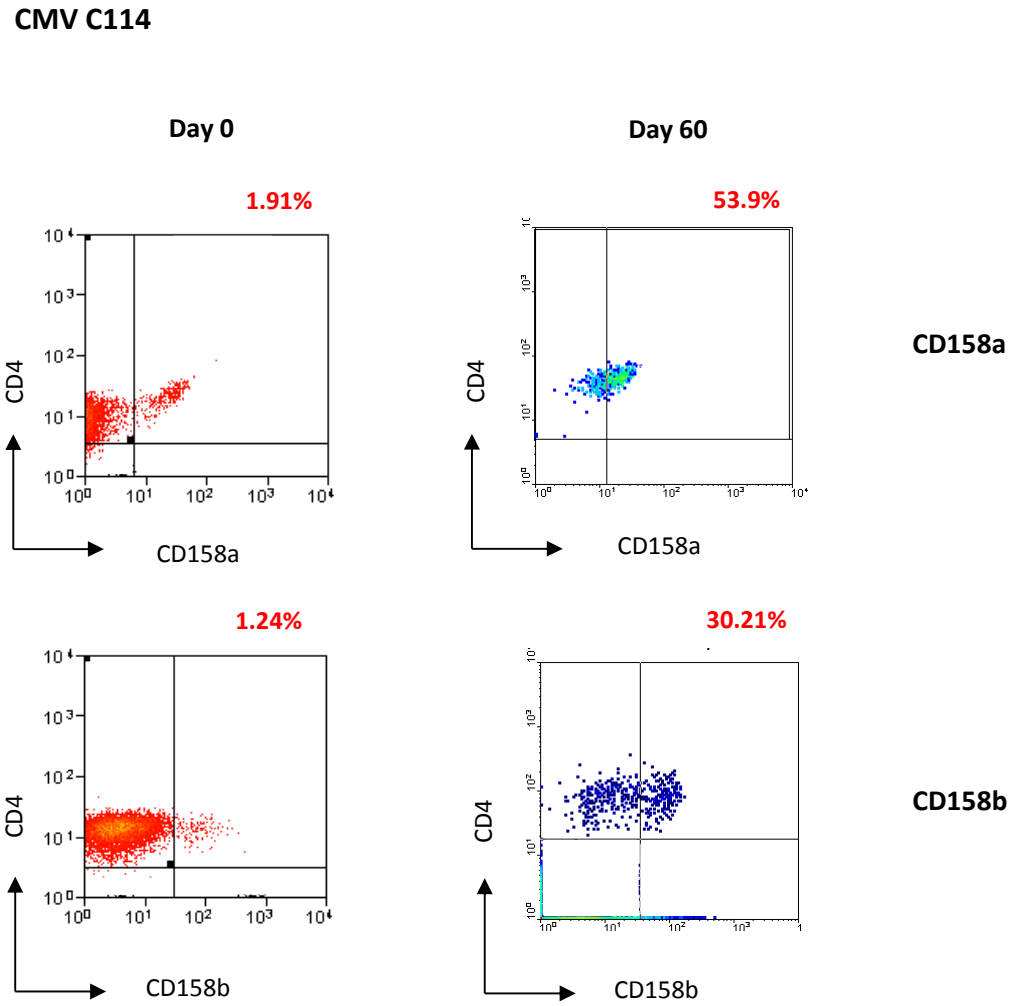

Figure 5-15: KIR expression on CD4$^+$ T cell clones increases with prolonged culture. Example staining from CMV-specific clone C114. T cell clones were stained with CD158a and CD158b antibodies on Day 0 and Day 60.

Having characterised KIR expression on CMV-specific $CD4^+$ T cell clones, I finally sought to investigate the functional consequence of this phenotype. At a point when the clonal populations contained a high proportion of KIR^+ cells, experiments could be carried out to investigate the effect of blocking KIR interactions. Antibodies to CD158a and CD158b were therefore incubated with the cell populations prior to IFNγ ELISAs and chromium release assays which were repeated as before (Figures 5-10 and 5-13). Figure 5-16 shows examples from 2 different clones, which are representative of all clones tested. Attempting to block KIRs on these clones using commercial antibodies had no significant effect on either recognition of processed antigen (Figure 5-16a) or killing of peptide-loaded LCL targets (Figure 5-16b). However it is unfair to draw conclusions from this data as it is not apparent whether mAbs HP-3E4 and GL183 block KIR function.

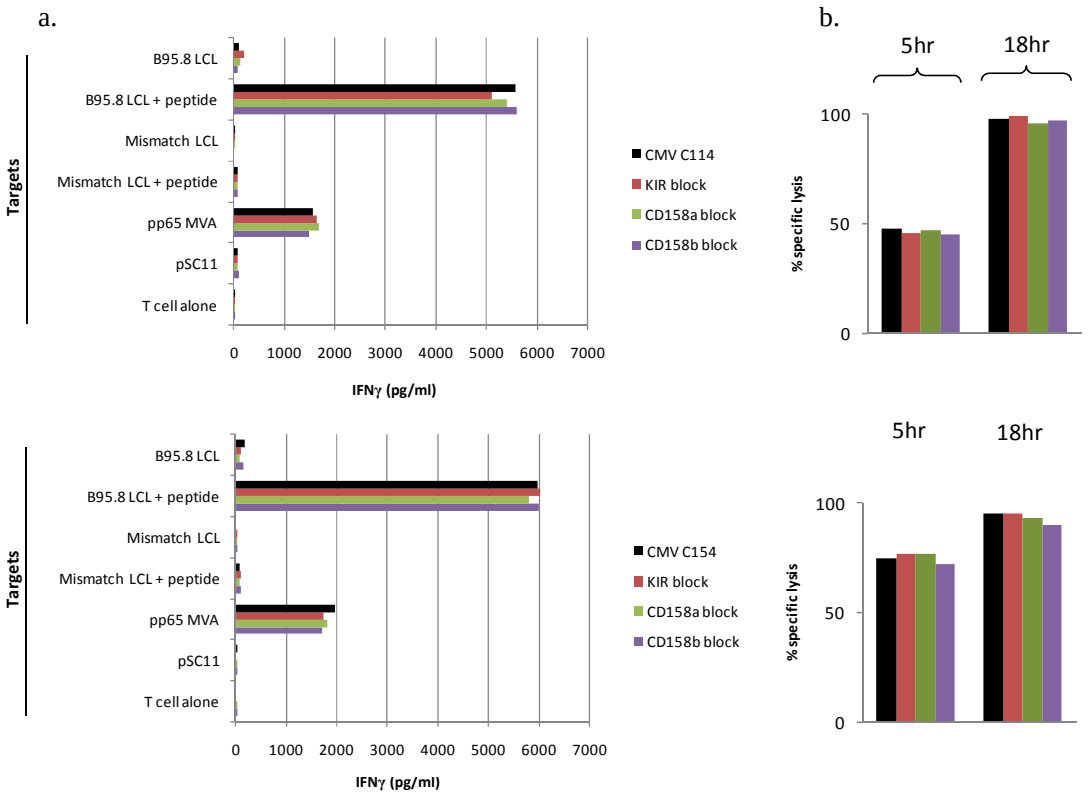

Figure 5-16: KIR blockade does not affect recognition or killing of LCL targets by A1 peptide-specific CD4$^+$ T cell clones. T cell clones were treated with either CD158a or CD158b antibodies or both before function was tested. (a) Recognition of A1 peptide was tested by IFNγ ELISA. (b) Killing of peptide-loaded targets was tested by chromium release. Results are expressed as the average from triplicate assays.

5.4 DISCUSSION

This chapter followed on from the data in the previous chapter to investigate, in more detail, the role of KIRs in the context of CMV infection. Initial experiments were carried out to determine whether CMV-specific T cells express KIRs. Once this was established, work was begun to try and generate KIR^+ $CD4^+$ T cell clones as a model to test function. This proved more difficult than first thought, and many complicating factors came to light.

CMV-specific T cells were shown to contain a higher frequency of KIR^+ cells than the general T cell pool. This was true for both $CD8^+$ and $CD4^+$ T cells although both should perhaps be considered individually when attempting to understand this observation. Huard and Karlsson suggested that repeated exposure to specific antigens would increase expression of NKRs on specific T cells and this work correlates with that (Huard *et al.* 2000). However there is some controversy as Gamadia *et al.* showed that $CD8^+$ CMV-specific T cells barely expressed KIRs at all (even when the rest of the $CD8^+$ T cell population did) (Gamadia *et al.* 2001). To reconcile these data, it is conceivable that KIR^+ and KIR^- T cells recognize distinct sets of CMV proteins. CMV-specific $CD8^+$ T cells in that study were identified by tetramer staining, using a single HLA-A2-restricted CMV peptide derived from pp65, one of the immunodominant CMV proteins (Gamadia *et al.* 2001; Anfossi *et al.* 2004). In contrast, in our assays CMV-specific T cells were identified after stimulation with a CMV peptide pool which contains a much larger number of potential T cell epitopes. It will therefore be of interest to test whether $CD8^+$ T cells specific for other CMV antigens express KIRs.

Since this work has been carried out, van Bergen *et al.* published a study looking at KIR expression on $CD4^+$ T cells in correlation with CMV. As with other studies published, the focus of the paper was not on healthy donors, but on rheumatoid arthritis patients. The group showed that there was a higher frequency of IFNγ producing cells in the $CD4^+$ KIR^+ subset of T cells than $CD4^+$ KIR^- (van Bergen *et al.* 2009). The data in this chapter fits in with this finding.

Despite the reduced frequency of KIR^+ cells in CMV seropositive donors, CMV-specific T cells do express KIRs. This apparent data, although conflicting, is credible as it backs up several other studies. In their paper, van Bergen *et al.* say they attempted to culture PBMCs in the presence of a CMV lysate and this invariably led to a reduction rather than an increase in the proportion of $CD4^+$ KIR^+ T cells, although this data was not shown (van Bergen *et al.* 2009). Cohort studies have indicated that the NKR profile on NK and T cells is imprinted by CMV (Guma *et al.* 2004). A recent study by van Stijn *et al.* demonstrated strong induction of NKRs on T cells as a direct result of CMV infection (van Stijn *et al.* 2008). A possible scenario may be that expression is regulated to provide essential control mechanisms for the activation of T cells by controlling costimulatory signals, dampening T cell activation when viral load diminishes, and controlling harmful activation to the host during latent viral infections. The stage of infection may well be relevant and it would be interesting to compare this between studies. Another possibility to consider is that CMV may itself have some kind of systemic effect of reducing KIR expression on cells of other specificities despite CMV-specific cells showing an increased pattern of KIR expression.

To add to the studies that are currently being carried out the frequency of expression of each individual KIR antigen was also addressed. Interestingly, not all KIR antigens were regulated in the same way. In the $CD8^+$ T cell compartment the frequency of CD158b expression was significantly downregulated on CMV-specific T cells, whereas both CD158e1 and CD158i were significantly upregulated. The frequency of $CD4^+$ cells expressing CD158e1 and CD158i were again found to be significantly higher in the CMV-specific subset. However, CD158b, in contrast to $CD8^+$ T cells was also higher in the $CD4^+$ CMV-specific compartment. The frequency of cells expressing CD158a remained unchanged for both $CD8^+$ and $CD4^+$ T cells. This adds to current knowledge of KIR expression and indicates that not only is each KIR antigen independently regulated, but expression is controlled according to cell type. One big question that needs to be addressed however is whether the same pattern of expression is observed for each specific KIR receptor on $CD8^+$ and $CD4^+$ T cells. As GL183 is a non-specific antibody it is hard to draw firm conclusions here. Is expression of all 3 KIRs (KIR2DL2, KIR2DL3, KIR2DS2) altered, or is it just one? Is this the same for $CD8^+$ and $CD4^+$ T cells or are they unrelated? These are just some of the questions that need to be addressed in the future.

Co-expression of KIRs on CMV-specific T cells was also investigated. It was found that CMV-specific T cells express a more diverse repertoire of KIRs than the remaining T cell pool within the same donor. This is true for both $CD8^+$ and $CD4^+$ T cells. So, despite lower frequency of KIR^+ T cells in CMV seropositive donors , their CMV-specific T cells contain a higher frequency of KIR^+ cells and these cells are more likely to show a diverse KIR phenotype expressing 2 or 3 different types of KIR. A possibility may be that CMV is shaping the KIR repertoire and downregulates KIRs to evade the immune system. However it

is plausible that CMV-specific T cells upregulate KIRs in an attempt to combat CMV infection. Again, a question that really needs to be addressed is which KIRs exactly are affected. α-CD158e1 and α-CD158i are specific antibodies (one for an inhibitory KIR and one an activating), but α-CD158a and α-CD158b detect both inhibitory and activating receptors so it is difficult to interpret how CMV is modulating the immune system. It would be of interest to carry out a longitudinal study of patients who experience primary CMV infection (although these donors are difficult to identify other than post-transplant) to observe the changes in KIR expression as infection progresses. The study by van Stijn *et al.* looked at 13 renal transplant patients who experienced a primary CMV infection over a period of one year. The study was rather limited as they only looked at $CD8^+$ T cells using NLV and TPR tetramers, and they only looked at 2 time points – 40-60 weeks post transplant, and 1 year post-infection. The frequency of KIR^+ cells at these time points were compared to naïve $CD8^+$ T cells, and they found KIR3DL3 expression remained unchanged whereas KIR2DL1, 2DL2, 2DL4, 3DL1, 2DS2, 2DS4 and 3DL5a were upregulated during peak CMV infection but returned to baseline levels in the latency stage (van Stijn *et al.* 2008). This study used microarray analysis to look at mRNA, but if the expression on the cell surface is correlated with this data then the findings could be relevant.

In view of these changes in KIR expression I went on to investigate the function of KIR proteins. I tried on several occasions to clone KIR^+ $CD4^+$ T cells and eventually antigen-specific clones were generated that were shown to express KIRs. Unfortunately only ~2% of the clone showed KIR expression at early time points. However, this is not surprising as KIR expression has been shown to occur after TCR rearrangement (Vely *et al.* 2001). What was observed was that CD158a and CD158b expression increased with prolonged culture. IL-2

has been shown to upregulate expression of CD158a and CD158b (Kogure *et al.* 1999), and this was probably the case in these cultures. Clones were cultured for several months to increase KIR expression so functional assays could be attempted. However, neither antigen recognition nor killing were affected by the blockade of CD158a (KIR2DL1 and 2DS1) and/or CD158b (KIR2DL2, 2DL3 and 2DS2). Assuming these antibodies block KIRs, one possibility for the results observed is that the effects are simply too subtle to be seen with my assays. Alternatively, the blockade that I achieved with these antibodies may be non-specific and lead to opposing influences on cell cytotoxicity. α-CD158a and CD158b antibodies bind a range of inhibitory and activating receptors and it is possible that these cancel each other out. What would be of real use to investigate KIR function effectively would be a T cell clone with high expression of a single KIR. Alternatively the generation of specific reagents to target each single KIR antigen may be a more practical step.

