A study of MHC class I binding viral and host derived peptides and natural killer cell function

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Declaration of Originality

The data presented in this thesis is the result of my own work carried out between 2009 and 2012, unless otherwise specified in the text. This work was carried out in Section of Gastroenterology and Hepatology, Department of Medicine, Faculty of Medicine, Imperial College under the supervision of Professor Salim Khakoo and Dr Marco Purbhoo.
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Abstract

Natural killer (NK) cells are a key component of innate immunity and have been implicated in determining the outcome of HCV infection in both genetic and functional studies. The last two decades have seen significant advances in the understanding of NK cell regulation with the discovery of a multitude of activating and inhibitory receptors. CD94:NKG2A operates in tandem with the polymorphic killer cell immunoglobulin-like receptors (KIR) and Ly49 systems to inhibit NK cells, however it is not clear as to the benefits of having two distinct inhibitory receptor:ligand systems. Down regulation or modification of MHC class I expression is a key feature of NK cell recognition of virus infected cells. However, viruses can subvert this mechanism of NK cell surveillance by encoding peptides that can bind to MHC class I. The aim of this thesis is to further our understanding of the interaction between viral and host derived MHC class I binding peptides and their effect on NK cell inhibition.

By using an MHC deficient cell line, we have shown that HCV core\textsubscript{35-44} peptide is capable of enhancing cell surface expression of MHC class I (HLA-C and HLA-E). Although this peptide stabilises HLA-E, the HLA-E:HCV core\textsubscript{35-44} complex alone is insufficient to inhibit at NKG2A positive NK cells. However, in the presence of HLA-E binding MHC class I signal peptides, HCV core\textsubscript{35-44} has a synergistic effect in suppressing the NKG2A\textsuperscript{+} NK cell population. Peptides derived from other viruses such as EBV and HIV, and the stress related peptide derived from heat shock protein 60 also augment inhibition of NKG2A\textsuperscript{+} NK cells, but only when in the presence of MHC class I signal peptides. This augmentation is caused by recruitment of the non-signalling CD94 molecule to the immune synapse in the absence of its inhibitory signalling partner NKG2A. Thus CD94 can function independently as an enhancer of inhibition.

The augmentation of inhibition of CD94:NKG2A by non-inhibitory peptides, contrasts with antagonism of inhibition of KIR by low affinity peptide:MHC complexes. We also show that KIR\textsuperscript{+} and NKG2A\textsuperscript{+} NK cells respond with differing stoichiometries to MHC class I down-regulation. Thus peptide selectivity and MHC class I sensitivity of natural killer cell receptors provides a rationale for the evolution of two distinct inhibitory systems for MHC class I.
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparaginase</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster designation</td>
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<tr>
<td>cIg</td>
<td>Control immunoglobulin</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLR</td>
<td>C-type lectin-like receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>EBV</td>
<td>Epstein barr virus</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERM</td>
<td>Erzin/ radixin/ moesin</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<td>Glutamine</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>Human cytomegalovirus</td>
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<td>Hepatitis C virus</td>
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<tr>
<td>HLA</td>
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<td>HLA-C1</td>
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<td>Heat shock protein 60</td>
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HSC  Hepatic stellate cell
HSV  Herpes simplex virus
ICAM-1  Intercellular adhesion molecule-1
IFN-α  Interferon alpha
IFN-γ  Interferon gamma
IgG  Immunoglobulin G
IL  Interleukin
Ile  Isoleucine
ILT-2  Immunoglobulin-like transcript-2 (also known as LILRB-1, LIR-1)
IS  Immune synapse
ITAM  Immunoreceptor tyrosine-based activation motif
ITIM  Immunoreceptor tyrosine-based inhibitory motif
KIR  Killer cell immunoglobulin-like receptor
LAMP  Lysosomal associated membrane protein
Leu  Leucine
LFA-1  Lymphocyte function-associated antigen-1
LILRB-1  Leukocyte immunoglobulin-like receptor subfamily B member 1 (also known as ILT-2, LIR-1)
Lys  Lysine
mAb  Monoclonal antibody
MCMV  Murine cytomegalovirus
MHC  Major histocompatibility complex
MFI  Mean fluorescent intensity
MTOC  microtubule organising centre
NCR  Natural cytotoxicity receptor
NK  Natural killer
PBMC  Peripheral blood mononuclear cell
PLC  Peptide loading complex
RANTES  Factor regulated on activation-normal T cells expressed and secreted
SEM  Standard error of the mean
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<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>SHP</td>
<td>src homology 2 domain bearing tyrosine phosphatases</td>
</tr>
<tr>
<td>sp</td>
<td>signal peptide</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>β2m</td>
<td>beta-2 microglobulin</td>
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List of peptide abbreviations, full name and sequences

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<tr>
<td>GILG</td>
<td>HLA-A2 specific influenza matrix peptide</td>
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<td>Hepatitis C core residues 35-44</td>
<td>YLLPRRGPRL</td>
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<td>hsp60&lt;sub&gt;sp&lt;/sub&gt;</td>
<td>Heat shock protein 60 signal peptide</td>
<td>QMRPVSRVL</td>
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<td>VAP-FA</td>
<td>variant of TIMP-1 derived peptide</td>
<td>VAPWNSFAL</td>
</tr>
</tbody>
</table>
Acknowledgements

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Chapter 1. Introduction

1.1 HCV infection

Hepatitis C virus (HCV) is a burgeoning worldwide public health problem with an estimated 170 million people currently believed to be infected (Lavanchy, 2009). This small enveloped positive-stranded RNA virus belonging to the Hepacivirus genus of the Flaviviridae family was first identified in 1989 as the infectious agent responsible for human non-A, non-B hepatitis (Choo et al., 1989). Phylogenetic analysis has since revealed HCV as a rapidly mutating virus with six major genotypes (along with numerous subtypes) which vary by up to 30% in nucleotide sequence. This heterogeneity may facilitate HCV to escape the host immune response with the majority (75-85%) of exposed individuals developing chronic infection. Chronic HCV carries the associated risks of cirrhosis, liver failure and hepatocellular carcinoma and is the leading indication for liver transplantation in Western countries (Hoofnagle, 2002). The current cornerstone of treatment for chronic HCV consists of pegylated interferon alpha and ribavirin. However, a significant number of patients who receive treatment are not cured or experience serious side effects. Two new direct acting antivirals -NS3/ 4A protease inhibitors - have recently been integrated into standard therapy with improved response rates but with exacerbation of adverse effects.

The factors associated with HCV clearance are incompletely understood. It is widely accepted that HCV-specific T cell responses are crucial for the spontaneous resolution of acute HCV infection and long-term immunity (Day et al., 2002; Thimme et al., 2001). The innate immune system primes this virus-specific adaptive immune response. Indeed in chronic HCV many of the shortcomings seen in adaptive immunity may be related to deficiencies in innate immune cells. It is understood that natural killer (NK) cells, dendritic cells (DC) and T cells communicate with one another via cytokines and direct cell-to-cell contact (Mailliard et al., 2003). This interplay has prompted immense interest in the role of NK cells, which may represent an interface between the innate and adaptive immune system, in determining the outcome of HCV infection.
1.2 Natural Killer cells

Natural killer (NK) cells are one component of the immune response associated with the outcome of HCV infection. They were first described in 1975 as a lymphocyte subset capable of cytotoxicity against leukaemia cells in vitro without previous sensitisation (Kiessling et al., 1975). These bone marrow derived large granular lymphocytes comprise 5-20% of peripheral blood mononuclear cells but make up a substantially greater proportion (30-50%) of lymphocytes in the liver (Corado et al., 1997). Human NK cells are defined by the presence of surface markers CD56 and/or CD16 (the low affinity receptor for IgG Fc) and are CD3 negative. In contrast to T and B cells, NK cells do not require priming, enabling them to provide early protection against tumour transformation and intracellular pathogens such as viruses (Biron et al., 1999). Their receptors do not show germ-line rearrangement and are not clonally distributed.

1.2.1 Mechanism for NK cell activity against target cells

NK cells possess a number of mechanisms to wield their effector functions on target cells. They can mediate direct cytotoxicity via the release of preformed lytic granules and by surface expression of ligands that activate death receptors on target cells. Furthermore, they are early producers of cytokines, predominantly of the T-helper 1 (Th1) type, which may exert a direct antiviral effect and shape downstream immune responses (Figure 1.1).
Natural killer (NK) cells have several potential effector mechanisms. Degranulation of cytotoxic granules containing perforin, granzyme and sulphated proteoglycans results in direct cytotoxicity to target cells. Additionally, NK cell surface upregulation of Fas ligand (FasL) and tumour necrosis factor related apoptosis inducing ligand (TRAIL) can trigger death-receptor mediated apoptosis in target cells. NK cells are also an important source of cytokines, such as IFN-γ and TNF-α, which have a direct antiviral effect and induce the adaptive immune response.
Equipped with this potent arsenal, NK cells must be tightly controlled to enable them to provide host defence whilst avoiding autoreactivity against healthy cells. Regulation of NK cell activity is achieved by a vast array of stimulatory, costimulatory and inhibitory receptors (Tables 1.1 and 1.2), signals from which are integrated to determine their activation status. Activating receptors expressed on human NK cells include the C-type lectin receptors NKG2D and NKG2C/E, natural cytotoxicity receptors (NCR) NKp44, NKp30, NKp46, and CD16 (FC-γ-RIII), the low affinity Fc receptor which mediates antibody dependant cytotoxicity (ADCC) (Bauer et al., 1999; Oehler et al., 1978; Pende et al., 1999; Sivori et al., 1999; Vitale et al., 1998).

Ligands for these activating receptors are frequently upregulated in viral infections and tumours; however, they can also be expressed habitually on healthy cells. Therefore to prevent alloreactivity against normal cells, NK cells receive a dominant inhibitory signal from receptors for major histocompatibility complex (MHC) class I molecules which must be overcome before NK cells are activated.
Table 1.1. Inhibitory NK cell receptors and their ligands

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KIR family</strong></td>
<td></td>
</tr>
<tr>
<td>2DL1</td>
<td>Group 2 HLA-C</td>
</tr>
<tr>
<td>2DL2/3</td>
<td>Group 1 HLA-C</td>
</tr>
<tr>
<td>2DL5</td>
<td>Unknown</td>
</tr>
<tr>
<td>3DL1</td>
<td>Bw4 + HLA-B</td>
</tr>
<tr>
<td>3DL2</td>
<td>HLA-A3/A11</td>
</tr>
<tr>
<td><strong>CTLR family</strong></td>
<td></td>
</tr>
<tr>
<td>CD94:NKG2A</td>
<td>HLA-E</td>
</tr>
<tr>
<td>NKR-P1A</td>
<td>LLT-1</td>
</tr>
<tr>
<td><strong>LIR/ILT family</strong></td>
<td></td>
</tr>
<tr>
<td>LILRB1/ILT2/LIR1</td>
<td>HLA-A, -B, -C</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>LAIR-1</td>
<td>Collagen</td>
</tr>
<tr>
<td>Siglec-7</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>KLRG-1/ MAFA</td>
<td>Cadherins</td>
</tr>
<tr>
<td>CEACAM 1</td>
<td>CEACAM 1</td>
</tr>
</tbody>
</table>

HLA, human leucocyte antigen; KIR, killer cell immunoglobulin-like receptor; CTLR, C-type lectin receptor; LIR/ILT, leucocyte immunoglobulin-like receptor/immunoglobulin-like transcript

Table 1.2. Activating NK cell receptors and their ligands

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NCR family</strong></td>
<td></td>
</tr>
<tr>
<td>NKp30</td>
<td>BAT-3</td>
</tr>
<tr>
<td>NKp44</td>
<td>Viral haemagglutinin</td>
</tr>
<tr>
<td>NKp46</td>
<td>Viral haemagglutinin</td>
</tr>
<tr>
<td><strong>CTLR family</strong></td>
<td></td>
</tr>
<tr>
<td>CD94:NKG2C</td>
<td>HLA-E</td>
</tr>
<tr>
<td>CD94:NKG2E</td>
<td>HLA-E</td>
</tr>
<tr>
<td>NKG2D</td>
<td>MIC-A/B, ULBP</td>
</tr>
<tr>
<td><strong>KIR family</strong></td>
<td></td>
</tr>
<tr>
<td>2DS1</td>
<td>Group 2 HLA-C</td>
</tr>
<tr>
<td>2DS2</td>
<td>Group 1 HLA-C</td>
</tr>
<tr>
<td>3DS1</td>
<td>Bw4 + ?HLA-B</td>
</tr>
<tr>
<td>2DS3</td>
<td>Unknown</td>
</tr>
<tr>
<td>2DS4</td>
<td>HLA-Cw4</td>
</tr>
<tr>
<td>2DS5</td>
<td>Unknown</td>
</tr>
<tr>
<td>2DL4</td>
<td>HLA-G</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>CD244 (2B4)</td>
<td>CD48</td>
</tr>
<tr>
<td>CD16</td>
<td>IgG</td>
</tr>
<tr>
<td>CD266 (DNAM-1)</td>
<td>CD112, CD155</td>
</tr>
<tr>
<td>CRACC</td>
<td>CRACC</td>
</tr>
<tr>
<td>NTB-A</td>
<td>NTB-A</td>
</tr>
</tbody>
</table>

NCR, natural cytotoxicity receptor
1.2.2. NK cell subsets

NK cells can be classified into two subsets depending on the level of cell surface CD56, CD56\textsuperscript{bright} and CD56\textsuperscript{dim}, each with distinct phenotypic properties (Cooper et al., 2001). CD56\textsuperscript{dim} NK cells express a moderate quantity of CD56 and represent the majority (>90%) of the peripheral blood NK cell population. Compared to the CD56\textsuperscript{bright} population, CD56\textsuperscript{dim} NK cells express higher levels of killer cell immunoglobulin-like receptors (KIR) and CD16. They also express a greater quantity of perforin and are regarded to have marked cytotoxic capacity. In contrast CD56\textsuperscript{bright} NK cells express high surface density of CD56 and contribute up to 10% of circulating NK cell pool. They are considered a less mature subset with the potential to differentiate into CD56\textsuperscript{dim} NK cells (Chan et al., 2007; Romagnani et al., 2007). CD56\textsuperscript{bright} NK cells express a high level of the inhibitory receptor CD94:NKG2A and are generally KIR and CD16. The primary role of the CD56\textsuperscript{bright} NK cell fraction is believed to be cytokine production, enabling them to have an immunoregulatory role at sites of inflammation where this population may be expanded. The functional distinction between these two subsets is not absolute; CD56\textsuperscript{bright} NK cells can express the degranulation marker CD107a, while CD56\textsuperscript{dim} NK cells can produce cytokines. Indeed the total interferon gamma (IFN-\(\gamma\)) production from CD56\textsuperscript{dim} NK cells can exceed that of the CD56\textsuperscript{bright} population (Korbel et al., 2009). Similarly CD56\textsuperscript{bright} NK cells are capable of cytotoxicity through the expression of TRAIL (Stegmann et al., 2010).

1.2.3. Role of cytokines in NK cell regulation

Signals derived from activating and inhibitory receptors are integrated by the NK cell to ultimately determine their functional outcome. However, a variety of cytokines such as IFN-\(\alpha\), IL-2, IL-12, IL-15, IL-18, IL-21 and IL-23 also play a role in NK cell development and function. To enable cytotoxicity of a target to occur a number of coordinated processes need to happen. These include cell adhesion, synapse formation, granule polarization and granule exocytosis. The relative contributions of these signals to activate an NK cell may vary according to the target cell involved, and it is clear that there is interplay between cytokine- and receptor-mediated signalling. For instance the adaptor molecule DAP10, which transduces activating signals from NKG2D, is associated with the IL-15 receptor b- and c-chains and this adaptor molecule can be phosphorylated by the IL-15-receptor-associated Jak-3
kinase (Horng et al., 2007). This system therefore provides a proximal molecular link between cytokine and receptor-mediated NK cell activation, which may extend to other cell surface activating receptors. IL-15 also plays an important role in NK cell development, promoting the differentiation of predominantly cytokine producing CD56\textsuperscript{bright} NK cells into cytotoxic CD56\textsuperscript{dim} NK cells (Huntington et al., 2009). IL-12 increases NK cell IFN-\gamma production while IL-2 enhances NK cell cytotoxicity (Biron et al., 1999).

1.2.4 “Missing-self” recognition, pathways to inhibition, and NK cell education

The critical role of inhibition as a means of regulating NK cell function was determined following a series of cytotoxicity experiments using MHC class I-deficient targets (Karre et al., 1986). The results of these were synthesized in the landmark ‘missing-self’ hypothesis (Ljunggren and Karre, 1990). In humans, MHC class I molecules comprise the classical human leukocyte antigen (HLA) HLA-A, -B, and -C (MHC class Ia), and the non-classical HLA-E, -F, and -G (MHC class Ib) molecules. They are key regulators of NK cell activity that are expressed ubiquitously on healthy cells and provide NK cells with a means of identifying ‘self’. Down-regulation or modification of MHC class I expression during viral infection, cellular stress or carcinogenesis releases the inhibitory signal to NK cells and permits their activation (Figure 1.2). Several inhibitory receptors for MHC class I have now been identified. The KIR family and the lectin-like CD94:NKG2A receptor play the most prominent role in NK regulation by MHC class I. These MHC specific receptors are also important for maturation of NK cells to a fully functional state in a process referred to interchangeably as ‘licensing’ or ‘education’ (Kim et al., 2005). There are several proposed theories for NK cell licensing, including the ‘arming’ and ‘disarming’ models (Raulet and Vance, 2006). The arming model proposes that, in the context of NK cell licensing, self-MHC inhibitory receptors serve similar functions to activating receptors providing positive signals that drive NK cell functional maturation (Yokoyama and Kim, 2006). The disarming model proposes that to acquire functional capacity NK cells require balanced activation and inhibitory signalling. In the absence of self-MHC class I recognition, NK cells are rendered anergic by downregulation of unopposed activation receptor signalling pathways. Therefore whilst these self-MHC receptors generate an inhibitory signal in the context of an effector response against targets, they emanate different signals to induce NK cell tolerance.
Figure 1.2. Receptor mediated regulation of NK cell activity and the “missing self” hypothesis

(a) In the absence of ligands for inhibitory or activating NK cell receptors, there is no activation of NK cells. Hyporesponsiveness is also seen in NK cells lacking inhibitory receptors for MHC class I which are necessary for NK cell education. (b) Healthy target cells express a normal quantity of MHC class I that interacts with inhibitory NK cell receptors conferring protection against NK cell mediated lysis. (c) In viral infection or tumour transformation there may be upregulation of activating ligands, with downregulation of MHC class I, leading to NK cell activation. (d) In the presence of activating and inhibitory ligands, NK cell activity is determined by signals integrated from activating and inhibitory signals.
1.3. KIR receptors

Killer cell immunoglobulin-like (KIR) receptors are type I transmembrane glycoproteins expressed on NK cells, γδ T cells and a minority of CD8 αβ T cells (Uhrberg et al., 2001). Encoded on chromosome 19 (19q13.4), they represent a tremendously polymorphic group of receptors, showing diversity at both locus and allelic levels (Middleton et al., 2008; Wilson et al., 2000; Wilson et al., 1997). There are 15 different KIR genes (13 expressed, KIR2DL1, KIR2DL2/L3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S1, KIR3DL2, KIR3DL3 and two pseudogenes, KIR2DP1 and KIR3DP1) expressed in a stochastic fashion to generate a repertoire of NK cells expressing different combinations of KIR in the same individual (Gardiner, 2008; Khakoo and Carrington, 2006; Robinson et al., 2013) (Figure 1.4). Their ligands, HLA-A, -B, and –C molecules, are encoded on chromosome 6 and are equally polymorphic. The rapid evolution and diversity of KIR/HLA genes supports the notion that they are subject to pathogen-mediated selection. This theory is reinforced by the observation that KIR receptor-ligand interactions may influence the outcome of viral infections such as HCV and HIV (Alter et al., 2007a; Khakoo et al., 2004; Martin et al., 2007).