It is now believed that CMV undergoes intermittent reactivation in immunocompetent hosts (Gillespie *et al.* 2000). If activating KIRs also play a significant role in controlling CMV reactivation in healthy donors this may contribute to the maintenance of their polymorphism within the population. Any potential benefit of inheriting multiple activating KIRs must be weighed against their potential contribution towards other disease. Indeed, the inheritance of activating KIRs in the absence of an inhibitory homologue has been shown to increase the risk of psoriatic arthritis and other autoimmune phenomena (Martin *et al.* 2002; Nelson *et al.* 2004).

A number of questions regarding the involvement of KIRs in the immune response to CMV remain open. The identification of KIR ligands becomes essential to define their putative participation in the response against CMV. The true ligands for activating KIRs remain to be determined. There is considerable homology between the extracellular sequence of activating and inhibitory KIRs which might suggest that they share ligands. However, activating KIRs demonstrate only a weak affinity for HLA class I although they may potentially respond to an increase in levels of HLA class I on the cell surface (Saulquin *et al.* 2003; Stewart *et al.* 2005). The interaction between activating KIRs and HLA class I may be dependent on the peptide presented within the peptide binding groove of the HLA class I molecule. There is certainly considerable evidence that peptides affect the interaction between inhibitory KIRs and HLA class I (Zappacosta *et al.* 1997). Alternatively the true ligand(s) for activating KIRs may not be HLA class I molecules but virally encoded homologues. In mice, the lectin-type activating receptor Ly49H confers resistance against murine CMV by engaging a major histocompatibility (MHC)-like protein encoded by the virus (Arase *et al.* 2002). In humans the KIR-related protein LILRB1 binds to the CMV encoded HLA class I homologue UL18 and shows increased expression on lymphocytes in lung transplant patients with CMV disease (Berg *et al.* 2003) and there is evidence that CMV seropositivity influences the cell surface expression of molecules encoded within the leukocyte receptor complex (LRC).

Chapter 6

Discussion

The biology of KIR proteins is an intriguing and complex subject and is further complicated by the considerable polymorphism within the KIR gene complex. Firstly, the number of KIR genes varies between individuals (Uhrberg *et al.* 1997), with potentially hundreds of discrete KIR genotypes. Secondly, some KIR sequences vary by the presence or absence of ITIM motifs. Thirdly, there are additional, more common, causes of sequence variation such as single nucleotide polymorphisms and gene insertions or deletions (Martin *et al.* 2000; Shilling *et al.* 2002). Fourthly, KIRs possess either two or three Ig domains and the reasons for this functional dimorphism are not understood. The result of this is that the KIR region on the leukocyte receptor complex (LRC) is highly plastic (Parham 2004).

KIR genes have been found in disparate species including humans, primates (Rajalingam *et al.* 2001) and ungulates (Dobromylskyj *et al.* 2007). Comparison of the KIR region between man and other primate species suggests that there has been a rapid expansion of the KIR region within the last few million years (Canavez *et al.* 2001; Hershberger *et al.* 2001). Three of the KIR lineages are conserved in humans and chimpanzees, suggesting that they predate divergence of the two species.

A fascinating aspect of HLA/KIR evolutionary biology is the very extreme divergence in haplotype frequencies between human populations, presumed to indicate regional population differences in pathogen driven selection (Parham 2005). The AA genotype is found in around 56% of Japanese individuals and around 15% of Australian Aboriginal individuals. While these frequencies must also be considered in the context of the corresponding HLA class I allelic frequencies, they suggest that different populations may possess NK cell systems of inherently different functional programming.

NK cells are known to play an important role in a wide range of disease settings and there have been many studies relating KIR genotypes to disease susceptibility (Jie *et al.* 2004; Rajagopalan *et al.* 2005; Williams *et al.* 2005; Khakoo *et al.* 2006). The disease studies encompass several conditions in which NK cell function might be expected to play a role. This includes viral infection, autoimmune and inflammatory conditions, tumour immunity, pre-eclampsia and recurrent spontaneous abortion.

A fundamental question needing to be addressed is what are the forces driving the variability of KIRs? One possibility is reproduction – it has been shown that the interaction between maternal KIR and trophoblast, rather than having an immune function, plays a physiological role related to placental development (Hiby *et al.* 2004). Certain HLA/KIR genotypes appear to have been selected against and so it is postulated that reproduction may have been a factor in the evolution of KIR polymorphisms. Another suggestion in the literature is that it is indeed pathogen-driven selection that occurs and maintained by balancing selection for heterozygote advantage. NK cells presumably express inhibitory KIR in order to set thresholds for cellular activation to ensure that only strong stimuli will elicit an effector

response. This system is advantageous in that it permits detection of virus-infected cells or tumours that have downregulated MHC class I. This provides the opportunity for viruses to acquire MHC class I homologues that engage inhibitory NK receptors, but these viral class I proteins would fail to interact with the T cell receptor, thus circumventing both NK and T cell-mediated immunity. The existence of m157 and its ability to bind Ly49 documents that this mechanism of subversion has been devised by a viral pathogen.

In this thesis, I sought to gain a better understanding of KIR biology in healthy donors. I was also keen to investigate the effect of CMV on KIR expression and was interested to determine exactly what the consequence of CMV infection is on pattern and number of KIR molecules found on lymphocyte subsets.

My initial work focussed on production of recombinant soluble proteins to study the biophysical interactions between 2DKIRs and their ligands. Both KIR2DS2 and KIR2DL2 were expressed to high purity as were HLA-C molecules Cw*0401 and Cw*0702. I was able to show that both KIR2DS2 and KIR2DL2 interacted with HLA-Cw*0702. I also observed an interaction between KIR2DL2 and HLA-Cw*0401. Initially this caused me to reconsider my primary data as KIR2DL2 has previously been shown to bind HLA-C1 group allotypes only, and not HLA-C2 due to a genetic polymorphism at residues 77 and 80 (Colonna *et al.* 1993). However, during a conversation with Paul Norman, I discovered that there is now evidence for KIR2DL2 interacting with some HLA-C2 alleles. In 2008 they observed binding to Cw*0501 and Cw*0202 (Moesta *et al.* 2008), and subsequently have found that polymorphisms at positions 16 (Pro→Arg) and 148 (Arg→Cys) lying opposite the binding

site induce a positive charge and result in a conformational change and the binding site opening (unpublished data).

One interesting finding from my work is that the HLA-C tetramers did not show significant staining of NK cells despite the presence of KIR proteins on these cells. In contrast, when cells were cultured *in vitro* in the presence of IL-2 for 1 week there was a marked increase in staining with HLA-C reagents. One possibility that I have considered is that the epitope for HLA-C binding is blocked in resting NK cells but is released by *in vitro* culture. There is a developing precedent for this theory with the realisation that membrane proteins are frequently involved in interactions with partners at the cell surface. These so called '*cis*' interactions can serve to block binding of ligand partners until released and has been observed in murine Ly49A and PIRB proteins, and human LIR-1 and LILRB2 (Masuda *et al.* 2007; Held *et al.* 2008; Chalifour *et al.* 2009). Due to time constraints I was unable to pursue this possibility further but it remains of considerable interest.

I performed a detailed FACs analysis of the expression of KIR receptors on lymphoid cells. One limitation of this when compared to techniques such as microarray is that only the proteins being expressed on the surface of the cell are measured. Also, some of the antibodies available are cross-reactive, specific for both activating and inhibitory forms of KIR proteins. However, observations from this comprehensive study have indicated several possible roles for KIRs to follow up. Healthy donors were seen to have low frequencies of T cells expressing KIRs. In the $CD8^+$ subset frequencies were highest in T_{CM} and T_{EMRA} populations, whereas in the $CD4^+$ subset only the T_{EMRA} population showed an increased frequency of KIR^+ cells. The $CD4^+$ T_{EMRA} population is cytotoxic (Appay *et al.* 2002) and therefore I

wonder whether KIRs play a role in protecting against cytotoxicity. Figure 6-1 illustrates a model whereby T_{CM} and T_{EMRA} $CD8^+$ T cells show increased frequencies of KIR^+ cells to protect against cytotoxicity in secondary lymphoid tissues where primary immune responses are induced, as well as in the periphery (respectively). $CD4^+$ T cells only show increased frequencies of KIR^+ cells in the cytotoxic T_{EMRA} subset. This theory is based on a dominant inhibitory function for KIR expression. α-CD158a and α-CD158b antibodies are cross-reactive and so may be measuring activating KIR2DS1 and KIR2DS2 as well as inhibitory KIRs. However the α-CD158e antibody is specific for inhibitory KIR3DL1. α-CD158i antibody detects activating KIR2DS4, although this allele is often inherited as a non-functional deletion variant (KIR1D) (Hsu *et al.* 2002; Maxwell *et al.* 2002). Therefore it is plausible to assume we are predominantly measuring inhibitory KIRs.

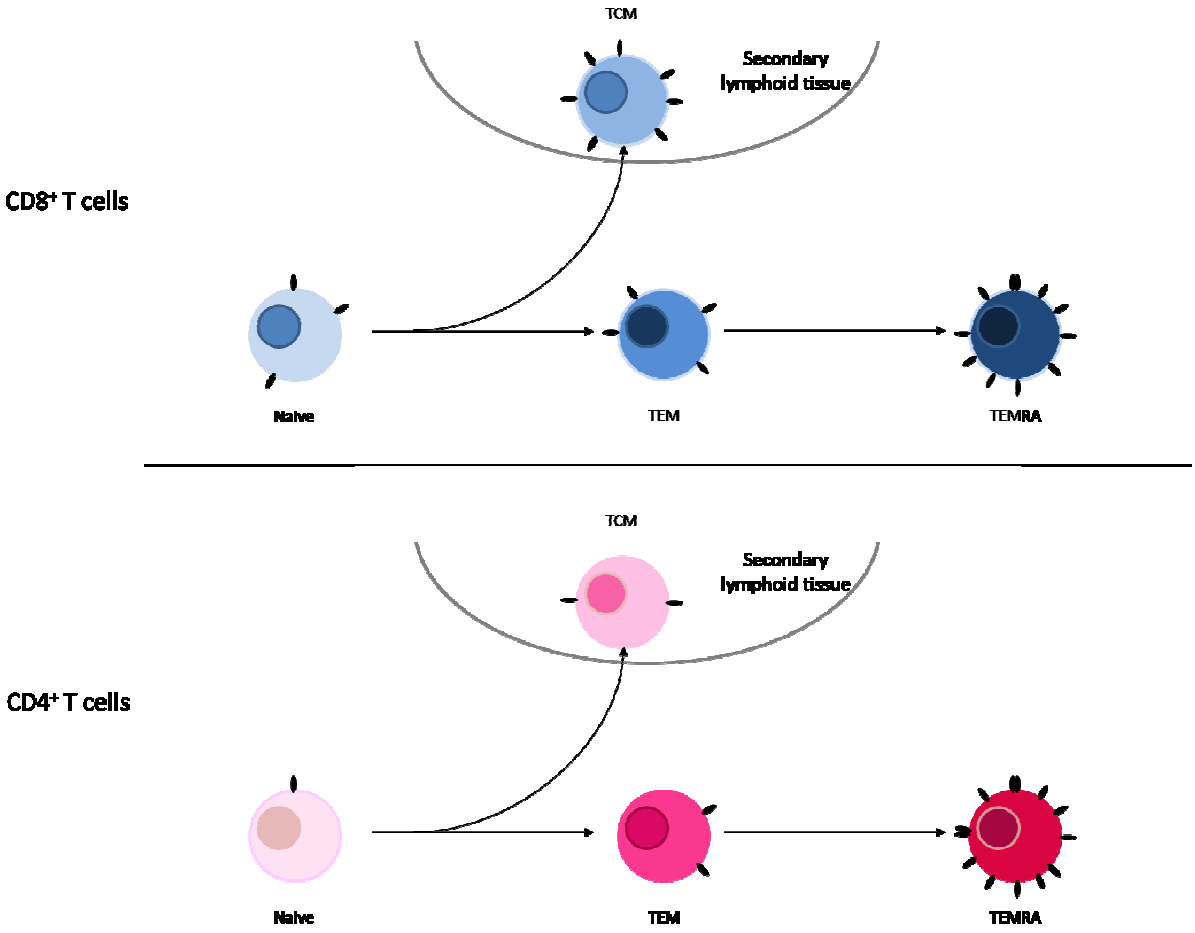

Figure 6-1: Proposed model of KIR expression on T cells protecting against cytotoxicity. A higher frequency of T_{CM} and T_{EMRA} $CD8^+$ T cells express KIRs when compared to other $CD8^+$ T cell memory subsets. The cytotoxic $CD4^+$ T_{EMRA} population is the only $CD4^+$ T cell memory subset to express a substantial frequency of KIR^+ cells.

This study is the first to identify the differential regulation of CD158e1 allotypes on each lymphocyte subset. Previously the DX9 antibody has been characterised and shown to detect different allotypes at two staining intensities but expression on T cells has never been investigated. I have shown that the CD158e1 receptor is heavily regulated and different allotypes are preferentially expressed according to the cell type. Interestingly, the binding properties of these subsets of allotypes differs and the resulting signals to the cell vary in strength (Carr *et al.* 2005). NK cells were shown to predominantly express allotypes that provide a weaker signal, whereas T cells (especially $CD4^+$) exhibited a higher proportion of $CD158e1^+$ cells staining for allotypes that generate a strong inhibitory signal upon binding their HLA-Bw4 ligand.

Another interesting finding from this study is that CMV-seropositive donors have a lower frequency of KIR^+ cells. In addition, from the KIR co-expression experiments I found that the KIR repertoire is more limited on these cells. It appears that CMV infection is driving the KIR expression to select for a specific repertoire. It would be interesting to investigate this further with a much larger cohort, and using KIR genotyped donors, to determine whether or not there are any individual KIR alleles that are preferentially targeted. This idea of CMV leaving an 'imprint' in the receptor repertoire of infected cells has been previously described for other NKRs (Guma *et al.* 2004) and it would be interesting to see if KIRs are regulated in the same manner. CMV infection is a much more common phenotype for *homo sapiens* than a seronegative state and thus might be regarded as the 'norm' in human evolution. As such we need to gain a clear understanding of the effect this virus has in altering receptor expression on lymphoid cells.

I finally investigated KIR expression on CMV-specific T cells and demonstrated that a higher frequency of these cells are KIR^+ than in the rest of the T cell pool. These cells were used as a model to examine KIR function, however I was unable to show any effect when these interactions were blocked. I do not doubt however that these receptors are playing an important role. It may be that the effects were too subtle to see a difference in cytokine production or killing. Also, the use of cross-reactive antibodies may mean that any effect seen was masked – α-CD158a and α-CD158b antibodies would have blocked both activating and inhibitory interactions and one may have cancelled the other out. Finally, this is an artificial target – peptide pulsed LCLs are not truly representative of the natural target *in vivo* and although routinely used to test T cell function we must not forget this.

Future work

This thesis has begun to characterise the pattern of KIR expression in healthy individuals and the functional consequence of this expression pattern. However there is a multitude of future work that could be continued. Clearly there is a need for more reagents to benefit KIR research. Unfortunately our attempts at producing a specific-antibody have, as yet, yielded no success. Although an extremely tall order, given the high degree of homology between receptors, the benefit of producing such a reagent would be invaluable. An increasingly popular way to circumvent this problem has been to KIR genotype donors and/or cells and determine which allotypes are being expressed via a process of elimination. In the absence of time constraints I would have genotyped all of my donors and undergone further analyses to try and determine the pattern of individual allele expression. Following on from this it would be of great benefit to have T cell clones expressing single KIR alleles so the true effect of

blocking this interaction could be measured. If not naturally obtained then transfecting KIR genes into cells would also be a possibility.

There is still conflicting data regarding KIR expression on CMV-specific T cells. It would be of real benefit to stain donors over a period of time ranging from the point of primary CMV infection to years or even decades afterwards as it is plausible that KIR expression may change throughout the course of CMV infection. This is something that I initiated by collecting samples from patients who were undergoing CMV reactivation post stem cell transplant, but they were extremely lymphopenic and so data was difficult to interpret. The optimum samples would be obtained from donors experiencing primary CMV infection, but these donors are difficult to identify. Not only is KIR expression important in the context of CMV, but also their ligands. It has been fairly well characterised that CMV downregulates HLA-A and HLA-B, but the effect on HLA-C is less well documented. As HLA-C molecules are ligands for the 2DKIRS it would be of benefit to investigate the effect of CMV on expression levels.

Finally, the work on 2DKIR and their interactions with HLA-C still needs pursuing. It would be useful to test interactions against HLA-C incorporating a larger panel of peptides. As HLA-C monomers appeared to be so unstable this may be easier to achieve at a cellular level. LCLs pulsed with peptides could be stained with KIR tetramers and differences in frequencies examined for each peptide.

Clearly there is still a lot of work to do in the field of KIR biology, but the work presented in this thesis provides a good foundation for future investigations.

List of references

Abbas,A.K. and Lichtman,A.H. (2003) Cellular and Molecular Immunology. Elsevier Science.

Abedin, S., J. J. Michel, B. Lemster and A. N. Vallejo (2005). "Diversity of NKR expression in aging T cells and in T cells of the aged: The new frontier into the exploration of protective immunity in the elderly." Experimental Gerontology **40**(7): 537-548.

Adam, E., J. L. Melnick, J. L. Probtsfield, B. L. Petrie, J. Burek, K. R. Bailey, C. H. McCollum and M. E. DeBakey (1987). "High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis." Lancet **2**(8554): 291-3.

Adam, S. G., A. Caraux, N. Fodil-Cornu, J. C. Loredo-Osti, S. Lesjean-Pottier, J. Jaubert, I. Bubic, S. Jonjic, J. L. Guenet, S. M. Vidal and F. Colucci (2006). "Cmv4, a new locus linked to the NK cell gene complex, controls innate resistance to cytomegalovirus in wild-derived mice." J Immunol **176**(9): 5478-85.

Ahlenstiel, G., M. P. Martin, X. Gao, M. Carrington and B. Rehermann (2008). "Distinct KIR/HLA compound genotypes affect the kinetics of human antiviral natural killer cell responses." J Clin Invest **118**(3): 1017-26.

Alp, N. J., T. D. Allport, J. Van Zanten, B. Rodgers, J. G. Sissons and L. K. Borysiewicz (1991). "Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein." J Virol **65**(9): 4812-20.

Alter, G., S. Rihn, H. Streeck, N. Teigen, A. Piechocka-Trocha, K. Moss, K. Cohen, A. Meier, F. Pereyra, B. Walker and M. Altfeld (2008). "Ligand-independent exhaustion of killer immunoglobulin-like receptor-positive CD8+ T cells in human immunodeficiency virus type 1 infection." J Virol **82**(19): 9668-77.

Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael and M. M. Davis (1996). "Phenotypic analysis of antigen-specific T lymphocytes." Science **274**(5284): 94-6.

Amyes, E., C. Hatton, D. Montamat-Sicotte, N. Gudgeon, A. B. Rickinson, A. J. McMichael and M. F. Callan (2003). "Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection." J Exp Med **198**(6): 903-11.

Amyes, E., A. J. McMichael and M. F. Callan (2005). "Human CD4+ T cells are predominantly distributed among six phenotypically and functionally distinct subsets." J Immunol **175**(9): 5765-73.

Anfossi, N., V. Pascal, E. Vivier and S. Ugolini (2001). "Biology of T memory type 1 cells." Immunological Reviews **181**(1): 269-278.

Anfossi, N., J. M. Doisne, M. A. Peyrat, S. Ugolini, O. Bonnaud, D. Bossy, V. Pitard, P. Merville, J. F. Moreau, J. F. Delfraissy, J. Dechanet-Merville, M. Bonneville, A. Venet and E. Vivier (2004). "Coordinated expression of Ig-like inhibitory MHC class I receptors and acquisition of cytotoxic function in human CD8+ T cells." J Immunol **173**(12): 7223-9.

Anfossi, N., P. Andre, S. Guia, C. S. Falk, S. Roetynck, C. A. Stewart, V. Breso, C. Frassati, D. Reviron, D. Middleton, F. Romagne, S. Ugolini and E. Vivier (2006). "Human NK cell education by inhibitory receptors for MHC class I." Immunity **25**(2): 331-42.

Appay, V., J. J. Zaunders, L. Papagno, J. Sutton, A. Jaramillo, A. Waters, P. Easterbrook, P. Grey, D. Smith, A. J. McMichael, D. A. Cooper, S. L. Rowland-Jones and A. D. Kelleher (2002). "Characterization of CD4(+) CTLs ex vivo." J Immunol **168**(11): 5954-8.

Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael and S. L. Rowland-Jones (2002). "Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections." Nat Med **8**(4): 379-85.

Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill and L. L. Lanier (2002). "Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors." **296**(5571): 1323.

Arase, H. and L. L. Lanier (2002). "Virus-driven evolution of natural killer cell receptors." Microbes Infect **4**(15): 1505-12.

Arlettaz, L., S. Degermann, C. De Rham, E. Roosnek and B. Huard (2004). "Expression of inhibitory KIR is confined to CD8+ effector T cells and limits their proliferative capacity." Eur J Immunol **34**(12): 3413-22.

Asanuma, H., M. Sharp, H. T. Maecker, V. C. Maino and A. M. Arvin (2000). "Frequencies of memory T cells specific for varicella-zoster virus, herpes simplex virus, and cytomegalovirus by intracellular detection of cytokine expression." J Infect Dis **181**(3): 859-66.

Badovinac, V. P., B. B. Porter and J. T. Harty (2002). "Programmed contraction of CD8(+) T cells after infection." Nat Immunol **3**(7): 619-26.

Bancroft, G. J., G. R. Shellam and J. E. Chalmer (1981). "Genetic influences on the augmentation of natural killer (NK) cells during murine cytomegalovirus infection: correlation with patterns of resistance." J Immunol **126**(3): 988-94.

Beninga, J., B. Kropff and M. Mach (1995). "Comparative analysis of fourteen individual human cytomegalovirus proteins for helper T cell response." J Gen Virol **76 (Pt 1)**: 153-60.

Beninga, J., H. Kalbacher and M. Mach (1996). "Analysis of T helper cell response to glycoprotein H (gpUL75) of human cytomegalovirus: evidence for strain-specific T cell determinants." J Infect Dis **173**(5): 1051-61.

Berg, L., G. C. Riise, D. Cosman, T. Bergstrom, S. Olofsson, K. Karre and E. Carbone (2003). "LIR-1 expression on lymphocytes, and cytomegalovirus disease in lung-transplant recipients." Lancet **361**(9363): 1099-101.

Betser-Cohen, G., G. Katz, T. Gonen-Gross, N. Stern, T. I. Arnon, H. Achdout, R. Gazit and O. Mandelboim (2006). "Reduced KIR2DL1 recognition of MHC class I molecules presenting phosphorylated peptides." J Immunol **176**(11): 6762-9.

Biassoni, R., C. Cantoni, D. Pende, S. Sivori, S. Parolini, M. Vitale, C. Bottino and A. Moretta (2001). "Human natural killer cell receptors and co-receptors." Immunol Rev **181**: 203-14.

Biron, C. A., K. S. Byron and J. L. Sullivan (1989). "Severe herpesvirus infections in an adolescent without natural killer cells." N Engl J Med **320**(26): 1731-5.

Bitmansour, A. D., S. L. Waldrop, C. J. Pitcher, E. Khatamzas, F. Kern, V. C. Maino and L. J. Picker (2001). "Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus." J Immunol **167**(3): 1151-63.

Boehme, K. W., M. Guerrero and T. Compton (2006). "Human cytomegalovirus envelope glycoproteins B and H are necessary for TLR2 activation in permissive cells." J Immunol **177**(10): 7094-102.

Bottino, C., R. Castriconi, D. Pende, P. Rivera, M. Nanni, B. Carnemolla, C. Cantoni, J. Grassi, S. Marcenaro, N. Reymond, M. Vitale, L. Moretta, M. Lopez and A. Moretta (2003). "Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule." J Exp Med **198**(4): 557-67.

Boutboul, F., D. Puthier, V. Appay, O. Pelle, H. Ait-Mohand, B. Combadiere, G. Carcelain, C. Katlama, S. L. Rowland-Jones, P. Debre, C. Nguyen and B. Autran (2005). "Modulation of interleukin-7 receptor expression characterizes differentiation of CD8 T cells specific for HIV, EBV and CMV." AIDS **19**(17): 1981-6.

Boyington, J. C., S. A. Motyka, P. Schuck, A. G. Brooks and P. D. Sun (2000). "Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand." Nature **405**(6786): 537-43.

Boyle, K. A., R. L. Pietropaolo and T. Compton (1999). "Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway." Mol Cell Biol **19**(5): 3607-13.

Boyton, R. J. and D. M. Altmann (2002). "Is selection for TCR affinity a factor in cytokine polarization?" Trends Immunol **23**(11): 526-9.

Brenchley, J. M., N. J. Karandikar, M. R. Betts, D. R. Ambrozak, B. J. Hill, L. E. Crotty, J. P. Casazza, J. Kuruppu, S. A. Migueles, M. Connors, M. Roederer, D. C. Douek and R. A. Koup (2003). "Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells." Blood **101**(7): 2711-20.

Britt, W. J. and S. Boppana (2004). "Human cytomegalovirus virion proteins." Hum Immunol **65**(5): 395-402.

Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo and W. M. Yokoyama (2001). "Vital involvement of a natural killer cell activation receptor in resistance to viral infection." Science **292**(5518): 934-7.

Burrows, S. R., R. A. Elkington, J. J. Miles, K. J. Green, S. Walker, S. M. Haryana, D. J. Moss, H. Dunckley, J. M. Burrows and R. Khanna (2003). "Promiscuous CTL recognition of viral epitopes on multiple human leukocyte antigens: biological validation of the proposed HLA A24 supertype." J Immunol **171**(3): 1407-12.

Callan, M. F., C. Fazou, H. Yang, T. Rostron, K. Poon, C. Hatton and A. J. McMichael (2000). "CD8(+) T-cell selection, function, and death in the primary immune response in vivo." J Clin Invest **106**(10): 1251-61.

Campbell, K. S., M. Cella, M. Carretero, M. Lopez-Botet and M. Colonna (1998). "Signaling through human killer cell activating receptors triggers tyrosine phosphorylation of an associated protein complex." Eur.J.Immunol. **28**(2): 599-609.

Canavez, F., N. T. Young, L. A. Guethlein, R. Rajalingam, S. I. Khakoo, B. P. Shum and P. Parham (2001). "Comparison of chimpanzee and human leukocyte Ig-like receptor genes reveals framework and rapidly evolving genes." J Immunol. **167**(10): 5786-5794.

Carr, W. H., A. M. Little, E. Mocarski and P. Parham (2002). "NK cell-mediated lysis of autologous HCMV-infected skin fibroblasts is highly variable among NK cell clones and polyclonal NK cell lines." Clin Immunol **105**(2): 126-40.

Carr, W. H., M. J. Pando and P. Parham (2005). "KIR3DL1 Polymorphisms That Affect NK Cell Inhibition by HLA-Bw4 Ligand." J Immunol **175**: 5222-5229.

Carrasco, J., D. Godelaine, A. Van Pel, T. Boon and P. van der Bruggen (2006). "CD45RA on human CD8 T cells is sensitive to the time elapsed since the last antigenic stimulation." Blood **108**(9): 2897-905.

Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek and R. A. Koup (2006). "Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation." J Exp Med **203**(13): 2865-77.

Cassatella, M. A., I. Anegon, M. C. Cuturi, P. Griskey, G. Trinchieri and B. Perussia (1989). "Fc gamma R(CD16) interaction with ligand induces Ca2+ mobilization and phosphoinositide turnover in human natural killer cells. Role of Ca2+ in Fc gamma R(CD16)-induced transcription and expression of lymphokine genes." J Exp Med **169**(2): 549-67.

Castle, S. C. (2000). "Clinical relevance of age-related immune dysfunction." Clin Infect Dis **31**(2): 578-85.

Cauda, R., E. F. Prasthofer, C. E. Grossi, R. J. Whitley and R. F. Pass (1987). "Congenital cytomegalovirus: immunological alterations." J Med Virol **23**(1): 41-9.

Cha, T. A., E. Tom, G. W. Kemble, G. M. Duke, E. S. Mocarski and R. R. Spaete (1996). "Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains." J Virol **70**(1): 78-83.

Chalifour, A., L. Scarpellino, J. Back, P. Brodin, E. Devevre, F. Gros, F. Levy, G. Leclercq, P. Hoglund, F. Beermann and W. Held (2009). "A Role for cis Interaction between the Inhibitory Ly49A receptor and MHC class I for natural killer cell education." Immunity **30**(3): 337-47.