1.3.1. KIR structure

KIR can be subcategorized by their number of extracellular immunoglobulin domains, either two or three (‘2D’ or ‘3D’), and by the length of their cytoplasmic tails, short or long (‘S’ or ‘L’) (D’Andrea et al., 1995; Wagtmann et al., 1995). In general, long cytoplasmic tails are associated with immunoreceptor tyrosine-based inhibitory motifs (ITIMs). In contrast KIR members with a short cytoplasmic tail contain a charged lysine in their transmembrane domain, allowing association with the adaptor protein DAP-12, consistent with an activating function. Receptor nomenclature would therefore imply that KIR2DL is composed of two immunoglobulin domains (2D), with a long (L) intracytoplasmic tail and an inhibitory function. The immunoglobulin domains, designated D0, D1 and D2, are the site of ligand recognition and can be used to further classify KIR receptors into three groups. Type I KIR2D receptors possess two immunoglobulin domains with a D1 and D2 conformation. The structurally divergent Type II KIR2D receptors have two extra-cellular domain proteins with a D0 and D2 conformation, where KIR3D receptors have three immunoglobulin domains (D0, D1 and D2) (Vilches and Parham, 2002). Crystal structure analysis of KIR2DL2 and KIR2DL3 has revealed variations in
the orientation of the D1 and D2 domains (Boyington et al., 2000a; Maenaka et al., 1999b). This difference in orientation may account for the higher avidity of KIR2DL2 compared to KIR2DL3 for HLA-C1 ligands (Moesta et al., 2008). Furthermore, the D1 and D2 domains contact the MHC class I molecule such that they overlap the C-terminal end of the bound peptide (Boyington et al., 2001). This may explain the peptide sensitivity of inhibitory KIR, particularly at positions 7 or 8 of the peptide (Fada et al., 2010).
Figure 1.3. Schematic diagram of how KIR diversity may influence NK cell function

From (Cheent and Khakoo, 2009). KIR diversity can impact NK cells at different levels. Variations in gene content and allelic diversity at the KIR locus results in different individuals having variable numbers of activating and inhibitory KIR. KIR are expressed stochastically on NK cells to generate a ‘KIR’ repertoire, which varies among different individuals. The presence or absence of human leucocyte antigen (HLA) class I ligands for these KIR may further impact on this repertoire, and on the functionality of these KIR-expressing subpopulations in different individuals.
1.3.2. KIR ligands

The various KIR receptors have discrete HLA ligands (Tables 1.1 and 1.2). For example, KIR2DL1 recognises HLA-C allotypes with a lysine at position 80 of the heavy chain (group 2 HLA-C, or HLA-C2), whereas KIR2DL2 and KIR2DL3, which segregate as alleles of the same locus, recognise the remaining HLA-C allotypes with asparagine at position 80 (group 1 HLA-C; HLA-C1) (Keaney et al., 2004; Mandelboim et al., 1996). Although KIR2DL2 and KIR2DL3 bind the same ligands, there are subtle differences. Disease association studies have shown that KIR2DL3 and HLA-C1, but not KIR2DL2, is protective against chronic HCV infection (Khakoo et al., 2004; Romero et al., 2008). This observation may be supported by the finding that KIR2DL3 binds more weakly to its ligands than KIR2DL2, therefore permitting a lower threshold for NK cell activation (Fadda et al., 2010; Winter et al., 1998). Whereas all HLA-C alleles encode KIR ligands, this is only the case for 46% of HLA-A alleles and 36% of HLA-B alleles (Parham et al., 2012). KIR3DL1 binds HLA-B allotypes carrying the Bw4 motif (Cella et al., 1994; Gumperz et al., 1995), while no KIR have been shown to bind to the alternative motif, Bw6. KIR3DL2 binds to HLA-A3 and HLA-A11 allotypes in a peptide specific manner (Dohring et al., 1996; Hansasuta et al., 2004). KIR2DL4, the only activating KIR receptor with a long tail, binds the non-classical MHC class I molecule HLA-G (Ponte et al., 1999).

1.3.3. Evolutionary aspects of KIR/ MHC class I

Simian primates and rodents utilise structurally diverse, distinctly evolved but functionally similar KIR and Ly49 as their receptors for polymorphic MHC class I molecules (Parham, 2008). Although KIR-like sequences have been found recently in ungulates and other mammals, cattle are the only non-primate species to have a diversified family of KIR (McQueen et al., 2002). Phylogenetic analysis suggest that KIR genes evolved in mammals from the ancestral KIR3D gene that duplicated to form a common ancestor of the 3DL gene and 3DX gene (Guethlein et al., 2007). In humans KIR3DL expanded rapidly, most likely driven by highly diversified MHC class I molecules and pathogen selection, whereas KIR3DX became redundant. Meanwhile, cattle diversified the KIR3DX gene while retaining a single copy of the KIR3DL gene. Few KIR remain orthologous in primates, the exception being 2DL4, which is present in humans, apes and monkeys (Sambrook et al., 2006).
The MHC class I molecule HLA-C is more recently evolved than its HLA-A and -B counterparts and likely diversified from an HLA-B-like ancestor possessing the C1 epitope (Moesta et al., 2009). Indeed HLA-C has evolved to be a more specialized ligand for KIR, as evidenced by the observation that all HLA-C allotypes can serve as KIR ligands, compared to only a fraction of HLA-A and B allotypes (Parham et al., 2012).

A unique feature of the human KIR is the segregation into two groups of KIR haplotype, designated groups A and B. Haplotype A is enriched for inhibitory KIRs, whereas B haplotype is more varied and is distinguished by the accumulation of genes encoding activating KIR receptors (Uhrberg et al., 1997). All human populations possess both A and B haplotypes, suggesting that they have balancing functions. Selection for KIR A haplotypes may be important for the successful outcome of viral infections. For example, KIR2DL3, an A haplotype allele, is protective against chronic HCV infection, whereas KIR2DL2, a B haplotype allele, is not (Khakoo et al., 2004). In addition to their importance immune defence, NK cells have a key role in reproductive health. Haplotype B selection may confer reproductive success, as evidenced by mothers who lack this haplotype (AA genotype) having higher rates of preeclampsia and miscarriage, particularly if the foetus expresses HLA-C2 (Hiby et al., 2008; Hiby et al., 2004). Such disorders of pregnancy have been linked with insufficient uterine invasion of the foetal trophoblast cells, a process that is guided by activated uterine NK cells from the mother. Thus activating KIR associated with the KIR B haplotype may be beneficial for promoting better placental development, thus maintaining selection of this group of alleles.

1.4. CD94 and NKG2 members

CD94 is a type 2 transmembrane glycoprotein belonging to the C-type lectin-like receptor (CTLR) family, which includes the inhibitory subunit NKG2A (which has a splice variant NKG2B) and the activating subunits NKG2C, NKG2E, and NKG2D. The genes for this family are encoded in the NK cell receptor complex on chromosome 12p13 (Plougastel and Trowsdale, 1998). CD94 exists on the cell surface primarily in a heterodimeric form with either NKG2A/C or E. NKG2D is somewhat distinct to the other CTLR molecules, operating as a homodimer. Whereas CD94/NKG2 bind the non-
classical MHC molecule HLA-E, human NKG2D recognises stress-induced and tumour ligands such as MHC class I-related chain A (MICA), MICB and UL-16 binding proteins (ULBP) 1-5 (Bauer et al., 1999).

1.4.1. CD94:NKG2A structure

CD94:NKG2A is a heterodimeric inhibitory receptor consisting of two C-type lectin subunits, CD94 (30 kDa) and NKG2A (43 kDa), linked by disulphide bonds (Lazetic et al., 1996). Typical C type lectins are defined by their binding to carbohydrates in a Ca\(^{2+}\) dependent manner. In contrast CD94:NKG2 molecules lack these Ca\(^{2+}\) binding residues and therefore do not bind carbohydrates (Day, 1994). CD94 also lacks one of two alpha helices known to be present in C-type lectins (Boyington et al., 1999). Instead of an a2 helix, CD94 possesses a long unstructured loop region that makes extensive intramolecular interaction with its counterpart. Although CD94 can exist as a homodimer, its preferred assembly is as a heterodimer with an NKG2A/C or E molecule (Boyington et al., 1999). This is partly explained by the fact that CD94 lacks signalling capacity as it does not possess a cytoplasmic tail, unlike NKG2A which has an ITIM containing cytoplasmic domain, and NGK2C/E which harbour the ITAM-containing adaptor protein DAP-12 conferring an activating function.

The structure of CD94:NKG2A interacting with HLA-E\(_{\text{peptide}}\) has been recently determined (Kaiser et al., 2008; Petrie et al., 2008). On binding with HLA-E\(_{\text{peptide}}\), no structural rearrangement of CD94:NKG2A is observed, indicating that a lock and key mechanism is used. The CD94 subunit forms the majority of the interactions with HLA-E. The peptide contributes 23% of the interface between receptor and ligand. CD94 remains dominant (80%) in the interaction with the peptide, making contact with P5, P6, and P8 residues whereas the NKG2A moiety only interacts with the P5 residue (Petrie et al., 2008). Thus CD94 may be crucial to interacting with ligand, while NKG2A serves to provide signalling capacity to the receptor. This theme is covered in greater detail in Chapter 4.
1.4.2. CD94:NKG2A ligand

Compared to KIR, CD94:NKG2A is relatively non-polymorphic as is its ligand, the MHC class Ib molecule HLA-E. HLA-E is expressed on virtually all healthy cells but at lower levels than MHC class Ia molecules (Braud et al., 1999). Its cell surface expression requires specific nonameric peptides derived from the leader sequence (amino acids 3–11) of MHC class Ia molecules HLA-A, -B and -C, and the MHC class Ib molecule HLA-G (Braud et al., 1998; Llano et al., 1998). HLA-E is unable to bind its own leader peptide; therefore HLA-E expression reflects the presence of the other MHC class I molecules, and the capacity of the cell to process and present antigen (Hoare et al., 2008). Only two variants of HLA-E have been described, HLA-E^R and HLA-E^G, differing only at one amino acid position with either an arginine or a glycine at position 107, respectively (Strong et al., 2003). This system enables NK cells to observe the presence of a diverse panel of polymorphic MHC-class I molecules using a single, highly-conserved receptor-ligand combination. The affinity and kinetics of CD94:NKG2A/HLA-E interaction are very similar to those seen between certain KIR receptors and their MHC class I ligands, characterised by remarkably fast association and dissociation rates and relatively weak affinities for MHC class I (Vales-Gomez et al., 1999; Vales-Gomez et al., 1998).

1.4.3. CD94 evolution

CD94:NKG2A has been conserved throughout evolution, with functional equivalents present in mice and primates (Shum et al., 2002; Vance et al., 1999). There are several benefits offered by the CD94:NKG2A receptor system. As the vast majority of NK cells express CD94:NKG2A, the presence of this highly conserved receptor:ligand pairing may safeguard against alloreactivity in face of the highly polymorphic KIR:HLA system. Furthermore, CD94:NKG2A provides a constancy of NK cell education as determined by response to an MHC deficient target compared to the variability of KIR education (Yawata et al., 2008). CD94 also plays a crucial role in the protection against mousepox, a lethal viral infection in mice (Fang et al., 2011).
1.5. MHC class I

MHC class I molecules were acknowledged as potential ligands for NK cell receptors following the discovery that target cell susceptibility to NK cell mediated lysis was inversely related to the level of MHC class I expressed by the target cell (Karre et al., 1986; Storkus et al., 1989). Subsequent studies revealed the existence of multiple NK cell receptors that recognised distinct MHC class I ligands. NK cells expressing "p58" molecules (58kDa proteins later identified as KIR2DL members), were shown to specifically interact with HLA-C (Ciccone et al., 1992; Moretta et al., 1993). Transfection of HLA-Cw3 into an MHC deficient P815 murine cell line protected these cells against p58+ NK cell lysis. However, transfection with other MHC class I alleles (HLA-A molecules) did not confer protection. Moreover, NK cells expressing receptors other than p58 still lysed HLA-Cw3 transfected targets. It is now accepted the various KIR receptors bind distinct HLA-A, -B and –C molecules, whereas the relatively conserved CD94:NKG2A receptor binds to HLA-E.

All functional MHC class I molecules are heterotrimeric complexes consisting of a polymorphic MHC-encoded heavy chain, a non-MHC-encoded light chain called β2-microglobulin and a short peptide fragment comprising 8-10 residues (Marsh et al., 2000; Schumacher et al., 1990; Townsend et al., 1989). In healthy cells, these tightly bound peptides are derived from a variety of intracellular proteins that are degraded by cytosolic proteases leading to the generation of an array of self peptides (Figure 1.4). MHC class Ia molecules (HLA-A, -B, and –C) bind a highly diverse group of peptides, in contrast to HLA-E which predominantly binds a restricted set of peptides derived from the leader sequence of MHC class Ia molecules and HLA-G (Braud et al., 1997; Braud et al., 1998; Lee et al., 1998). The peptides are translocated to the endoplasmic reticulum via ATP-dependent transporter associated with antigen processing (TAP) molecules where they associate with assembling MHC-class I to form a stable complex which can then be expressed at the cell surface. Each MHC-class I molecule is capable of binding a different peptide, leading to the presentation of a broad range of self peptides at the cell surface. Utilising their α/β T cell receptor (TCR), cytotoxic T lymphocytes (CTL) are able to survey a cells cytoplasmic contents via MHC-class I, monitoring for the presence of non-self peptides. In viral infections, the peptide repertoire of MHC-Class I may change as viral proteins are degraded in the cytosol (Hickman et al., 2003; Meiring et al., 2006). Presentation of viral peptides by MHC class I
may therefore represent an important mechanism by which CTLs identify virus infected cells. However, such peptide-MHC complexes can also influence NK cell activity.

Each MHC class I molecule has a strict preference for the peptide sequence it will bind. There are key residue positions that anchor the peptide to MHC class I, therefore alterations to the peptide sequence can promote or interfere with binding to the MHC class I molecule. However, both KIR and CD94:NKG2A recognition of MHC class I is also sensitive to the particular peptide bound to the class I molecule. Consequently not all MHC class I bound peptides will be recognised by NK cell receptors.

Degradation of cellular contents by cytosolic proteases, such as the multicatalytic proteasome complex, leads to the generation of a variety of self peptides. These peptides are translocated from the cytoplasm into lumen of the endoplasmic reticulum (ER) by a transporter associated with antigen processing (TAP) dependent mechanism. Here they bind to a complex of β2-microglobulin and MHC class I heavy chain to form a stable complex which then leaves the ER via the secretory pathway to reach the cell surface. Adapted from O’Callaghan, (2000).

Figure 1.4. Peptide loading onto MHC class I

Degradation of cellular contents by cytosolic proteases, such as the multicatalytic proteasome complex, leads to the generation of a variety of self peptides. These peptides are translocated from the cytoplasm into lumen of the endoplasmic reticulum (ER) by a transporter associated with antigen processing (TAP) dependent mechanism. Here they bind to a complex of β2-microglobulin and MHC class I heavy chain to form a stable complex which then leaves the ER via the secretory pathway to reach the cell surface. Adapted from O’Callaghan, (2000).
1.6. NK cells and viruses

Although NK cells were first described on the basis of their anti-tumour functions, they play a central role in defence against pathogens, particularly viruses (Brandstadter and Yang, 2011). NK cell deficiencies have been reported in individuals with human cytomegalovirus (HCMV), Epstein Barr virus (EBV), Human immunodeficiency virus (HIV), Hepatitis B virus and Hepatitis C virus; however, it is often difficult to establish whether such deficiencies are a cause or consequence of infection (Bonavida et al., 1986; Corado et al., 1997; Dunn et al., 2009; Merino et al., 1986). Mice that are depleted or deficient of NK cells have increased susceptibility to herpes simplex virus (HSV), murine cytomegalovirus (MCMV), coxsackie virus and influenza (Bukowski et al., 1984; Godeny and Gauntt, 1987; Habu et al., 1984; Stein-Streilein and Guffee, 1986). The crucial role of NK cells in viral infections is perhaps best illustrated by a small number of individuals in whom NK cells are absent. For example, a longitudinal follow-up of a female patient with an isolated deficiency affecting NK cells demonstrated recurrent severe herpesvirus infections, including varicella zoster, human cytomegalovirus (HCMV) and herpes simplex virus (HSV) (Biron et al., 1989). Thus, the critical function of NK cells in viral infections is unequivocal, and is further supported by the various strategies viruses have evolved to subvert NK cell immune surveillance (Figure 1.5).
Figure 1.5. Viral mechanisms for evading NK cells

The strategies by which viruses evade NK cells fall into five categories and are depicted in the interaction between a virus infected target cell (left) and an NK cell (right). (a) NK cells can be inhibited by a viral MHC class I homologue with structural similarity to endogenous host class I that binds to inhibitory class I receptors on NK cells. (b) Viruses can inhibit expression of HLA-A and HLA-B, resulting in a relative increase in HLA-C and HLA-E on the surface of the target cell; these inhibit NK cells through the class I inhibitory receptors CD94:NKG2A and KIR, respectively. Alternatively, viral gene expression can result in selectively increased expression of HLA-E, which inhibits NK cells through CD94:NKG2A. (c) Virus-encoded proteins can function as cytokine binding proteins that block the action of NK cell activating cytokines. In addition, viruses can produce homologues, or increase host production of cytokines that inhibit NK cells. (d) NK cell activities can also be avoided by decreased expression of NK cell–activating ligands in virus-infected target cells, which prevent signal transduction via NK cell–activating receptors. To achieve the same end, viruses can encode antagonists of the activating receptor–ligand interaction. (e) Viruses can also directly inhibit NK cells by infecting them or using envelope proteins to ligate NK cell inhibitory receptors. Proteins outlined in red are virally encoded. From Orange et al, (2002).
1.6.1 NK cells in HIV infection