Chalupny, N. J., A. Rein-Weston, S. Dosch and D. Cosman (2006). "Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142." Biochem Biophys Res Commun **346**(1): 175-81.

Chandler, S. H. and J. K. McDougall (1986). "Comparison of restriction site polymorphisms among clinical isolates and laboratory strains of human cytomegalovirus." J Gen Virol **67 (Pt 10)**: 2179-92.

Chapman, T. L., A. P. Heikeman and P. J. Bjorkman (1999). "The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18." Immunity **11**(5): 603-13.

Chapman, T. L., A. P. Heikema, A. P. West, Jr. and P. J. Bjorkman (2000). "Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2)." Immunity **13**(5): 727-36.

Chaux, P., V. Vantomme, V. Stroobant, K. Thielemans, J. Corthals, R. Luiten, A. M. Eggermont, T. Boon and P. van der Bruggen (1999). "Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4(+) T lymphocytes." J Exp Med **189**(5): 767-78.

Chen, G., P. Shankar, C. Lange, H. Valdez, P. R. Skolnik, L. Wu, N. Manjunath and J. Lieberman (2001). "CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection." Blood **98**(1): 156-64.

Cohen, G. B., R. T. Gandhi, D. M. Davis, O. Mandelboim, B. K. Chen, J. L. Strominger and D. Baltimore (1999). "The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells." Immunity **10**(6): 661-71.

Colonna, M., T. Spies, J. L. Strominger, E. Ciccone, A. Moretta, L. Moretta, D. Pende and O. Viale (1992). "Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene." Proc Natl Acad Sci U S A **89**(17): 7983-5.

Colonna, M., G. Borsellino, M. Falco, G. B. Ferrara and J. L. Strominger (1993). "HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells." Proc Natl Acad Sci U S A **90**(24): 12000-4.

Colonna, M., H. Nakajima, F. Navarro and M. Lopez-Botet (1999). "A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells." J Leukoc Biol **66**(3): 375-81.

Cook, M. A., P. A. Moss and D. C. Briggs (2003). "The distribution of 13 killer-cell immunoglobulin-like receptor loci in UK blood donors from three ethnic groups." Eur.J Immunogenet. **30**(3): 213-221.

Cook, M. A., D. W. Milligan, C. D. Fegan, P. J. Darbyshire, P. Mahendra, C. F. Craddock, P. A. H. Moss and D. C. Briggs (2004). "The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling hematopoietic stem cell transplantation for myeloid leukemia." Blood **103**(4): 1521-1526.

Cook, M., D. Briggs, C. Craddock, P. Mahendra, D. Milligan, C. Fegan, P. Darbyshire, S. Lawson, E. Boxall and P. Moss (2005). "Donor KIR genotype has a major influence on the rate of cytomegalovirus reactivation following T-cell replete stem cell transplantation." Blood: 2005-03.

Cooley, S., F. Xiao, M. Pitt, M. Gleason, V. McCullar, T. L. Bergemann, K. L. McQueen, L. A. Guethlein, P. Parham and J. S. Miller (2007). "A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature." Blood **110**(2): 578-86.

Cooper, M. A., T. A. Fehniger, A. Fuchs, M. Colonna and M. A. Caligiuri (2004). "NK cell and DC interactions." Trends Immunol **25**(1): 47-52.

Correa, I. and D. H. Raulet (1995). "Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells." Immunity **2**(1): 61-71.

Costello, R. T., S. Sivori, E. Marcenaro, M. Lafage-Pochitaloff, M. J. Mozziconacci, D. Reviron, J. A. Gastaut, D. Pende, D. Olive and A. Moretta (2002). "Defective expression and function of natural killer cell-triggering receptors in patients with acute myeloid leukemia." Blood **99**(10): 3661-7.

Cranage, M. P., T. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, H. Hart, S. E. Bell, A. C. Minson and et al. (1986). "Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus." EMBO J **5**(11): 3057-63.

Crum, K. A., S. E. Logue, M. D. Curran and D. Middleton (2000). "Development of a PCR-SSOP approach capable of defining the natural killer cell inhibitory receptor (KIR) gene sequence repertoires." Tissue Antigens **56**(4): 313-26.

Daniels, K. A., G. Devora, W. C. Lai, C. L. O'Donnell, M. Bennett and R. M. Welsh (2001). "Murine cytomegalovirus is regulated by a discrete subset of natural killer cells reactive with monoclonal antibody to Ly49H." J Exp Med **194**(1): 29-44.

David, G., M. Morvan, K. Gagne, N. Kerdudou, C. Willem, A. Devys, M. Bonneville, G. Follea, J. D. Bignon and C. Retiere (2009). "Discrimination between the main activating and inhibitory killer cell immunoglobulin-like receptor positive natural killer cell subsets using newly characterized monoclonal antibodies." Immunology **128**(2): 172-84.

Davignon, J. L., P. Castanie, J. A. Yorke, N. Gautier, D. Clement and C. Davrinche (1996). "Anti-human cytomegalovirus activity of cytokines produced by CD4+ T-cell clones specifically activated by IE1 peptides in vitro." J Virol **70**(4): 2162-9.

Davrinche, C., C. Pasquier, M. Cerutti, L. Serradell, D. Clement, G. Devauchelle, S. Michelson and J. L. Davignon (1993). "Expression of human cytomegalovirus immediate early protein IE1 in insect cells: splicing of RNA and recognition by CD4+ T-cell clones." Biochem Biophys Res Commun **195**(1): 469-77.

Day, C. L., N. P. Seth, M. Lucas, H. Appel, L. Gauthier, G. M. Lauer, G. K. Robbins, Z. M. Szczepiorkowski, D. R. Casson, R. T. Chung, S. Bell, G. Harcourt, B. D. Walker, P. Klenerman and K. W. Wucherpfennig (2003). "Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers." J Clin Invest **112**(6): 831-42.

De Maria, A., M. Fogli, P. Costa, G. Murdaca, F. Puppo, D. Mavilio, A. Moretta and L. Moretta (2003). "The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44)." Eur J Immunol **33**(9): 2410-8.

de Wit, T. P., H. C. Morton, P. J. Capel and J. G. van de Winkel (1995). "Structure of the gene for the human myeloid IgA Fc receptor (CD89)." J Immunol **155**(3): 1203-9.

Dechanet, J., P. Merville, A. Lim, C. Retiere, V. Pitard, X. Lafarge, S. Michelson, C. Meric, M. M. Hallet, P. Kourilsky, L. Potaux, M. Bonneville and J. F. Moreau (1999). "Implication of gammadelta T cells in the human immune response to cytomegalovirus." J Clin Invest **103**(10): 1437-49.

Denduluri, N. and W. B. Ershler (2004). "Aging biology and cancer." Semin Oncol **31**(2): 137-48.

Dobromylskyj, M. and S. Ellis (2007). "Complexity in cattle KIR genes: transcription and genome analysis." Immunogenetics **59**(6): 463-72.

Dolan, A., C. Cunningham, R. D. Hector, A. F. Hassan-Walker, L. Lee, C. Addison, D. J. Dargan, D. J. McGeoch, D. Gatherer, V. C. Emery, P. D. Griffiths, C. Sinzger, B. P. McSharry, G. W. Wilkinson and A. J. Davison (2004). "Genetic content of wild-type human cytomegalovirus." J Gen Virol **85**(Pt 5): 1301-12.

Dong, H., L. Nilsson and C. G. Kurland (1996). "Co-variation of tRNA abundance and codon usage in Escherichia coli at different growth rates." J Mol Biol **260**(5): 649-63.

Dunn, R. V., V. Reat, J. Finney, M. Ferrand, J. C. Smith and R. M. Daniel (2000). "Enzyme activity and dynamics: xylanase activity in the absence of fast anharmonic dynamics." Biochem J **346 Pt 2**: 355-8.

Dunne, P. J., J. M. Faint, N. H. Gudgeon, J. M. Fletcher, F. J. Plunkett, M. V. Soares, A. D. Hislop, N. E. Annels, A. B. Rickinson, M. Salmon and A. N. Akbar (2002). "Epstein-Barr virus-specific CD8(+) T cells that re-express CD45RA are apoptosis-resistant memory cells that retain replicative potential." Blood **100**(3): 933-40.

Elkington, R., S. Walker, T. Crough, M. Menzies, J. Tellam, M. Bharadwaj and R. Khanna (2003). "Ex vivo profiling of CD8+-T-cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers." J Virol **77**(9): 5226-40.

Elkington, R., N. H. Shoukry, S. Walker, T. Crough, C. Fazou, A. Kaur, C. M. Walker and R. Khanna (2004). "Cross-reactive recognition of human and primate cytomegalovirus sequences by human CD4 cytotoxic T lymphocytes specific for glycoprotein B and H." Eur J Immunol **34**(11): 3216-26.

Elkington, R. and R. Khanna (2005). "Cross-recognition of human alloantigen by cytomegalovirus glycoprotein-specific CD4+ cytotoxic T lymphocytes: implications for graft-versus-host disease." Blood **105**(3): 1362-4.

Ellefsen, K., A. Harari, P. Champagne, P. A. Bart, R. P. Sekaly and G. Pantaleo (2002). "Distribution and functional analysis of memory antiviral CD8 T cell responses in HIV-1 and cytomegalovirus infections." Eur J Immunol **32**(12): 3756-64.

Fan, Q. R., D. N. Garboczi, C. C. Winter, N. Wagtmann, E. O. Long and D. C. Wiley (1996). "Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule." Proc Natl Acad Sci U S A **93**(14): 7178-83.

Fan, Q. R., E. O. Long and D. C. Wiley (2001). "Crystal structure of the human natural killer cell inhibitory receptor KIR2DL1-HLA-Cw4 complex." Nat Immunol **2**(5): 452-60.

Fauriat, C., S. Just-Landi, F. Mallet, C. Arnoulet, D. Sainty, D. Olive and R. T. Costello (2007). "Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction." Blood **109**(1): 323-30.

Ferlazzo, G., D. Thomas, S. L. Lin, K. Goodman, B. Morandi, W. A. Muller, A. Moretta and C. Munz (2004). "The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic." J Immunol **172**(3): 1455-62.

Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux and D. H. Raulet (2005). "A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules." Blood **105**(11): 4416-23.

Fletcher, J. M., M. Vukmanovic-Stejic, P. J. Dunne, K. E. Birch, J. E. Cook, S. E. Jackson, M. Salmon, M. H. Rustin and A. N. Akbar (2005). "Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion." J Immunol **175**(12): 8218-25.

Frankenberg, N., S. Pepperl-Klindworth, R. G. Meyer and B. Plachter (2002). "Identification of a conserved HLA-A2-restricted decapeptide from the IE1 protein (pUL123) of human cytomegalovirus." Virology **295**(2): 208-16.

Fuchs, A., M. Cella, E. Giurisato, A. S. Shaw and M. Colonna (2004). "Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155)." J Immunol **172**(7): 3994-8.

Gamadia, L. E., R. J. Rentenaar, P. A. Baars, E. B. Remmerswaal, S. Surachno, J. F. Weel, M. Toebes, T. N. Schumacher, I. J. ten Berge and R. A. van Lier (2001). "Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers." Blood **98**(3): 754-61.

Gamadia, L. E., E. B. Remmerswaal, J. F. Weel, F. Bemelman, R. A. van Lier and I. J. Ten Berge (2003). "Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease." Blood **101**(7): 2686-92.

Gamadia, L. E., R. J. Rentenaar, R. A. van Lier and I. J. ten Berge (2004). "Properties of CD4(+) T cells in human cytomegalovirus infection." Hum Immunol **65**(5): 486-92.

Garboczi, D. N., D. T. Hung and D. C. Wiley (1992). "HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in Escherichia coli and complexed with single antigenic peptides." Proc Natl Acad Sci U S A **89**(8): 3429-33.

Garcia-Sastre, A. and C. A. Biron (2006). "Type 1 interferons and the virus-host relationship: a lesson in detente." Science **312**(5775): 879-82.

Garcia, S., J. DiSanto and B. Stockinger (1999). "Following the development of a CD4 T cell response in vivo: from activation to memory formation." Immunity **11**(2): 163-71.

Gardiner, C. M., L. A. Guethlein, H. G. Shilling, M. Pando, W. H. Carr, R. Rajalingam, C. Vilches and P. Parham (2001). "Different NK cell surface phenotypes defined by the DX9 antibody are due to KIR3DL1 gene polymorphism." J.Immunol. **166**(5): 2992-3001.

Gautier, N., E. Chavant, E. Prieur, B. Monsarrat, H. Mazarguil, C. Davrinche, J. E. Gairin and J. L. Davignon (1996). "Characterization of an epitope of the human cytomegalovirus protein IE1 recognized by a CD4+ T cell clone." Eur J Immunol **26**(5): 1110-7.

Gazit, R., B. Z. Garty, Y. Monselise, V. Hoffer, Y. Finkelstein, G. Markel, G. Katz, J. Hanna, H. Achdout, R. Gruda, T. Gonen-Gross and O. Mandelboim (2004). "Expression of KIR2DL1 on the entire NK cell population: a possible novel immunodeficiency syndrome." Blood **103**(5): 1965-6.

Geginat, J., F. Sallusto and A. Lanzavecchia (2001). "Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells." J Exp Med **194**(12): 1711-9.

Gillespie, G. M., M. R. Wills, V. Appay, C. O'Callaghan, M. Murphy, N. Smith, P. Sissons, S. Rowland-Jones, J. I. Bell and P. A. Moss (2000). "Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors." J Virol **74**(17): 8140-50.

Gillespie, G. M., A. Bashirova, T. Dong, D. W. McVicar, S. L. Rowland-Jones and M. Carrington (2007). "Lack of KIR3DS1 binding to MHC class I Bw4 tetramers in complex with CD8+ T cell epitopes." AIDS Res Hum Retroviruses **23**(3): 451-5.

Golden-Mason, L., J. R. Burton, Jr., N. Castelblanco, J. Klarquist, S. Benlloch, C. Wang and H. R. Rosen (2006). "Loss of IL-7 receptor alpha-chain (CD127) expression in acute HCV infection associated with viral persistence." Hepatology **44**(5): 1098-109.

Goldrath, A. W., P. V. Sivakumar, M. Glaccum, M. K. Kennedy, M. J. Bevan, C. Benoist, D. Mathis and E. A. Butz (2002). "Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells." J Exp Med **195**(12): 1515-22.

Gomez-Lozano, N., C. M. Gardiner, P. Parham and C. Vilches (2002). "Some human KIR haplotypes contain two KIR2DL5 genes: KIR2DL5A and KIR2DL5B." Immunogenetics **54**(5): 314-9.

Grayson, J. M., A. J. Zajac, J. D. Altman and R. Ahmed (2000). "Cutting edge: increased expression of Bcl-2 in antigen-specific memory CD8+ T cells." J Immunol **164**(8): 3950-4.

Grendell, R. L., A. L. Hughes and T. G. Golos (2001). "Cloning of rhesus monkey killer-cell Ig-like receptors (KIRs) from early pregnancy decidua." Tissue Antigens **58**(5): 329-334.

Guethlein, L. A., L. R. Flodin, E. J. Adams and P. Parham (2002). "NK Cell Receptors of the Orangutan (Pongo pygmaeus): A Pivotal Species for Tracking the Coevolution of Killer Cell Ig-Like Receptors with MHC-C." J.Immunol. **169**(1): 220-229.

Guma, M., A. Angulo, C. Vilches, N. Gomez-Lozano, N. Malats and M. Lopez-Botet (2004). "Imprint of human cytomegalovirus infection on the NK cell receptor repertoire." Blood **104**(12): 3664-71.

Gupta, S. (2005). "Molecular mechanisms of apoptosis in the cells of the immune system in human aging." Immunol Rev **205**: 114-29.

Gussow, D., R. S. Rein, I. Meijer, W. de Hoog, G. H. Seemann, F. M. Hochstenbach and H. L. Ploegh (1987). "Isolation, expression, and the primary structure of HLA-Cw1 and HLA-Cw2 genes: evolutionary aspects." Immunogenetics **25**(5): 313-22.

Hamann, D., P. A. Baars, M. H. Rep, B. Hooibrink, S. R. Kerkhof-Garde, M. R. Klein and R. A. van Lier (1997). "Phenotypic and functional separation of memory and effector human CD8+ T cells." J Exp Med **186**(9): 1407-18.

Hansasuta, P., T. Dong, H. Thananchai, M. Weekes, C. Willberg, H. Aldemir, S. Rowland-Jones and V. M. Braud (2004). "Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific." Eur J Immunol **34**(6): 1673-9.

Harari, A., F. Vallelian and G. Pantaleo (2004). "Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load." Eur J Immunol **34**(12): 3525-33.

Harcourt, G. C., T. J. Scriba, N. Semmo, S. Bounds, E. Taylor and P. Klenerman (2006). "Identification of key peptide-specific CD4+ T cell responses to human cytomegalovirus: implications for tracking antiviral populations." Clin Exp Immunol **146**(2): 203-10.

Held, W., J. Roland and D. H. Raulet (1995). "Allelic exclusion of Ly49-family genes encoding class I MHC-specific receptors on NK cells." Nature **376**(6538): 355-358.

Held, W. and R. A. Mariuzza (2008). "Cis interactions of immunoreceptors with MHC and non-MHC ligands." Nat Rev Immunol **8**(4): 269-78.

Hershberger, K. L., R. Shyam, A. Miura and N. L. Letvin (2001). "Diversity of the killer cell Ig-like receptors of rhesus monkeys." J.Immunol. **166**(7): 4380-4390.

Hiby, S. E., J. J. Walker, M. O'Shaughnessy K, C. W. Redman, M. Carrington, J. Trowsdale and A. Moffett (2004). "Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success." J Exp Med **200**(8): 957-65.

Hiby, S. E., L. Regan, W. Lo, L. Farrell, M. Carrington and A. Moffett (2008). "Association of maternal killer-cell immunoglobulin-like receptors and parental HLA-C genotypes with recurrent miscarriage." Hum Reprod **23**(4): 972-6.

Hirose, J., T. Kouro, H. Igarashi, T. Yokota, N. Sakaguchi and P. W. Kincade (2002). "A developing picture of lymphopoiesis in bone marrow." Immunol Rev **189**: 28-40.

Hislop, A. D., N. E. Annels, N. H. Gudgeon, A. M. Leese and A. B. Rickinson (2002). "Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection." J Exp Med **195**(7): 893-905.

Hooper, M., E. G. Kallas, D. Coffin, D. Campbell, T. G. Evans and R. J. Looney (1999). "Cytomegalovirus seropositivity is associated with the expansion of CD4+CD28- and CD8+CD28- T cells in rheumatoid arthritis." J Rheumatol **26**(7): 1452-7.

Hopkins, J. I., A. N. Fiander, A. S. Evans, M. Delchambre, D. Gheysen and L. K. Borysiewicz (1996). "Cytotoxic T cell immunity to human cytomegalovirus glycoprotein B." J Med Virol **49**(2): 124-31.

Horvath, R., J. Cerny, J. Benedik, Jr., J. Hokl, I. Jelinkova and J. Benedik (2000). "The possible role of human cytomegalovirus (HCMV) in the origin of atherosclerosis." J Clin Virol **16**(1): 17-24.

Hsu, K. C., S. Chida, D. E. Geraghty and B. Dupont (2002a). "The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism." Immunological Reviews **190**(1): 40-52.

Hsu, K. C., X. R. Liu, A. Selvakumar, E. Mickelson, R. J. O'Reilly and B. Dupont (2002c). "Killer Ig-Like Receptor Haplotype Analysis by Gene Content: Evidence for Genomic Diversity with a Minimum of Six Basic Framework Haplotypes, Each with Multiple Subsets." The Journal of Immunology **169**(9): 5118-5129.

Huard, B. and L. Karlsson (2000). "KIR expression on self-reactive CD8+ T cells is controlled by T-cell receptor engagement." Nature **403**(6767): 325-8.

Huard, B. and K. Fruh (2000). "A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells." Eur J Immunol **30**(2): 509-15.

Huntington, N. D., C. A. Vosshenrich and J. P. Di Santo (2007). "Developmental pathways that generate natural-killer-cell diversity in mice and humans." Nat Rev Immunol **7**(9): 703-14.

Iezzi, G., E. Scotet, D. Scheidegger and A. Lanzavecchia (1999). "The interplay between the duration of TCR and cytokine signaling determines T cell polarization." Eur J Immunol **29**(12): 4092-101.

Inoue, H., H. Nojima and H. Okayama (1990). "High efficiency transformation of Escherichia coli with plasmids." Gene **96**(1): 23-8.Jenkins, M. K., A. Khoruts, E. Ingulli, D. L. Mueller, S. J. McSorley, R. L. Reinhardt, A. Itano and K. A. Pape (2001). "In vivo activation of antigen-specific CD4 T cells." Annu Rev Immunol **19**: 23-45.

Jie, H. B. and N. Sarvetnick (2004). "The role of NK cells and NK cell receptors in autoimmune disease." Autoimmunity **37**(2): 147-53.

Jonjic, S., I. Pavic, P. Lucin, D. Rukavina and U. H. Koszinowski (1990). "Efficacious control of cytomegalovirus infection after long-term depletion of CD8+ T lymphocytes." J Virol **64**(11): 5457-64.

Kaech, S. M. and R. Ahmed (2001). "Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells." Nat Immunol **2**(5): 415-22.

Kaech, S. M., S. Hemby, E. Kersh and R. Ahmed (2002). "Molecular and functional profiling of memory CD8 T cell differentiation." Cell **111**(6): 837-51.

Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh and R. Ahmed (2003). "Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells." Nat Immunol **4**(12): 1191-8.

Karre, K., H. G. Ljunggren, G. Piontek and R. Kiessling (1986). "Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy." Nature **319**(6055): 675-8.

Karrer, U., S. Sierro, M. Wagner, A. Oxenius, H. Hengel, U. H. Koszinowski, R. E. Phillips and P. Klenerman (2003). "Memory inflation: continuous accumulation of antiviral CD8+ T cells over time." J Immunol **170**(4): 2022-9.

Katz, G., R. Gazit, T. I. Arnon, T. Gonen-Gross, G. Tarcic, G. Markel, R. Gruda, H. Achdout, O. Drize, S. Merims and O. Mandelboim (2004). "MHC class I-independent recognition of NK-activating receptor KIR2DS4." J Immunol **173**(3): 1819-25.

Katz, G., G. Markel, S. Mizrahi, T. I. Arnon and O. Mandelboim (2001). "Recognition of HLA-Cw4 but not HLA-Cw6 by the NK cell receptor killer cell Ig-like receptor two-domain short tail number 4." J Immunol **166**(12): 7260-7.

Kelley, J., L. Walter and J. Trowsdale (2005). "Comparative genomics of natural killer cell receptor gene clusters." PLoS Genet **1**(2): 129-39.

Kern, F., E. Khatamzas, I. Surel, C. Frommel, P. Reinke, S. L. Waldrop, L. J. Picker and H. D. Volk (1999a). "Distribution of human CMV-specific memory T cells among the CD8pos. subsets defined by CD57, CD27, and CD45 isoforms." Eur J Immunol **29**(9): 2908-15.

Kern, F., I. P. Surel, N. Faulhaber, C. Frommel, J. Schneider-Mergener, C. Schonemann, P. Reinke and H. D. Volk (1999b). "Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited." J Virol **73**(10): 8179-84.

Kern, F., T. Bunde, N. Faulhaber, F. Kiecker, E. Khatamzas, I. M. Rudawski, A. Pruss, J. W. Gratama, R. Volkmer-Engert, R. Ewert, P. Reinke, H. D. Volk and L. J. Picker (2002). "Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals." J Infect Dis **185**(12): 1709-16.

Khakoo, S. I., R. Geller, S. Shin, J. A. Jenkins and P. Parham (2002). "The D0 domain of KIR3D acts as a major histocompatibility complex class I binding enhancer." J Exp Med **196**(7): 911-21.

Khakoo, S. I., C. L. Thio, M. P. Martin, C. R. Brooks, X. Gao, J. Astemborski, J. Cheng, J. J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A. M. Little, G. J. Alexander, M. E. Cramp, S. J. O'Brien, W. M. Rosenberg, D. L. Thomas and M. Carrington (2004). "HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection." Science **305**(5685): 872-4.

Khakoo, S. I. and M. Carrington (2006). "KIR and disease: a model system or system of models?" Immunol Rev **214**: 186-201.

Khan, N., N. Shariff, M. Cobbold, R. Bruton, J. A. Ainsworth, A. J. Sinclair, L. Nayak and P. A. Moss (2002). "Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals." J Immunol **169**(4): 1984-92.

Khan, N., A. Hislop, N. Gudgeon, M. Cobbold, R. Khanna, L. Nayak, A. B. Rickinson and P. A. Moss (2004). "Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection." J Immunol **173**(12): 7481-9.

Khanna, R., S. R. Burrows, P. M. Steigerwald-Mullen, S. A. Thomson, M. G. Kurilla and D. J. Moss (1995). "Isolation of Cytotoxic T Lymphocytes from Healthy Seropositive Individuals Specific for Peptide Epitopes from Epstein-Barr Virus Nuclear Antigen 1: Implications for Viral Persistence and Tumor Surveillance1." Virology **214**(2): 633-637.

Khanna, R. and S. R. Burrows (2000). "Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases." Annu Rev Microbiol **54**: 19-48.

Khanna, R. and D. J. Diamond (2006). "Human cytomegalovirus vaccine: time to look for alternative options." Trends Mol Med **12**(1): 26-33.

Khattab, B. A., W. Lindenmaier, R. Frank and H. Link (1997). "Three T-cell epitopes within the C-terminal 265 amino acids of the matrix protein pp65 of human cytomegalovirus recognized by human lymphocytes." J Med Virol **52**(1): 68-76.

Kiessling, R., E. Klein and H. Wigzell (1975). ""Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype." Eur J Immunol **5**(2): 112-7.

Kikuchi-Maki, A., S. Yusa, T. L. Catina and K. S. Campbell (2003). "KIR2DL4 is an IL-2-regulated NK cell receptor that exhibits limited expression in humans but triggers strong IFN-gamma production." The Journal of Immunology **171**(7): 3415-3425.

Kikuchi-Maki, A., T. L. Catina and K. S. Campbell (2005b). "Cutting Edge: KIR2DL4 Transduces Signals into Human NK Cells through Association with the Fc Receptor Protein." J Immunol **174**: 3859-3863.

Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen and W. M. Yokoyama (2005). "Licensing of natural killer cells by host major histocompatibility complex class I molecules." Nature **436**(7051): 709-13.

Klimpel, G. R., D. W. Niesel, M. Asuncion and K. D. Klimpel (1988). "Natural killer cell activation and interferon production by peripheral blood lymphocytes after exposure to bacteria." Infect Immun **56**(6): 1436-41.

Kogure, T., H. Fujinaga, A. Niizawa, L. X. Hai, Y. Shimada, H. Ochiai and K. Terasawa (1999). "Killer-cell inhibitory receptors, CD158a/b, are upregulated by interleukin-2, but not interferon-gamma or interleukin-4." Mediators Inflamm **8**(6): 313-8.

Komanduri, K. V., S. M. Donahoe, W. J. Moretto, D. K. Schmidt, G. Gillespie, G. S. Ogg, M. Roederer, D. F. Nixon and J. M. McCune (2001). "Direct measurement of CD4+ and CD8+ T-cell responses to CMV in HIV-1-infected subjects." Virology **279**(2): 459-70.

Kondo, K., H. Kaneshima and E. S. Mocarski (1994). "Human cytomegalovirus latent infection of granulocyte-macrophage progenitors." Proc Natl Acad Sci U S A **91**(25): 11879-83.

Kondo, E., Y. Akatsuka, K. Kuzushima, K. Tsujimura, S. Asakura, K. Tajima, Y. Kagami, Y. Kodera, M. Tanimoto, Y. Morishima and T. Takahashi (2004). "Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles." Blood **103**(2): 630-8.

Krmpotic, A., I. Bubic, B. Polic, P. Lucin and S. Jonjic (2003). "Pathogenesis of murine cytomegalovirus infection." Microbes Infect **5**(13): 1263-77.

Lanier, L. L. (2005). "NK cell recognition." Annu Rev Immunol **23**: 225-74.

Lau, L. L., B. D. Jamieson, T. Somasundaram and R. Ahmed (1994). "Cytotoxic T-cell memory without antigen." Nature **369**(6482): 648-52.

Li, C. R., P. D. Greenberg, M. J. Gilbert, J. M. Goodrich and S. R. Riddell (1994). "Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis." Blood **83**(7): 1971-9.

Li, B., H. C. Bisgaard and V. E. Forbes (2004). "Identification and expression of two novel cytochrome P450 genes, belonging to CYP4 and a new CYP331 family, in the polychaete Capitella capitata sp.I." Biochem Biophys Res Commun **325**(2): 510-7.

Lian, R. H., Y. Li, S. Kubota, D. L. Mager and F. Takei (1999). "Recognition of class I MHC by NK receptor Ly-49C: identification of critical residues." J.Immunol. **162**(12): 7271-7276.