Emerging evidence from both in vitro and animal studies demonstrate the importance of NK cells in limiting viral activity during the early stages of human immunodeficiency virus (HIV)-1 infection (Alter and Altfeld, 2009; Alter et al., 2007b). This role is emphasised by the influence of variegated KIR on the outcome of HIV infection. Martin et al. provided the first demonstration of the role of KIR polymorphisms in determining the outcome of a viral infection when they reported that the activating receptor KIR3DS1 in combination with HLA-B alleles that have the Bw4 serological epitope with isoleucine at position 80 (HLA-Bw4\(^{80I}\)) were associated with a delayed progression of HIV infection (Martin et al., 2002). More recently, it has been shown that inhibitory KIR/HLA incompatibility between sexual partners confers protection against HIV-1 transmission possibly due to alloreactive NK cells from the exposed partner killing KIR ligand-mismatched target cells from an HIV-1 infected partner (Jennes et al., 2013). NK cells can suppress HIV replication through direct cytotoxic activity against infected cells as well as the production of anti-viral cytokines such as IFN-γ. Consistent with this, exposed uninfected individuals exhibit greater in-vitro NK cell cytotoxicity and IFN-γ production (Ravet et al., 2007; Scott-Algara et al., 2003). However, HIV appears to have the ability to escape NK cell immune responses (Alter et al., 2005; Fogli et al., 2004; Kottlil et al., 2003; Nattermann et al., 2005b). NK-cell cytotoxicity and cytokine secretion are impaired during chronic HIV infection, but these deficiencies likely occur earlier in the course of infection (Naranbhai et al., 2013). Most studies assessing immune responses in HIV infection compare infected individuals with an uninfected control group of individuals. Naranbhai et al. (2013) analysed samples from 41 high-risk heterosexual women enrolled in a randomised controlled trial of Tenofovir microbicide gel. Paired samples were collected from initially HIV-uninfected individuals prior to HIV acquisition, and during viraemic acute HIV-1 infection. They demonstrate that NK cells are activated during acute HIV-1 infection with IFN-γ production correlating with viral load. Alter et al. report an initial expansion of NK cells during acute HIV-1, particularly of the cytotoxic CD56\(^{dim}\) subset that may account for the early fall in viraemia. An increase in KIR\(^+\) but not NKG2A\(^+\) NK cells is observed during acute HIV infection which suppresses NK cell cytotoxicity compared to the KIR\(^-\) population (Mavilio et al., 2003; Naranbhai et al., 2013).

In chronic HIV infection there is an expansion of a functionally defective CD56\(^{-}\) CD16\(^+\) NK cell subset with an associated reduction in both the CD56\(^{bright}\) and CD56\(^{dim}\) NK populations (Alter et al., 2005;
Mavilio et al., 2005; Mavilio et al., 2006). HIV also targets MHC class I expression to escape recognition by CTLs while maintain protection against NK cells. For example the HIV protein Nef selectively targets HLA-A and HLA-B, while leaving HLA-C and HLA-E relatively intact (Bonaparte and Barker, 2004; Cohen et al., 1999; Williams et al., 2002). Consequently, NK cells expressing KIR3DL1 are more likely to be released from inhibition by HIV-infected targets than are NK cells that express other inhibitory receptors, or even NK cells expressing KIR3DL1 in combination with an inhibitory receptor for HLA-C. There is also greater complexity at the HLA-B locus for the Bw4-positive allotypes and KIR3DL1. Different KIR3DL1 alleles are expressed on the cell surface in varying quantities and individual KIR3DL1 allotypes bind to diverse HLA-B allotypes with different avidities. The clinical correlate of this observation is that in HIV infection KIR3DL1 alleles that are expressed at high levels confer the greatest protection (Martin et al., 2007).

Blocking the interaction between HLA-C and -E and their corresponding KIR and CD94:NKG2A inhibitory receptors increases NK cell killing of CD4+ T cells infected with HIV strains that reduce MHC class I expression (Bonaparte and Barker, 2004). However, HIV may potentially modulate CD94:NKG2A mediated NK cell regulation by encoding for a peptide that increases HLA-E expression. Peptide HIVp24 aa14-22 (AISPRTLNA; HIVp2414-22) is a well recognised T cell epitope that was identified using a motif based approach at a potential ligand for HLA-E (Nattermann et al., 2005b). Nattermann et al. (2005b) showed that HIVp2414-22 increased cell surface HLA-E expression leading to a subsequent inhibition of cytotoxicity of an NKL cell line via the CD94:NKG2A receptor. Furthermore, this study demonstrated that lymphocytes from HIV infected individuals have a significantly greater expression of HLA-E compared to healthy control lymphocytes, and that in-vitro infection of PBMCs with HIV-1 leads to an increase in HLA-E expression protecting these cells from NK cell mediated lysis despite down-regulation of classical MHC class I molecules. Thus, increased cell surface HLA-E on HIV-infected cells may play a part in the immune escape from NK cell surveillance.
1.6.2. NK cells in CMV infection

Human Cytomegalovirus (HCMV), a double stranded DNA virus belonging to the β-herpesvirus family, is highly prevalent in humans. Seropositivity detected in up to 80% of UK adults and potentially higher prevalence in the developing world (Vyse et al., 2009). Infection is usually acquired at a young age and the majority of immunocompetent hosts are asymptomatic (Jost and Altfeld, 2013). However, reactivation of latent CMV infection is a significant cause of morbidity and mortality following solid organ and haemopoetic transplantation, immunosuppressive treatment and acquired immune deficiency syndrome (AIDS). Reactivation or acquisition of infection in pregnancy is a major cause of congenital CMV which may result in sensorineural deafness and developmental delay. Most of the animal models to assess CMV infection have used murine CMV (MCMV), a closely related virus sharing approximately 50% homology to HCMV. Both MCMV and HCMV have evolved a number of mechanisms to escape NK cell recognition of infected cells. For example, CMV can down-regulate ligands for the NK cell activating receptor NKG2D. In mice, the ligands for NKG2D include the stress induced molecules RAE-1, MULT1 and H60 (Diefenbach et al., 2000). The MCMV proteins m152, m145 and m155 down-modulate expression of RAE-1, MULT1 and H60, respectively (Arapovic et al., 2009; Krmpotic et al., 2005; Lodoen et al., 2004). The human NKG2D ligands, MICB, ULBP-1 and ULBP-2 are down-regulated by the HCMV glycoprotein UL16, whilst UL142 reduces expression of MICA (Ashiru et al., 2009; Bacon et al., 2004; Cosman et al., 2001; Wills et al., 2005). Thus, attenuated expression of NKG2D ligands mediated by CMV may impair NK cell activation and cytolytic activity against infected cells.

HCMV can also interfere with signalling at the NK cell inhibitory receptors CD94:NKG2A and LILRB1 (ILT-2). The HCMV UL40 glycoprotein contains a nonameric peptide, VMAPRTLIL, which is an exact homologue to an HLA-E binding HLA-C derived leader peptide. This peptide therefore increases cell surface HLA-E expression leading to inhibition of CD94:NKG2A+ NK cells (Tomasec et al., 2000; Ulbrecht et al., 2000). HCMV also possesses the glycoprotein UL18, a viral mimic of MHC Class I that can bind to β2m (Beck and Barrell, 1988). Cell surface UL18 binds to LILRB1 with greater affinity compared to MHC Class I, thus inhibiting activation of NK cells expressing this receptor (Chapman et al., 1999; Cosman et al., 1997; Prod'homme et al., 2007).
One of the most consistent findings in CMV infection is an expansion and persistence of NKG2C⁺ NK cells (Beziat et al., 2012; Foley et al., 2012; Guma et al., 2004; Guma et al., 2006; Lopez-Verges et al., 2011). The activating receptor NKG2C is expressed in under 2% of HCMV seronegative individuals, compared to up to 25% in those with positive HCMV serology (Guma et al., 2004; Guma et al., 2006). This NKG2C⁺ subset represent a more differentiated phenotype as evidenced by the acquisition of the maturation marker CD57, KIR positivity, and the absence of the inhibitory receptor NKG2A (Della Chiesa et al., 2012; Foley et al., 2012; Lopez-Verges et al., 2011). Foley et al. (2012) also demonstrate this specific NK-cell subset persists and expands on HCMV reactivation, while a similar memory has also been observed in Ly49 expressing NK cells in MCMV infection (Sun et al., 2009). Thus, at least in CMV infection, there is evidence that NK cells develop a memory phenotype previously thought to be specific to adaptive immune cells. NKG2C acquisition is therefore likely represents a protective host response to CMV infection. However, the precise mechanism by which NKG2C⁺NK cells recognises HCMV has yet to be elucidated. NKG2C binds HLA-E with a six-fold lower affinity that its inhibitory counterpart NKG2A (Kaiser et al., 2005), and at present an HCMV-encoded protein that directly binds NKG2C has not been identified.

1.7. NK cells in the liver

The liver is enriched in NK cells. This intrahepatic NK cell population embedded in the endothelial lining of the liver sinusoids were originally termed ‘pit cells’ (Wisse et al., 1976). These are large granular lymphocytes that are capable of spontaneous cytotoxicity against an MHC deficient cell line without prior sensitisation, a defining NK cell characteristic (Bouwens et al., 1987; Kiessling et al., 1975). They contain distinctive granules and, uniquely, rod-core vesicles (Kaneda and Wake, 1983). They can be sub-classified based on the density and size of their granules which are either low-density and small or high-density and large, with NK cells expressing the latter resembling peripheral blood NK cells. It has been shown in rats that peripheral blood high-density large granular cells migrate to the liver and differentiate into liver specific low-density small granular NK cells (Vanderkerken et al., 1993).
Intrahepatic NK cells may behave differently to NK cells in other areas due to the tolerogenic environment in the liver. Murine intrahepatic NK cells are hyporesponsive. They are less cytotoxic and have an altered cytokine profile producing lower levels of IFN-γ and greater levels of immunoregulatory cytokines, such as IL-10, compared to peripheral blood and splenic NK cells (Lassen et al., 2010). This hyporesponsive state has been described in the early stages of hepatitis B virus infection and may contribute to the establishment of chronic infection (Dunn et al., 2009).