Liu, N., T. Phillips, M. Zhang, Y. Wang, J. T. Opferman, R. Shah and P. G. Ashton-Rickardt (2004). "Serine protease inhibitor 2A is a protective factor for memory T cell development." Nat Immunol **5**(9): 919-26.

Ljunggren, H. G. and K. Karre (1990). "In search of the 'missing self': MHC molecules and NK cell recognition." Immunol Today **11**(7): 237-44.

Llano, M., M. Guma, M. Ortega, A. Angulo and M. Lopez-Botet (2003). "Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets." Eur J Immunol **33**(10): 2744-54.

Lodoen, M. B. and L. L. Lanier (2005). "Viral modulation of NK cell immunity." Nat Rev Microbiol **3**(1): 59-69.

Loh, J., D. T. Chu, A. K. O'Guin, W. M. Yokoyama and H. W. t. Virgin (2005). "Natural killer cells utilize both perforin and gamma interferon to regulate murine cytomegalovirus infection in the spleen and liver." J Virol **79**(1): 661-7.

Long, E. O., D. F. Barber, D. N. Burshtyn, M. Faure, M. Peterson, S. Rajagopalan, V. Renard, M. Sandusky, C. C. Stebbins, N. Wagtmann and C. Watzl (2001). "Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158)." Immunol Rev **181**: 223-33.

Long, H. M., T. A. Haigh, N. H. Gudgeon, A. M. Leen, C. W. Tsang, J. Brooks, E. Landais, E. Houssaint, S. P. Lee, A. B. Rickinson and G. S. Taylor (2005). "CD4+ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines." J Virol **79**(8): 4896-907.

Lopez-Botet, M. and T. Bellon (1999). "Natural killer cell activation and inhibition by receptors for MHC class I." Curr Opin Immunol **11**(3): 301-7.

Lowe, D. P., M. A. Cook, S. J. Bowman and D. C. Briggs (2009). "Association of killer cell immunoglobulin-like receptors with primary Sjogren's syndrome." Rheumatology (Oxford) **48**(4): 359-62.

Lucas, M., C. L. Day, J. R. Wyer, S. L. Cunliffe, A. Loughry, A. J. McMichael and P. Klenerman (2004). "Ex vivo phenotype and frequency of influenza virus-specific CD4 memory T cells." J Virol **78**(13): 7284-7.

Luszczek, W., M. Manczak, M. Cislo, P. Nockowski, A. Wisniewski, M. Jasek and P. Kusnierczyk (2004). "Gene for the activating natural killer cell receptor, KIR2DS1, is associated with susceptibility to psoriasis vulgaris." Hum Immunol **65**(7): 758-66.

Macallan, D. C., D. Wallace, Y. Zhang, C. De Lara, A. T. Worth, H. Ghattas, G. E. Griffin, P. C. Beverley and D. F. Tough (2004). "Rapid turnover of effector-memory CD4(+) T cells in healthy humans." J Exp Med **200**(2): 255-60.

Makoff, A. J., M. D. Oxer, M. A. Romanos, N. F. Fairweather and S. Ballantine (1989). "Expression of tetanus toxin fragment C in E. coli: high level expression by removing rare codons." Nucleic Acids Res **17**(24): 10191-202.

Mandelboim, O., H. T. Reyburn, M. Vales-Gomez, L. Pazmany, M. Colonna, G. Borsellino and J. L. Strominger (1996). "Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules." J Exp Med **184**(3): 913-22.

Marsh, S. G., P. Parham, B. Dupont, D. E. Geraghty, J. Trowsdale, D. Middleton, C. Vilches, M. Carrington, C. Witt, L. A. Guethlein, H. Shilling, C. A. Garcia, K. C. Hsu and H. Wain (2003). "Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002." Tissue Antigens **62**(1): 79-86.

Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia and F. Sallusto (2004). "Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming." Nat Immunol **5**(12): 1260-5.

Martin, A. M., E. M. Freitas, C. S. Witt and F. T. Christiansen (2000). "The genomic organization and evolution of the natural killer immunoglobulin-like receptor (KIR) gene cluster." Immunogenetics **51**(4-5): 268-80.

Martin, M. P., G. Nelson, J. H. Lee, F. Pellett, X. Gao, J. Wade, M. J. Wilson, J. Trowsdale, D. Gladman and M. Carrington (2002). "Cutting edge: susceptibility to psoriatic arthritis: influence of activating killer Ig-like receptor genes in the absence of specific HLA-C alleles." J Immunol **169**(6): 2818-22.

Martin, M. P., Y. Qi, X. Gao, E. Yamada, J. N. Martin, F. Pereyra, S. Colombo, E. E. Brown, W. L. Shupert, J. Phair, J. J. Goedert, S. Buchbinder, G. D. Kirk, A. Telenti, M. Connors, S. J. O'Brien, B. D. Walker, P. Parham, S. G. Deeks, D. W. McVicar and M. Carrington (2007). "Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1." Nat Genet **39**(6): 733-40.

Marx, J. L. (1980). "Natural killer cells help defend the body." Science **210**(4470): 624-6.

Masopust, D., V. Vezys, A. L. Marzo and L. Lefrancois (2001). "Preferential localization of effector memory cells in nonlymphoid tissue." Science **291**(5512): 2413-7.

Masuda, A., A. Nakamura, T. Maeda, Y. Sakamoto and T. Takai (2007). "Cis binding between inhibitory receptors and MHC class I can regulate mast cell activation." J Exp Med **204**(4): 907-20.

Maxwell, L. D., A. Wallace, D. Middleton and M. D. Curran (2002). "A common KIR2DS4 deletion variant in the human that predicts a soluble KIR molecule analogous to the KIR1D molecule observed in the rhesus monkey." Tissue Antigens **60**(3): 254-8.

McLaughlin-Taylor, E., H. Pande, S. J. Forman, B. Tanamachi, C. R. Li, J. A. Zaia, P. D. Greenberg and S. R. Riddell (1994). "Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes." J Med Virol **43**(1): 103-10.

Mercado, R., S. Vijh, S. E. Allen, K. Kerksiek, I. M. Pilip and E. G. Pamer (2000). "Early programming of T cell populations responding to bacterial infection." J Immunol **165**(12): 6833-9.

Middleton, D., A. S. Diler, A. Meenagh, C. Sleator and P. A. Gourraud (2009). "Killer immunoglobulin-like receptors (KIR2DL2 and/or KIR2DS2) in presence of their ligand (HLA-C1 group) protect against chronic myeloid leukaemia." Tissue Antigens **73**(6): 553-60.

Mingari, M. C., F. Schiavetti, M. Ponte, C. Vitale, E. Maggi, S. Romagnani, J. Demarest, G. Pantaleo, A. S. Fauci and L. Moretta (1996). "Human CD8+ T lymphocyte subsets that express HLA class I-specific inhibitory receptors represent oligoclonally or monoclonally expanded cell populations." Proc Natl Acad Sci U S A **93**(22): 12433-8.

Mingari, M. C., M. Ponte, C. Vitale, R. Bellomo and L. Moretta (2000). "Expression of HLA class I-specific inhibitory receptors in human cytolytic T lymphocytes: a regulated mechanism that controls T-cell activation and function." Hum Immunol **61**(1): 44-50.

Mitchell, D. A., W. Xie, R. Schmittling, C. Learn, A. Friedman, R. E. McLendon and J. H. Sampson (2008). "Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma." Neuro Oncol **10**(1): 10-8.

Mocarski, E. S., Jr. (2002). "Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion." Trends Microbiol **10**(7): 332-9.

Moesta, A. K., P. J. Norman, M. Yawata, N. Yawata, M. Gleimer and P. Parham (2008). "Synergistic polymorphism at two positions distal to the ligand-binding site makes KIR2DL2 a stronger receptor for HLA-C than KIR2DL3." J Immunol **180**(6): 3969-79.

Momot, T., S. Koch, N. Hunzelmann, T. Krieg, K. Ulbricht, R. E. Schmidt and T. Witte (2004). "Association of killer cell immunoglobulin-like receptors with scleroderma." Arthritis Rheum **50**(5): 1561-5.

Moretta, A., G. Tambussi, E. Ciccone, D. Pende, G. Melioli and L. Moretta (1989). "CD16 surface molecules regulate the cytolytic function of CD3CD16+ human natural killer cells." Int J Cancer **44**(4): 727-30.

Moretta, L., R. Biassoni, C. Bottino, C. Cantoni, D. Pende, M. C. Mingari and A. Moretta (2002). "Human NK cells and their receptors." Microbes Infect **4**(15): 1539-44.

Moretta, L. and A. Moretta (2004). "Killer immunoglobulin-like receptors." Curr Opin Immunol **16**(5): 626-33.

Munz, C., K. L. Bickham, M. Subklewe, M. L. Tsang, A. Chahroudi, M. G. Kurilla, D. Zhang, M. O'Donnell and R. M. Steinman (2000). "Human CD4(+) T lymphocytes consistently respond to the latent Epstein-Barr virus nuclear antigen EBNA1." J Exp Med **191**(10): 1649-60.

Murali-Krishna, K., L. L. Lau, S. Sambhara, F. Lemonnier, J. Altman and R. Ahmed (1999). "Persistence of memory CD8 T cells in MHC class I-deficient mice." Science **286**(5443): 1377-81.

Namekawa, T., M. R. Snyder, J. H. Yen, B. E. Goehring, P. J. Leibson, C. M. Weyand and J. J. Goronzy (2000). "Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis." J Immunol **165**(2): 1138-45.

Nelson, G. W., M. P. Martin, D. Gladman, J. Wade, J. Trowsdale and M. Carrington (2004). "Cutting edge: heterozygote advantage in autoimmune disease: hierarchy of protection/susceptibility conferred by HLA and killer Ig-like receptor combinations in psoriatic arthritis." J Immunol **173**(7): 4273-6.

Nieto, F. J., E. Adam, P. Sorlie, H. Farzadegan, J. L. Melnick, G. W. Comstock and M. Szklo (1996). "Cohort study of cytomegalovirus infection as a risk factor for carotid intimal-medial thickening, a measure of subclinical atherosclerosis." Circulation **94**(5): 922-7.

Norman, P. J., H. A. Stephens, D. H. Verity, D. Chandanayingyong and R. W. Vaughan (2001). "Distribution of natural killer cell immunoglobulin-like receptor sequences in three ethnic groups." Immunogenetics **52**(3-4): 195-205.

Norman, P. J., L. Abi-Rached, K. Gendzekhadze, D. Korbel, M. Gleimer, D. Rowley, D. Bruno, C. V. Carrington, D. Chandanayingyong, Y. H. Chang, C. Crespi, G. Saruhan-Direskeneli, P. A. Fraser, K. Hameed, G. Kamkamidze, K. A. Koram, Z. Layrisse, N. Matamoros, J. Mila, M. H. Park, R. M. Pitchappan, D. D. Ramdath, M. Y. Shiau, H. A. Stephens, S. Struik, D. H. Verity, R. W. Vaughan, D. Tyan, R. W. Davis, E. M. Riley, M. Ronaghi and P. Parham (2007). "Unusual selection on the KIR3DL1/S1 natural killer cell receptor in Africans." Nat Genet **39**(9): 1092-9.

Nurieva, R. I., Y. Chung, D. Hwang, X. O. Yang, H. S. Kang, L. Ma, Y. H. Wang, S. S. Watowich, A. M. Jetten, Q. Tian and C. Dong (2008). "Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages." Immunity **29**(1): 138-49.

O'Callaghan C, A., M. F. Byford, J. R. Wyer, B. E. Willcox, B. K. Jakobsen, A. J. McMichael and J. I. Bell (1999). "BirA enzyme: production and application in the study of membrane receptor-ligand interactions by site-specific biotinylation." Anal Biochem **266**(1): 9-15.

Palmer, B. E., N. Blyveis, A. P. Fontenot and C. C. Wilson (2005). "Functional and phenotypic characterization of CD57+CD4+ T cells and their association with HIV-1-induced T cell dysfunction." J Immunol **175**(12): 8415-23.

Parham, P. (2004). "Killer cell immunoglobulin-like receptor diversity: balancing signals in the natural killer cell response." Immunol Lett **92**(1-2): 11-3.

Parham, P. (2005). "MHC CLASS I MOLECULES AND KIRS IN HUMAN HISTORY, HEALTH AND SURVIVAL." Nature Reviews Immunology **5**(3): 201-214.

Pende, D., R. Biassoni, C. Cantoni, S. Verdiani, M. Falco, D. C. di, L. Accame, C. Bottino, A. Moretta and L. Moretta (1996). "The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer." J.Exp.Med. **184**(2): 505-518.

Phillips, J. L. (1986). "Transferrin receptors and natural killer cell lysis. A study using Colo 205 cells exposed to 60 Hz electromagnetic fields." Immunol Lett **13**(6): 295-9.

Phillips, J. H., J. E. Gumperz, P. Parham and L. L. Lanier (1995). "Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes." Science **268**(5209): 403-5.

Polic, B., H. Hengel, A. Krmpotic, J. Trgovcich, I. Pavic, P. Luccaronin, S. Jonjic and U. H. Koszinowski (1998). "Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection." J Exp Med **188**(6): 1047-54.

Ponte, M., C. Cantoni, R. Biassoni, A. Tradori-Cappai, G. Bentivoglio, C. Vitale, S. Bertone, A. Moretta, L. Moretta and M. C. Mingari (1999). "Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor." Proc Natl Acad Sci U S A **96**(10): 5674-9.

Quinnan, G. V., Jr., N. Kirmani, A. H. Rook, J. F. Manischewitz, L. Jackson, G. Moreschi, G. W. Santos, R. Saral and W. H. Burns (1982). "Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients." N Engl J Med **307**(1): 7-13.

Quinnan, G. V., Jr., W. H. Burns, N. Kirmani, A. H. Rook, J. Manischewitz, L. Jackson, G. W. Santos and R. Saral (1984). "HLA-restricted cytotoxic T lymphocytes are an early immune response and important defense mechanism in cytomegalovirus infections." Rev Infect Dis **6**(2): 156-63.

Rajagopalan, S. and E. O. Long (1997). "The direct binding of a p58 killer cell inhibitory receptor to human histocompatibility leukocyte antigen (HLA)-Cw4 exhibits peptide selectivity." J.Exp.Med. **185**(8): 1523-1528.

Rajagopalan, S. and E. O. Long (1999). "A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells." J Exp Med **189**(7): 1093-100.

Rajagopalan, S. and E. O. Long (2005). "Understanding how combinations of HLA and KIR genes influence disease." J Exp Med **201**(7): 1025-9.

Rajalingam, R., M. Hong, E. J. Adams, B. P. Shum, L. A. Guethlein and P. Parham (2001). "Short KIR haplotypes in pygmy chimpanzee (Bonobo) resemble the conserved framework of diverse human KIR haplotypes." J Exp Med **193**(1): 135-46.