In addition to their role in protection against pathogens and tumour transformation, intrahepatic NK cells have been demonstrated to have anti-fibrotic functions via inhibition of hepatic stellate cells (HSC). They are capable of directly inducing HSC apoptosis, and producing IFN-γ which inhibits HSC activation (Baroni et al., 1996; Melhem et al., 2006; Notas et al., 2009). Moreover, greater levels of peripheral blood NK cell cytotoxicity have been associated with less liver fibrosis in patients with chronic HCV, consistent with the lysis of activated hepatic stellate cells (Morishima et al., 2006).

1.7.1. NK cells in acute HCV infection

NK cells have been recently been implicated in the acute phase of HCV infection. Nowadays, these patients rarely come to the attention of clinicians as they are frequently asymptomatic and therefore do not seek medical assistance. Amadei et al. reported an increase in CD56\textsuperscript{bright} NK cells (with associated reduction in CD56\textsuperscript{dim} subset) in acute HCV patients compared to healthy individuals (Amadei et al., 2010). Individuals who spontaneously cleared the virus show a decline in the CD56\textsuperscript{bright} population, with levels comparable to healthy control individuals after 1-3 months, indicating a return to baseline which is not observed in those who progressed to chronic infection. Expression of the activating receptor NKG2D is also increased in the acute phase of infection. Functional experiments show augmented IFN-γ production and cytotoxicity in these patients and a trend for more NK cell degranulation in individuals expressing HLA-C1 specific KIR receptors, which is maximal in those with self-limiting infection. Thus in the acute phase of HCV infection there is activation of NK cells indicating their role in the immune response at this stage (Figure 1.6). Pelletier et al. have also studied individuals in the acute phase of HCV infection (Pelletier et al., 2010). They also found increased activity of NK cells in the acute phase of infection, as determined by a degranulation assay.
However, they show that the NK cells from intravenous drug users have generally lower levels of IFN-γ secretion compared to healthy control NK cells, postulating this may be connected to opioid use. They demonstrated that the quantity of the inhibitory receptor NKG2A declined on CD56^{bright} NK cells during the follow-up phase only in spontaneously resolvers. Moreover, they were able to correlate NK cell activity with T cell activity, implying a co-ordinated innate and adaptive immune response to HCV in the acute phase of infection. Thus there is activation of NK cells in the acute phase of HCV infection which declines in those clearing HCV and persists in those remaining chronically infected.
Figure 1.6. NK cells in acute HCV infection

As a component of the innate immune system, natural killer (NK) cells represent the primary effector population in the early stages of acute hepatitis C virus (HCV) infection. Downregulation of major histocompatibility complex (MHC) class I on virus infected hepatocytes reduces the inhibitory signal to NK cells, shifting the balance towards NK cell activation. Dendritic cells (DC) engage with NK cells via the NKp30 receptor, and produce cytokines that boost NK cell proliferation towards an “NK1” phenotype. In addition to direct cytotoxicity of target cells, activated NK1 cells produce cytokines such as IFN-γ and TNF-α which suppress HCV replication, reciprocally activate DCs, and prime naive CD4 T-cells inducing a T-helper (Th)-1 response.
1.7.2. NK cells in chronic HCV infection

NK cells are more amenable to study in individuals with chronic HCV infection. Comparison with healthy donors reveals perturbations in NK cell frequency, phenotype and function (Figure 1.7). Peripheral blood NK cell frequencies, both absolute number and percentage of total lymphocyte population, are reduced in chronic HCV compared to healthy individuals (Bonorino et al., 2009; Dessouki et al., 2010; Meier et al., 2005; Morishima et al., 2006; Oliviero et al., 2009). The reduction in NK frequency may either be a consequence of HCV infection or a predisposing factor to chronic HCV infection, and both hypotheses have some support. In individuals with chronic HCV infection, NK cell frequency increases following successful antiviral therapy while reduction in peripheral blood NK cell frequency in individuals with chronic HCV as compared to spontaneous resolvers has also been noted. IL-15, a pivotal cytokine for NK cell development, proliferation and function, may be relevant here. Meier et al. demonstrate a significant reduction in serum IL-15 levels in HCV patients as compared to healthy controls and show that exogenous IL-15 rescues HCV NK cells from apoptosis, increasing ex-vivo proliferation and function (Meier et al., 2005). Furthermore, DCs are an important source of IL-15 and have been shown to cross talk with NK cells. In chronic HCV infection, IL-15 production by IFN-α-stimulated DCs is deficient (Jinushi et al., 2003). Thus a downstream consequence of DC dysfunction could be a failure of production or proliferation of NK cells.
In chronic HCV infection, changes occur in NK cell frequency, phenotype and function. HCV core encoded peptides upregulate MHC class I (particularly HLA-E) on virus infected cells, which may impair NK cell surveillance. Upregulation of the inhibitory receptor CD94:NKG2A binding to its ligand HLA-E leads to skewing of the NK cell cytokine profile towards the immunoregulatory cytokines IL-10 and TGF-β. These NK2 cytokines influence DC and T cell function, leading to further production of immunoregulatory cytokines and generation of a T-helper (Th)-2 response. Altered production of cytokines crucial for NK cell development leads to a reduction in NK cell frequency. These changes collectively provide an environment permissive for HCV infection. Additionally, upregulation of NK cell tumour necrosis factor related apoptosis inducing ligand (TRAIL) contributes to liver injury while increased TGF-β and attenuated IFN-γ production promotes activation of hepatic stellate cells (HSC) leading to fibrosis. Regulatory T cells (Treg) may suppress the tumour surveillance function of NK cells, increasing the risk of hepatocellular carcinoma.

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1.7.3. Skewing of subset distribution

A number of studies have documented a relative increase in circulating CD56^{bright} but not CD56^{dim} NK cells in individuals with chronic HCV compared to healthy individuals and spontaneous resolvers (Bonorino et al., 2009; De Maria et al., 2007; Golden-Mason et al., 2008; Lee et al., 2010; Lin et al., 2004; Morishima et al., 2006). This is unlikely to be due to preferential retention of the CD56^{dim} NK cells in the liver as Bonorino et al. (2009) demonstrate that the intrahepatic CD56^{dim} population as a percentage of total NK cells is also reduced (80.5% intrahepatic compared to 94% in peripheral blood), while the CD56^{bright} population is expanded (19.5% intrahepatic compared to 6.0% in peripheral blood). CD56^-CD16^+ NK cells appear to represent a more terminally differentiated NK cell subset and there is an expansion of this population in chronic HCV infection (Gonzalez et al., 2009). CD56^-CD16^+ NK cells have reduced perforin expression compared with CD56^{dim} NK cells and have been shown to be hypofunctional, particularly in their interactions with DCs (Mavilio et al., 2006). In HCV, chemokine production by this subset is skewed towards MIP-1β, and there is also a reduction in IFN-γ and TNF-α secretion compared to the CD56^+ NK population. Thus overall there is a skewing of NK cells away from the CD56^{dim} CD16^+ subset, thought to be the main cytotoxic subset of NK cells. This is may be an effect of IFN-α, as there is a strong IFN-α response to HCV infection, while therapy with PEGylated IFN-α and ribavirin leads to an increase in CD56^{bright} and a decline in CD56^{dim} NK cells (de Oliveira Crispim et al., 2012; Su et al., 2002).

1.7.4. Alterations in Phenotype

Changes in phenotype may reflect changes in subset distribution and also the effect of cytokines on specific subsets of NK cells. The most consistent finding in chronic HCV infection has been an increase in NKG2A expression (Jinushi et al., 2004; Nattermann et al., 2006; Nattermann et al., 2005a). This occurs on both intrahepatic and peripheral blood NK populations (Bonorino et al., 2009), and likely represents expansion of CD56^{bright} NK cells that are KIR-negative and NKG2A-positive (Dessouki et al., 2010; Jinushi et al., 2004; Oliviero et al., 2009; Su et al., 2002). Expression of activating receptors is also increased. Such receptors include NKG2C, NKp44, NKp46 and NKp30.
Initial reports of decreased expression of NKp46 have not been subsequently confirmed (Nattermann et al., 2006). Similarly, there is conflicting evidence with respect to NKG2D expression which has been reported to be up-regulated, down-regulated and also unchanged (De Maria et al., 2007; Harrison et al., 2010; Jinushi et al., 2004; Takehara and Hayashi, 2005). Such contrasting data may relate to genuine differences in NK cell phenotype and function, difference in the populations studied, or technical issues such as sample preparation, cytokine stimulation or freezing. However, taken together there appears to be an activation of NK cells in the acute phase of HCV infection, which persists into the chronic phase.

1.7.5. Altered function

Initial reports suggested diminished natural cytotoxicity in chronic HCV that was restored by successful HCV clearance with IFN-α and ribavirin therapy (Bonavita et al., 1993; Corado et al., 1997; Crotta et al., 2002; Par et al., 2002). However the number of cytotoxic CD56dim NK cells in the peripheral blood is depressed and hence, recent studies which take into account cytotoxicity per NK cell have demonstrated normal or increased NK cell cytotoxicity in chronic HCV (Ahlenstiel et al., 2010; De Maria et al., 2007; Duesberg et al., 2001; Golden-Mason et al., 2008; Morishima et al., 2006; Oliviero et al., 2009; Yoon et al., 2009). There is greater expression of activation markers such as CD122 (a subunit of IL-2 receptor that is crucial for IL-2 and IL-15 signalling), CD69, and NKp44 (Ahlenstiel et al., 2010; Oliviero et al., 2009). Up-regulation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) on NK cells also may be an important mechanism underlying the anti-HCV effect of NK cells. Liver NK cells expressing TRAIL kill autologous hepatocytes in mice (Ochi et al., 2004), and may therefore contribute to liver injury. NK cell TRAIL expression is increased in chronic HCV, and these cells have a phenotype consistent with IFN-α stimulation (Ahlenstiel et al., 2010; Stegmann et al., 2010).

A change in the cytokine profile of NK cells in chronic HCV may be relevant to the persistence of HCV infection. Failure of NK cell production of IFN-γ in chronic HCV has been reported (Ahlenstiel et al., 2010; Dessouki et al., 2010; Oliviero et al., 2009). IFN-γ has potent anti-HCV properties, directly blocking HCV replication in a dose-dependent manner (Crotta et al., 2010; Jo et al., 2009; Wang et
IFN-γ also indirectly suppresses HCV activity by polarising T cell differentiation towards a virus specific Th1 phenotype. Additionally, increased HCV-NK cell production of Th2 cytokines such as IL-10 and TGF-β, and the chemokine IL-8, may skew the cytokine profile to environment which is more permissive for HCV (Crotta et al., 2010; De Maria et al., 2007; Jinushi et al., 2004; Oliviero et al., 2009; Polyak et al., 2001). Cytokines may also affect the differentiation and maturation of NK cells (Loza and Perussia, 2001). In particular NK cells can be polarised towards an NK2 phenotype under the influence of IL-4 and IL-13 (Peritt et al., 1998). Thus the cytokine microenvironment regulates both NK cell phenotype and function. In chronic HCV infection the dominant effect on NK cells appears to be of IFN-α, but the roles of IL-4, IL-10 and IL-13 warrants further investigation.

NK cells may also be modulated by direct cellular interactions, especially with DCs. All mature NK cells express the activating receptor NKG2D, the ligand for which are MHC class I chain related (MIC) proteins. In HCV infection there is an impairment of MIC-A/B expression which may impair NK cell activation (Jinushi et al., 2003). NK cell activation of DCs may be reciprocally perturbed. When co-cultured with human hepatic cells, healthy control NK cells enhance maturation and activation of DCs to promote a Th1-polarised CD4 T-cell response (Jinushi et al., 2004). HCV-NK cells have a reduced capacity to activate DCs due to CD94:NKG2A-mediated NK cell inhibition. This has a reciprocal effect of skewing NK cell cytokine expression towards the immunoregulatory cytokines IL-10 and TGF-β, which promote Th2 type differentiation. Interestingly, inhibition of NKG2A restored the ability of HCV-NKs to activate DCs, and also the production of the Th1 cytokines IFN-γ and TNF-α. This may be important as HCV can up-regulate cell surface HLA-E, the ligand for NKG2A, in vivo (Nattermann et al., 2005a).

The activation status of NK cells correlates with liver inflammation. Increased expression of NKG2A, CD69 and CD107a (a marker of NK cell degranulation) on peripheral blood NK cells have all been linked with disease-related elevation of ALT levels. Additionally, Oliveiro et al showed an inverse correlation of peripheral NK cell NKG2D expression with ALT (Ahlenstiel et al., 2010; Bonorino et al., 2009; Oliviero et al., 2009; Stegmann et al., 2010). NK cells have been localised to necrotic areas in liver biopsy specimens in chronic HCV, but not chronic HBV (Bonorino et al., 2009). These intrahepatic NK cells express higher levels of TRAIL and the activating receptor NKp46 (Ahlenstiel et al., 2010). Bonorino et al have shown an inverse correlation between NKG2A-positive NK cells and
viral load (Bonorino et al., 2009). However although correlations with disease and RNA levels have significant p values, they have relatively low values for the correlation coefficient “r” indicating that much of the variability in inflammation and RNA levels is accounted for by factors other than those specifically studied.

1.8. NK cells and the outcome of HCV infection

NK cells were originally implicated in determining the outcome of HCV infection in an immunogenetic study of the KIR genes and their HLA-C ligands (Khakoo et al., 2004). By comparing the presence or absence of KIR genes and their HLA ligands in over 1000 individuals exposed to HCV it was shown that the specific combination of the inhibitory receptor KIR2DL3 and its group 1 HLA-C ligand (HLA-C1) was protective against chronic HCV infection (Khakoo et al., 2004). The protective effect of this gene combination was limited to individuals infected by intravenous drug use or accidental needle-stick injury. No protective effect was observed in subjects infected by transfusion of blood products in whom the innate immune response was thought to be overwhelmed by the higher infecting inoculum. The association of KIR2DL3 and HLA-C1 has subsequently been confirmed in a smaller study of intravenous drug users of Puerto-Rican origin, indicating that this protective effect is consistent across different populations (Romero et al., 2008). It has been postulated that this gene combination is protective because the KIR2DL3 binds HLA-C1 with a lower avidity than other inhibitory KIR, and thus NK cells expressing this specific inhibitory receptor have a lower threshold for activation (Fadda et al., 2010; Moesta et al., 2008). Consistent with this, NK cells expressing KIR2DL3 respond more rapidly to influenza infected cells in vitro (Ahlenstiel et al., 2008).

The oligomorphic MHC class I molecule HLA-E has also been associated with the outcome of HCV. In a genetic study homozygosity for the HLA-E\(^R\) allele has been shown to be protective against chronic infection with HCV genotypes 2 and 3 (Schulte et al., 2009). This protective effect was thought to be due to an effect on HLA-E restricted T cells, although the HLA-E\(^R\) allele may have a lower affinity for peptides and hence be expressed at lower levels. This could therefore lead to less inhibition of NK cells via the CD94:NKG2A receptor (Strong et al., 2003).
1.9. Background to this study

1.9.1. Viral peptide mediated up-regulation of MHC class I and immune escape

Up-regulation of cell surface MHC-class I expression in viral infection has been shown to impair NK cell cytotoxicity, and may represent a mechanism employed by viruses to subvert the innate immune response (Herzer et al., 2003; Nattermann et al., 2005a; Nattermann et al., 2005b; Tomasec et al., 2000; Ulbrecht et al., 2000). A peptide derived from HCV core protein, YLLPRRGPRL (HCV core amino acids 35-44; HCV core35-44), is a well recognised HLA-A2 epitope that is also capable of binding to HLA-E in-vivo (Nattermann et al., 2005a). The NK cell receptor for HLA-E is CD94:NKG2A, which is also up-regulated in chronic HCV infection (Harrison et al., 2010; Jinushi et al., 2004; Nattermann et al., 2006; Nattermann et al., 2005a). Thus, increased expression of HLA-E and CD94:NKG2A in chronic HCV may represent an important mechanism by which HCV escapes NK cell immune surveillance. HIV, EBV and CMV also encode for HLA-E binding peptides therefore this mechanism may also be relevant to other viruses.

1.9.2. Changes in peptide repertoire and NK cell activation

In addition to binding MHC class I-derived and virus-derived peptides, HLA-E can also bind stress related peptides such as the heat shock protein 60 (hsp60) signal peptide. hsp60 is a mitochondrial chaperone protein found in all cells, expression of which is increased when cells are exposed stressful stimuli (Michaelsson et al., 2002). hsp60SP (QMRPVSRVL) shares key anchor residues with MHC class I derived signal peptides, favouring binding to HLA-E. However, P5 valine (compared to P5 arginine in MHC Class I derived signal peptides) abrogates recognition by CD94:NKG2A, and may represent a mechanism by which NK cells can recognise stressed cells in a peptide dependent manner. A recent publication by our group revealed that HLA-C bound peptides that induce weak KIR binding can antagonise the effects of peptides that bind more strongly to KIR (Fadda et al., 2010). In this study, VAPWNSFAL (VAP-FA) and VAPWNSDAL (VAP-DA) were both shown to induce similar levels of HLA-Cw*0102 stabilisation on a TAP-deficient cell line. The subtle differences at P7 and P8 of these peptides led to differences in their interaction with KIR receptors: VAP-FA strongly inhibited degranulation of CD158b+ NK cells, while VAP-DA did not inhibit degranulation, despite promoting
clustering at the interface of effector and target cells. Interestingly, when the two peptides were mixed, the VAP-DA peptide appeared to antagonise the inhibitory effect of VAP-FA. This was a novel finding where non-inhibitory KIR binding MHC class I peptides can depress the inhibitory effect of a strong KIR binding peptide, thus acting as altered peptide ligands. Similarly, hsp60sp could potentially act as an altered peptide ligand at CD94:NKG2A and antagonise the inhibitory effect of HCV core35-44.

1.9.3. Aims and objectives

In this thesis, we aimed to:

1) Confirm the peptide specificity of HLA-E/CD94:NKG2A and HLA-Cw0102/KIR2DL2/3 looking particularly at:
   a. HCV core35-44 peptide
   b. Other viral peptides derived from EBV and HIV
   c. MHC class I derived signal peptides

2) Determine whether changes in peptide repertoire at HLA-E lead to antagonism at CD94:NKG2A in analogy to KIR antagonism induced by altered peptide ligands of HLA-C.

3) Analyse the differences between the KIR and CD94:NKG2A receptor systems.
Chapter 2. Materials and Methods

2.1. Cell lines and culture methods

2.1.1. 721.174 (.174)

The mutant human B-cell line LBL 721.174 (.174) is capable of synthesising MHC-class I molecules but has lost its ability to present intracellular self or viral peptides due to deficiency in transporter associated with antigen processing (TAP) (Cerundolo et al., 1990; Spies and DeMars, 1991). MHC class I molecules that reach the cell surface are most likely bound to low affinity peptides and therefore dissociate rapidly, leading to five-fold reduction in MHC class I expression at the .174 cell surface (Weinzierl et al., 2008). However, .174 cells can be loaded with exogenous peptide leading to stabilisation of MHC-class I at the cell surface when incubated at 26˚C (Correa and Raulet, 1995).