Rajalingam, R., P. Krausa, H. G. Shilling, J. B. Stein, A. Balamurugan, M. D. McGinnis, N. W. Cheng, N. K. Mehra and P. Parham (2002). "Distinctive KIR and HLA diversity in a panel of north Indian Hindus." Immunogenetics **53**(12): 1009-19.

Rajalingam, R., P. Parham and L. Abi-Rached (2004). "Domain Shuffling Has Been the Main Mechanism Forming New Hominoid Killer Cell Ig-Like Receptors." J Immunol **172**: 356-369.

Rasmussen, L., A. Geissler, C. Cowan, A. Chase and M. Winters (2002). "The genes encoding the gCIII complex of human cytomegalovirus exist in highly diverse combinations in clinical isolates." J Virol **76**(21): 10841-8.

Ramos-Casals, M., M. Garcia-Carrasco, M. P. Brito, A. Lopez-Soto and J. Font (2003). "Autoimmunity and geriatrics: clinical significance of autoimmune manifestations in the elderly." Lupus **12**(5): 341-55.

Raulet, D. H. (2004). "Interplay of natural killer cells and their receptors with the adaptive immune response." Nat Immunol **5**(10): 996-1002.

Ravkov, E. V., C. M. Myrick and J. D. Altman (2003). "Immediate early effector functions of virus-specific CD8(+)CCR7(+) memory cells in humans defined by HLA and CC chemokine ligand 19 tetramers." Journal of Immunology **170**(5): 2461-2468.

Reddehase, M. J., W. Mutter, K. Munch, H. J. Buhring and U. H. Koszinowski (1987). "CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity." J Virol **61**(10): 3102-8.

Reddehase, M. J. (2000). "The immunogenicity of human and murine cytomegaloviruses." Curr Opin Immunol **12**(6): 738.

Reddehase, M. J. (2002). "Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance." Nat Rev Immunol **2**(11): 831-44.

Reddy, K. R., H. Lilie, R. Rudolph and C. Lange (2005). "L-Arginine increases the solubility of unfolded species of hen egg white lysozyme." Protein Sci **14**(4): 929-35.

Reinhardt, R. L., A. Khoruts, R. Merica, T. Zell and M. K. Jenkins (2001). "Visualizing the generation of memory CD4 T cells in the whole body." Nature **410**(6824): 101-5.

Rentenaar, R. J., L. E. Gamadia, N. van DerHoek, F. N. van Diepen, R. Boom, J. F. Weel, P. M. Wertheim-van Dillen, R. A. van Lier and I. J. ten Berge (2000). "Development of virus-specific CD4(+) T cells during primary cytomegalovirus infection." J Clin Invest **105**(4): 541-8.

Retiere, C., B. M. Imbert, G. David, P. Courcoux and M. M. Hallet (1998). "A polymorphism in the major immediate-early gene delineates groups among cytomegalovirus clinical isolates." Virus Res **57**(1): 43-51.

Reusser, P., S. R. Riddell, J. D. Meyers and P. D. Greenberg (1991). "Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease." Blood **78**(5): 1373-80.

Reusser, P., G. Cathomas, R. Attenhofer, M. Tamm and G. Thiel (1999). "Cytomegalovirus (CMV)-specific T cell immunity after renal transplantation mediates protection from CMV disease by limiting the systemic virus load." J Infect Dis **180**(2): 247-53.

Reuter, J. D., J. H. Wilson, K. E. Idoko and A. N. van den Pol (2005). "CD4+ T-cell reconstitution reduces cytomegalovirus in the immunocompromised brain." J Virol **79**(15): 9527-39.

Roos, M. T., R. A. van Lier, D. Hamann, G. J. Knol, I. Verhoofstad, D. van Baarle, F. Miedema and P. T. Schellekens (2000). "Changes in the composition of circulating CD8+ T cell subsets during acute epstein-barr and human immunodeficiency virus infections in humans." J Infect Dis **182**(2): 451-8.

Rufer, N., A. Zippelius, P. Batard, M. J. Pittet, I. Kurth, P. Corthesy, J. C. Cerottini, S. Leyvraz, E. Roosnek, M. Nabholz and P. Romero (2003). "Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions." Blood **102**(5): 1779-87.

Sallusto, F., D. Lenig, R. Forster, M. Lipp and A. Lanzavecchia (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature **401**(6754): 708-12.

Sallusto, F. and A. Lanzavecchia (2001). "Exploring pathways for memory T cell generation." J Clin Invest **108**(6): 805-6.

Sambrook, J. G., A. Bashirova, H. Andersen, M. Piatak, G. S. Vernikos, P. Coggill, J. D. Lifson, M. Carrington and S. Beck (2006). "Identification of the ancestral killer immunoglobulin-like receptor gene in primates." BMC Genomics **7**: 209.

Santoli, D. and H. Koprowski (1979). "Mechanisms of activation of human natural killer cells against tumor and virus-infected cells." Immunol Rev **44**: 125-163.

Sauce, D., M. Larsen, S. Fastenackels, A. Duperrier, M. Keller, B. Grubeck-Loebenstein, C. Ferrand, P. Debre, D. Sidi and V. Appay (2009). "Evidence of premature immune aging in patients thymectomized during early childhood." J Clin Invest **119**(10): 3070-8.

Saulquin, X., L. N. Gastinel and E. Vivier (2003). "Crystal structure of the human natural killer cell activating receptor KIR2DS2 (CD158j)." J Exp Med **197**(7): 933-8.

Scalzo, A. A., N. A. Fitzgerald, A. Simmons, A. B. La Vista and G. R. Shellam (1990). "Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen." J Exp Med **171**(5): 1469-83.

Schofield, D. J., A. R. Pope, V. Clementel, J. Buckell, S. Chapple, K. F. Clarke, J. S. Conquer, A. M. Crofts, S. R. Crowther, M. R. Dyson, G. Flack, G. J. Griffin, Y. Hooks, W. J. Howat, A. Kolb-Kokocinski, S. Kunze, C. D. Martin, G. L. Maslen, J. N. Mitchell, M. O'Sullivan, R. L. Perera, W. Roake, S. P. Shadbolt, K. J. Vincent, A. Warford, W. E. Wilson, J. Xie, J. L. Young and J. McCafferty (2007). "Application of phage display to high throughput antibody generation and characterization." Genome Biol **8**(11): R254.

Schust, D. J., D. Tortorella, J. Seebach, C. Phan and H. L. Ploegh (1998). "Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11." J Exp Med **188**(3): 497-503.

Schwendemann, J., C. Choi, V. Schirrmacher and P. Beckhove (2005). "Dynamic differentiation of activated human peripheral blood CD8+ and CD4+ effector memory T cells." J Immunol **175**(3): 1433-9.

Sester, M., U. Sester, B. Gartner, G. Heine, M. Girndt, N. Mueller-Lantzsch, A. Meyerhans and H. Kohler (2001). "Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation." Transplantation **71**(9): 1287-94.

Sester, M., U. Sester, B. Gartner, B. Kubuschok, M. Girndt, A. Meyerhans and H. Kohler (2002). "Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus." J Virol **76**(8): 3748-55.

Sester, M., B. C. Gartner, U. Sester, M. Girndt, N. Mueller-Lantzsch and H. Kohler (2003). "Is the cytomegalovirus serologic status always accurate? A comparative analysis of humoral and cellular immunity." Transplantation **76**(8): 1229-30.

Shilling, H. G., L. A. Guethlein, N. W. Cheng, C. M. Gardiner, R. Rodriguez, D. Tyan and P. Parham (2002). "Allelic polymorphism synergizes with variable gene content to individualize human KIR genotype." J Immunol **168**(5): 2307-15.

Shilling, H. G., N. Young, L. A. Guethlein, N. W. Cheng, C. M. Gardiner, D. Tyan and P. Parham (2002). "Genetic Control of Human NK Cell Repertoire." The Journal of Immunology **169**(1): 239-247.

Simmen, K. A., J. Singh, B. G. Luukkonen, M. Lopper, A. Bittner, N. E. Miller, M. R. Jackson, T. Compton and K. Fruh (2001). "Global modulation of cellular transcription by human cytomegalovirus is initiated by viral glycoprotein B." Proc Natl Acad Sci U S A **98**(13): 7140-5.

Simonsen, L., T. A. Reichert, C. Viboud, W. C. Blackwelder, R. J. Taylor and M. A. Miller (2005). "Impact of influenza vaccination on seasonal mortality in the US elderly population." Arch Intern Med **165**(3): 265-72.

Sinclair, J. and P. Sissons (2006). "Latency and reactivation of human cytomegalovirus." J Gen Virol **87**(Pt 7): 1763-79.

Sinzger, C., A. Grefte, B. Plachter, A. S. Gouw, T. H. The and G. Jahn (1995). "Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues." J Gen Virol **76 (Pt 4)**: 741-50.

Sinzger, C., A. L. Bissinger, R. Viebahn, H. Oettle, C. Radke, C. A. Schmidt and G. Jahn (1999). "Hepatocytes are permissive for human cytomegalovirus infection in human liver cell culture and In vivo." J Infect Dis **180**(4): 976-86.

Snary, D., C. J. Barnstable, W. F. Bodmer and M. J. Crumpton (1977). "Molecular structure of human histocompatibility antigens: the HLA-C series." Eur J Immunol **7**(8): 580-5.

Snyder, G. A., A. G. Brooks and P. D. Sun (1999). "Crystal structure of the HLA-Cw3 allotype-specific killer cell inhibitory receptor KIR2DL2." Proc Natl Acad Sci U S A **96**(7): 3864-9.

Snyder, M. R., M. Lucas, E. Vivier, C. M. Weyand and J. J. Goronzy (2003). "Selective activation of the c-Jun NH2-terminal protein kinase signaling pathway by stimulatory KIR in the absence of KARAP/DAP12 in CD4+ T cells." J Exp Med **197**(4): 437-49.Snyder, M. R., T. Nakajima, P. Leibson, C. Weyand and J. J. Goronzy (2004). "Stimulatory Killer Ig-Like Receptors Modulate T Cell Activation through DAP12-Dependent and DAP12-Independent Mechanisms." J Immunol **173**: 3725-3731.

Soderberg-Naucler, C., K. N. Fish and J. A. Nelson (1997). "Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors." Cell **91**(1): 119-26.

Soderberg-Naucler, C., D. N. Streblow, K. N. Fish, J. Allan-Yorke, P. P. Smith and J. A. Nelson (2001). "Reactivation of latent human cytomegalovirus in CD14(+) monocytes is differentiation dependent." J Virol **75**(16): 7543-54.

Sodoyer, R., M. Damotte, T. L. Delovitch, J. Trucy, B. R. Jordan and T. Strachan (1984). "Complete nucleotide sequence of a gene encoding a functional human class I histocompatibility antigen (HLA-CW3)." EMBO J **3**(4): 879-85.

Sprent, J. and C. D. Surh (2002). "T cell memory." Annu Rev Immunol **20**: 551-79.

Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman and R. D. Schreiber (1998). "How cells respond to interferons." Annu Rev Biochem **67**: 227-64.

Steffens, H. P., S. Kurz, R. Holtappels and M. J. Reddehase (1998). "Preemptive CD8 T-cell immunotherapy of acute cytomegalovirus infection prevents lethal disease, limits the burden of latent viral genomes, and reduces the risk of virus recurrence." J Virol **72**(3): 1797-804.

Stewart, C. A., F. Laugier-Anfossi, F. Vely, X. Saulquin, J. Riedmuller, A. Tisserant, L. Gauthier, F. Romagne, G. Ferracci, F. A. Arosa, A. Moretta, P. D. Sun, S. Ugolini and E. Vivier (2005). "Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors." Proc Natl Acad Sci U S A **102**(37): 13224-9.

Storkus, W. J., J. Alexander, J. A. Payne, J. R. Dawson and P. Cresswell (1989). "Reversal of natural killing susceptibility in target cells expressing transfected class I HLA genes." Proc Natl Acad Sci U S A **86**(7): 2361-4.

Studier, F. W. and B. A. Moffatt (1986). "Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes." J Mol Biol **189**(1): 113-30.

Suzuki, Y., Y. Hamamoto, Y. Ogasawara, K. Ishikawa, Y. Yoshikawa, T. Sasazuki and M. Muto (2004). "Genetic polymorphisms of killer cell immunoglobulin-like receptors are associated with susceptibility to psoriasis vulgaris." J Invest Dermatol **122**(5): 1133-6.

Swain, S. L., H. Hu and G. Huston (1999). "Class II-independent generation of CD4 memory T cells from effectors." Science **286**(5443): 1381-3.

Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, J. A. Nelson and L. J. Picker (2005). "Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects." J Exp Med **202**(5): 673-85.

Tabor, S. and C. C. Richardson (1985). "A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes." Proc Natl Acad Sci U S A **82**(4): 1074-8.

Tan, J. T., B. Ernst, W. C. Kieper, E. LeRoy, J. Sprent and C. D. Surh (2002). "Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells." J Exp Med **195**(12): 1523-32.

Tanchot, C., F. A. Lemonnier, B. Perarnau, A. A. Freitas and B. Rocha (1997). "Differential requirements for survival and proliferation of CD8 naive or memory T cells." Science **276**(5321): 2057-62.

Tarazona, R., O. DelaRosa, C. Alonso, B. Ostos, J. Espejo, J. Pena and R. Solana (2000). "Increased expression of NK cell markers on T lymphocytes in aging and chronic activation of the immune system reflects the accumulation of effector/senescent T cells." Mech Ageing Dev **121**(1-3): 77-88.

Tarkkanen, J., H. Saxen, M. Nurminen, P. H. Makela and E. Saksela (1986). "Bacterial induction of human activated lymphocyte killing and its inhibition by lipopolysaccharide (LPS)." J Immunol **136**(7): 2662-9.

Taswell, C., H. R. MacDonald and J. C. Cerottini (1980). "Clonal analysis of cytolytic T lymphocyte specificity. I. Phenotypically distinct sets of clones as the cellular basis of cross-reactivity to alloantigens." J Exp Med **151**(6): 1372-85.

Tay, C. H. and R. M. Welsh (1997). "Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells." J Virol **71**(1): 267-75.

Taylor-Wiedeman, J., J. G. Sissons, L. K. Borysiewicz and J. H. Sinclair (1991). "Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells." J Gen Virol **72 (Pt 9)**: 2059-64.

Taylor-Wiedeman, J., P. Sissons and J. Sinclair (1994). "Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers." J Virol **68**(3): 1597-604.

Tomasec, P., E. C. Wang, A. J. Davison, B. Vojtesek, M. Armstrong, C. Griffin, B. P. McSharry, R. J. Morris, S. Llewellyn-Lacey, C. Rickards, A. Nomoto, C. Sinzger and G. W. Wilkinson (2005). "Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141." Nat Immunol **6**(2): 181-8.

Toneva, M., V. Lepage, G. Lafay, N. Dulphy, M. Busson, S. Lester, A. Vu-Trien, A. Michaylova, E. Naumova, J. McCluskey and D. Charron (2001). "Genomic diversity of natural killer cell receptor genes in three populations." Tissue Antigens **57**(4): 358-62.

Tormo, J., K. Natarajan, D. H. Margulies and R. A. Mariuzza (1999). "Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand." Nature **402**(6762): 623-31.

Toro, A. I. and J. Ossa (1996). "PCR activity of CMV in healthy CMV-seropositive individuals: does latency need redefinition?" Res Virol **147**(4): 233-8.

Trompeter, H. I., N. Gomez-Lozano, S. Santourlidis, B. Eisermann, P. Wernet, C. Vilches and M. Uhrberg (2005). "Three structurally and functionally divergent kinds of promoters regulate expression of clonally distributed killer cell Ig-like receptors (KIR), of KIR2DL4, and of KIR3DL3." J Immunol **174**(7): 4135-43.

Trowsdale, J., R. Barten, A. Haude, C. A. Stewart, S. Beck and M. J. Wilson (2001). "The genomic context of natural killer receptor extended gene families." Immunol Rev **181**: 20-38.

Trundley, A. E., S. E. Hiby, C. Chang, A. M. Sharkey, S. Santourlidis, M. Uhrberg, J. Trowsdale and A. Moffett (2006). "Molecular characterization of KIR3DL3." Immunogenetics **57**(12): 904-16.

Tu, W., S. Chen, M. Sharp, C. Dekker, A. M. Manganello, E. C. Tongson, H. T. Maecker, T. H. Holmes, Z. Wang, G. Kemble, S. Adler, A. Arvin and D. B. Lewis (2004). "Persistent and selective deficiency of CD4+ T cell immunity to cytomegalovirus in immunocompetent young children." J Immunol **172**(5): 3260-7.

Uhrberg, M., N. M. Valiante, B. P. Shum, H. G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L. L. Lanier and P. Parham (1997). "Human diversity in killer cell inhibitory receptor genes." Immunity **7**(6): 753-63.

Uhrberg, M., P. Parham and P. Wernet (2002). "Definition of gene content for nine common group B haplotypes of the Caucasoid population: KIR haplotypes contain between seven and eleven KIR genes." Immunogenetics **54**(4): 221-9.

Urbani, S., C. Boni, G. Missale, G. Elia, C. Cavallo, M. Massari, G. Raimondo and C. Ferrari (2002). "Virus-specific CD8+ lymphocytes share the same effector-memory phenotype but exhibit functional differences in acute hepatitis B and C." J Virol **76**(24): 12423-34.

Vallejo, A. N., J. C. Brandes, C. M. Weyand and J. J. Goronzy (1999). "Modulation of CD28 expression: distinct regulatory pathways during activation and replicative senescence." J Immunol **162**(11): 6572-9.

Vallejo, A. N. (2006). "Age-dependent alterations of the T cell repertoire and functional diversity of T cells of the aged." Immunol Res **36**(1-3): 221-8.

Vallejo, A. N. (2007). "Immune remodeling: lessons from repertoire alterations during chronological aging and in immune-mediated disease." Trends Mol Med **13**(3): 94-102.

van Bergen, J., A. Thompson, A. van der Slik, T. H. Ottenhoff, J. Gussekloo and F. Koning (2004). "Phenotypic and functional characterization of CD4 T cells expressing killer Ig-like receptors." J Immunol **173**(11): 6719-26.

van Bergen, J., E. M. Kooy-Winkelaar, H. van Dongen, F. A. van Gaalen, A. Thompson, T. W. Huizinga, M. C. Feltkamp, R. E. Toes and F. Koning (2009). "Functional killer Ig-like receptors on human memory CD4+ T cells specific for cytomegalovirus." J Immunol **182**(7): 4175-82.

van der Merwe, P. A. and A. N. Barclay (1994). "Transient intercellular adhesion: the importance of weak protein-protein interactions." Trends Biochem Sci **19**(9): 354-8.

van der Merwe, P. A. and A. N. Barclay (1996). "Analysis of cell-adhesion molecule interactions using surface plasmon resonance." Current Opinion in Immunology **8**(2): 257-261.

van der Slik, A. R., B. P. Koeleman, W. Verduijn, G. J. Bruining, B. O. Roep and M. J. Giphart (2003). "KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects." Diabetes **52**(10): 2639-42.

van Leeuwen, E. M., E. B. Remmerswaal, M. T. Vossen, A. T. Rowshani, P. M. Wertheim-van Dillen, R. A. van Lier and I. J. ten Berge (2004). "Emergence of a CD4+CD28- granzyme B+, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection." J Immunol **173**(3): 1834-41.

van Leeuwen, E. M., G. J. de Bree, E. B. Remmerswaal, S. L. Yong, K. Tesselaar, I. J. ten Berge and R. A. van Lier (2005). "IL-7 receptor alpha chain expression distinguishes functional subsets of virus-specific human CD8+ T cells." Blood **106**(6): 2091-8.

van Leeuwen, E. M., E. B. Remmerswaal, M. H. Heemskerk, I. J. ten Berge and R. A. van Lier (2006). "Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection." Blood **108**(9): 3121-7.

van Stijn, A., A. T. Rowshani, S. L. Yong, F. Baas, E. Roosnek, I. J. ten Berge and R. A. van Lier (2008). "Human cytomegalovirus infection induces a rapid and sustained change in the expression of NK cell receptors on CD8+ T cells." J Immunol **180**(7): 4550-60.

van Stipdonk, M. J., E. E. Lemmens and S. P. Schoenberger (2001). "Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation." Nat Immunol **2**(5): 423-9.

Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley and B. Stockinger (2006). "TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells." Immunity **24**(2): 179-89.

Vely, F., M. Peyrat, C. Couedel, J. Morcet, F. Halary, F. Davodeau, F. Romagne, E. Scotet, X. Saulquin, E. Houssaint, N. Schleinitz, A. Moretta, E. Vivier and M. Bonneville (2001). "Regulation of inhibitory and activating killer-cell Ig-like receptor expression occurs in T cells after termination of TCR rearrangements." J Immunol **166**(4): 2487-94.

Vilches, C., M. J. Pando and P. Parham (2000a). "Genes encoding human killer-cell Ig-like receptors with D1 and D2 extracellular domains all contain untranslated pseudoexons encoding a third Ig-like domain." Immunogenetics **51**(8-9): 639-646.

Vilches, C., R. Rajalingam, M. Uhrberg, C. M. Gardiner, N. T. Young and P. Parham (2000b). "KIR2DL5, a novel killer-cell receptor with a D0-D2 configuration of Ig-like domains." J Immunol **164**(11): 5797-804.

Vilches, C. and P. Parham (2002). "KIR: Diverse, Rapidly Evolving Receptors of Innate and Adaptive Immunity." Annual Review of Immunology **20**(1): 217-251.

Vivier, E. and N. Anfossi (2004). "Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future." Nat Rev Immunol **4**(3): 190-8.

Vogelzang, A., H. M. McGuire, D. Yu, J. Sprent, C. R. Mackay and C. King (2008). "A fundamental role for interleukin-21 in the generation of T follicular helper cells." Immunity **29**(1): 127-37.

Waldrop, S. L., C. J. Pitcher, D. M. Peterson, V. C. Maino and L. J. Picker (1997). "Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency." J Clin Invest **99**(7): 1739-50.

Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas and S. R. Riddell (1995). "Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor." N Engl J Med **333**(16): 1038-44.

Wang, E. C. and L. K. Borysiewicz (1995). "The role of CD8+, CD57+ cells in human cytomegalovirus and other viral infections." Scand J Infect Dis Suppl **99**: 69-77.

Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub and E. S. Huang (2003). "Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus." Nature **424**(6947): 456-61.

Wang, X., D. Y. Huang, S. M. Huong and E. S. Huang (2005). "Integrin alphavbeta3 is a coreceptor for human cytomegalovirus." Nat Med **11**(5): 515-21.

Weekes, M. P., M. R. Wills, K. Mynard, R. Hicks, J. G. Sissons and A. J. Carmichael (1999). "Large clonal expansions of human virus-specific memory cytotoxic T lymphocytes within the CD57+ CD28- CD8+ T-cell population." Immunology **98**(3): 443-9.

Weekes, M. P., M. R. Wills, J. G. Sissons and A. J. Carmichael (2004). "Long-term stable expanded human CD4+ T cell clones specific for human cytomegalovirus are distributed in both CD45RAhigh and CD45ROhigh populations." J Immunol **173**(9): 5843-51.

Weinberger, B., L. Lazuardi, I. Weiskirchner, M. Keller, C. Neuner, K. H. Fischer, B. Neuman, R. Wurzner and B. Grubeck-Loebenstein (2007). "Healthy aging and latent infection with CMV lead to distinct changes in CD8+ and CD4+ T-cell subsets in the elderly." Hum Immunol **68**(2): 86-90.

Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian and R. Ahmed (2003). "Lineage relationship and protective immunity of memory CD8 T cell subsets." Nat Immunol **4**(3): 225-34.

Willcox, B. E., G. F. Gao, J. R. Wyer, J. E. Ladbury, J. I. Bell, B. K. Jakobsen and P. A. van der Merwe (1999). "TCR binding to peptide-MHC stabilizes a flexible recognition interface." Immunity **10**(3): 357-65.

Willcox, B. E., L. M. Thomas, T. L. Chapman, A. P. Heikema, A. P. West, Jr. and P. J. Bjorkman (2002). "Crystal structure of LIR-2 (ILT4) at 1.8 A: differences from LIR-1 (ILT2) in regions implicated in the binding of the Human Cytomegalovirus class I MHC homolog UL18." BMC Struct Biol **2**: 6.

Williams, D. P., D. Regier, D. Akiyoshi, F. Genbauffe and J. R. Murphy (1988). "Design, synthesis and expression of a human interleukin-2 gene incorporating the codon usage bias found in highly expressed Escherichia coli genes." Nucleic Acids Res **16**(22): 10453-67.

Williams, F., L. D. Maxwell, I. A. Halfpenny, A. Meenagh, C. Sleator, M. D. Curran and D. Middleton (2003). "Multiple copies of KIR 3DL/S1 and KIR 2DL4 genes identified in a number of individuals." Hum Immunol **64**(7): 729-32.

Williams, A. P., A. R. Bateman and S. I. Khakoo (2005). "Hanging in the balance. KIR and their role in disease." Mol Interv **5**(4): 226-40.

Wills, M. R., A. J. Carmichael, K. Mynard, X. Jin, M. P. Weekes, B. Plachter and J. G. Sissons (1996). "The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL." J Virol **70**(11): 7569-79.

Wills, M. R., O. Ashiru, M. B. Reeves, G. Okecha, J. Trowsdale, P. Tomasec, G. W. Wilkinson, J. Sinclair and J. G. Sissons (2005). "Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis." J Immunol **175**(11): 7457-65.

Wilson, M. J., M. Torkar and J. Trowsdale (1997). "Genomic organization of a human killer cell inhibitory receptor gene." Tissue Antigens. **49**(6): 574-579.

Wilson, M. J., M. Torkar, A. Haude, S. Milne, T. Jones, D. Sheer, S. Beck and J. Trowsdale (2000). "Plasticity in the organization and sequences of human KIR/ILT gene families." Proc Natl Acad Sci U S A **97**(9): 4778-83.

Winter, C. C., J. E. Gumperz, P. Parham, E. O. Long and N. Wagtmann (1998). "Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition." J.Immunol. **161**(2): 571-577.

Witt, C. S., C. Dewing, D. C. Sayer, M. Uhrberg, P. Parham and F. T. Christiansen (1999). "Population frequencies and putative haplotypes of the killer cell immunoglobulin-like receptor sequences and evidence for recombination [see comments]." Transplantation **68**(11): 1784-1789.

Yamashita, N., L. Nguyen, J. L. Fahey and L. T. Clement (1993). "Phenotypic properties and cytotoxic functions of human CD8+ cells expressing the CD57 antigen." Nat Immun **12**(2): 79-91.

Yen, J. H., B. E. Moore, T. Nakajima, D. Scholl, D. J. Schaid, C. M. Weyand and J. J. Goronzy (2001). "Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis." J Exp Med **193**(10): 1159-67.

Young, J. L., J. M. Ramage, J. S. Gaston and P. C. Beverley (1997). "In vitro responses of human CD45R0brightRA- and CD45R0-RAbright T cell subsets and their relationship to memory and naive T cells." Eur J Immunol **27**(9): 2383-90.

Yu, Y. Y., T. George, J. R. Dorfman, J. Roland, V. Kumar and M. Bennett (1996). "The role of Ly49A and 5E6(Ly49C) molecules in hybrid resistance mediated by murine natural killer cells against normal T cell blasts." Immunity **4**(1): 67-76.

Yue, F. Y., C. M. Kovacs, R. C. Dimayuga, P. Parks and M. A. Ostrowski (2004). "HIV-1-specific memory CD4+ T cells are phenotypically less mature than cytomegalovirus-specific memory CD4+ T cells." J Immunol **172**(4): 2476-86.

Zappacosta, F., F. Borrego, A. G. Brooks, K. C. Parker and J. E. Coligan (1997). "Peptides isolated from HLA-Cw*0304 confer different degrees of protection from natural killer cell-mediated lysis." Proc Natl Acad Sci U S A **94**(12): 6313-8.

Zaunders, J. J., W. B. Dyer, B. Wang, M. L. Munier, M. Miranda-Saksena, R. Newton, J. Moore, C. R. Mackay, D. A. Cooper, N. K. Saksena and A. D. Kelleher (2004). "Identification of circulating antigen-specific CD4+ T lymphocytes with a CCR5+, cytotoxic phenotype in an HIV-1 long-term nonprogressor and in CMV infection." Blood **103**(6): 2238-47.

Zhou, L., M. M. Chong and D. R. Littman (2009). "Plasticity of CD4+ T cell lineage differentiation." Immunity **30**(5): 646-55.

Zhu, H., J. P. Cong, G. Mamtora, T. Gingeras and T. Shenk (1998). "Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays." Proc Natl Acad Sci U S A **95**(24): 14470-5.

Zimmerman, C., K. Brduscha-Riem, C. Blaser, R. M. Zinkernagel and H. Pircher (1996). "Visualization, characterization, and turnover of CD8+ memory T cells in virus-infected hosts." J Exp Med **183**(4): 1367-75.