2.1.2. K562

The K562 cell line is a human erythroleukaemic cell line derived from patient with chronic myeloid leukaemia. K562s represent a sensitive NK cell target as they fail to express MHC-class I due to a lack of β2-microglobulin.

2.1.3. NKL

NKLs represent a CD3⁺ CD56⁺ CD16⁺ cell line first isolated from the peripheral blood of a patient with large granular lymphocyte leukaemia (Robertson et al., 1996). They are KIR⁺ but express endogenous CD94 and NKG2A.

2.1.4. Jurkat cells and CD94-transfected Jurkats

Jurkat cells represent an leukaemic T cell line isolated from the peripheral blood of a 14 year old boy with T cell leukemia (Schneider et al., 1977). Jurkat cells stably transfected with CD94 (CD94 Jurkats) were obtained from Miguel López-Botet.
2.1.4. Cell culture

K562, Jurkat and CD94 Jurkat cells were cultured and maintained in R10 medium containing RPMI 1640 (Biowhittaker, U.K.) supplemented with 10% heat-inactivated fetal calf serum (Lonza), 1% penicillin/ streptomycin (Biowhittaker, U.K.) and L-glutamine (Biowhittaker, U.K.) at 37°C, 5% CO2. Cell line cultures were maintained in logarithmic phase at neutral pH by performing a 1:10 dilution every 72 hours. NKL cell lines were cultured in R10 medium supplemented with 1ng/ml recombinant human IL-2 (National Cancer Institute Biometric Research Branch; Fisher BioServices) at 37°C, 5% CO2.

2.1.5. Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were obtained from patients with haemochromatosis and healthy control donors at St Mary's Hospital, London. Each patient provided informed consent. PBMCs were isolated using Ficoll-Hypaque (Amersham Biosciences, U.K.) density centrifugation, suspended in 90% FCS/ 10% DMSO, and stored at -80°C in a Mr Frosty Cryo 1°C freezing container (Nalgene) for 24 hours, before transferring to liquid nitrogen (-140°C) until use.

2.1.6. NK cell isolation

Purified untouched NK cells were isolated from PBMCs using a negative selection kit (Dynabeads, Invitrogen, U.K). The kit depletes non-NK cells (T cells, B cells, monocytes, platelets, dendritic cells, granulocytes and erythrocytes) leaving negatively isolated human NK cells. An antibody mix towards the non-NK cells is added to PBMCs and allowed to bind to the cells before adding Dynabeads which will bind to the antibody-labelled cells. The bead-bound cells are then separated on a magnet and discarded, leaving untouched human NK cells, which were analysed for purity using flow cytometry.

2.2. Peptide Synthesis

Peptides were purchased from Peptide Protein Research Ltd (Hampshire, UK), with identity confirmed by MS (>95% purity). They were dissolved in DMSO and then added at a final concentration of 0-100 μM.
2.3. Antibodies and staining reagents

The following monoclonal antibodies were used for flow cytometry. Anti-human HLA-E phycoerythrin-conjugated (3D12; eBioscience), anti-human HLA-C*0102 (VP6G3; provided by Arend Mulder, Leiden, Netherlands), fluorescein isothiocyanate conjugated goat anti-human IgM antibody (AbDSerotec), fluorescein isothiocyanate conjugated anti HLA-A2 (BB7.2; Abcam), anti-CD3 eFluor 450 (OKT3; eBioscience), anti-CD56 Cy7 coupled to phycoerythrin (HCD56, Biolegend), anti-CD159a conjugated to phycoerythrin (Z199; Beckman Coulter), fluorescein isothiocyanate conjugated anti-CD158b (CH-L; BD Pharmingen), Alexa Fluor 647–conjugated antibody to human CD107a (H4A3; eBioscience), Alexa Fluor-488 goat anti-mouse IgG (Invitrogen), anti-NKG2C Peridinin-Chlorophyll-Protein-Complex (PerCP) (134591; R& D Systems, U.K.). For blocking experiments and microscopy: anti-human CD94 (DX22; eBioscience), anti-human CD85j (HP-F1; eBioscience), anti-CD158b (GL183 Abcam, U.K.) anti-CD159a (Z199; Beckman Coulter), Alexa Fluor-488 goat anti-mouse IgG (Invitrogen).

2.4. MHC class I stabilisation assay

1 x 10^5 .174 cells were incubated overnight at 26°C in R10 medium alone or R10 containing 0-100 micromolar (μM) of the specified peptide (HPLC tested >95% purity, Peptide Protein Research Ltd., U.K.). The cells were then stained for HLA-E (3D12), or HLA-A2 (BB7.2), or the corresponding isotype control, maintained at 4 °C and in the dark for 30 minutes before washing twice with FACS wash (PBS with 0.002% NaN₃ and 1% BSA) and fixing in 1% paraformaldehyde (PFA). For HLA-Cw*0102 detection, VP6G3 antibody was used at room temperature for 30 min. After washing twice to remove unbound antibody, cells were stained with F-ab goat anti-human IgM-FITC for 30 minutes at 4 °C and in the dark. As a negative control, .174 cells were incubated with the secondary antibody in the absence of the VP6G3. After two further washes the cells were fixed in 1% PFA and analysed by on a BD FACS Canto II analyzer (Becton Dickinson). Mean fluorescent intensity (MFI) of 10,000 cells gated by forward and side scatter was recorded.
2.5. CD107a/ LAMP-1 Degranulation assay

2.5.1. Effector cell preparation

Frozen PBMCs were washed in RPMI 1640 and resuspended in R10 at final concentration 1.5x10^6 cells/ml. Interleukin (IL)-15 (R&D Systems U.K.) was added at 1ng/ml prior to incubating 200 μl (3x10^5 cells/well overnight in a 96 well plate for 16 hours at 37˚C, 5% CO₂.

2.5.2. Target cell preparation

6x10^4 .174 cells alone or with varying concentration of peptide (0-100 μM) were incubated in a 96 well plate at 26˚C, 5% CO₂ for 16 hours. For peptide mix experiments, target cells were simultaneously loaded with two peptides with final concentration as indicated. MHC class I negative K562 cells were used as a positive control.

2.5.3. Degranulation Assay

The next day, IL-15 containing media was removed from the PBMCs before resuspending in CD107a-Alexa Fluor 647 (H4A3), or Mouse IgG1-k Alexa Fluor647 isotype control. The target cells were also resuspended in fresh R10 media +/- peptide. PBMCs were stimulated with target cells at an effector to target (E:T) ratio of 5:1 for a total of 4 hours at 26˚C, 5% CO₂. Optimal E:T ratio was determined by an E:T titration from 50:1 to 1:1, with additional assays performed to ascertain optimal incubation time and temperature. K562 target cells were used as a positive control. Spontaneous expression of CD107a was determined for PBMCs in the absence of target cells. After 1 hour of incubation, 7 μl of monensin was added to each well (Golgi-Stop, BD Biosciences, U.K.) at a final concentration of 7 μg/ml before incubating for a further 3 hours at 26˚C, 5% CO₂. Monensin reduces degradation of re-internalised CD107a by preventing acidification of endocytic vesicles, allowing visualisation of CD107a following stimulation. After the 4 hour incubation, PBMCs were resuspended in blocking-buffer (10% human serum (Cambrex, U.K.) in FACS wash) at 4˚C for 30 minutes. PMBCs were then stained using CD3 eFluor-450, CD56-PECy7, CD158b-FITC (identifies KIR2DL2/ KIR2DL3 subset) and NKG2A-PE (Beckman Coulter, U.K.). BD Compbeads (B.D. Biosciences, U.K.) were used for compensation controls. Cells were stained at 4˚C for 30 minutes in the dark before washing twice.
with FACS wash and fixing in 1% PFA. Flow cytometric analysis was performed using a FACS Canto analytical flow cytometer (B.D. Biosciences, U.K.). I gated on the lymphocyte population, before gating on CD3\(^+\) CD56\(^+\) cells to define the NK cell population. A total of 10,000 NK cell events were acquired and analysed using BD FACS Diva software (B.D. Biosciences, U.K.). CD107a expression by the various NK cell subsets was analysed. CD107a expression was defined as a percentage of the NK population expressing CD107a, minus spontaneous CD107a expression by PBMCs incubated in the absence of target cells.

2.5.4. Blocking experiments

For the blocking experiments, PBMCs were incubated with 25 μg/mL DX22 (anti-CD94), HP-F1 (anti LIRB1), GL183 (anti-CD158b) or IgG1 control antibody for 30 minutes at room temperature, before addition of .174 cells. Remainder of protocol as per NK cell degranulation assay, except degranulation gated on either CD3\(^-\) CD56\(^+\) or CD3\(^-\) CD56\(^+\) CD158b\(^-\) NK cells as stated.

2.6. Microscopy

0.5 x 10\(^6\) .174 cells were incubated overnight at 26 °C alone or with 0-100 μM peptide, then co-incubated with NKL cells, primary NK cells or CD94-Jurkat transfectants at an E:T ratio of 2:1 at 37 °C for 10 min. Cells were fixed in pre-warmed 2% PFA for 30 min at 37 °C before permeabilising with 0.01% Triton X-100 for 1 min. Cells were then stained with CD94 (DX22) or NKG2A (Z199), washed, stained with Alexa Fluor-488 goat anti-mouse IgG (staining performed at 4 °C in dark) followed by a further wash. Cells were imaged using a Leica SP2 upright microscope (Leica Microsystems) with transmission images and FITC emission collected in separate channels. Data were processed using Leica imaging software (Leica Microsystems) and Image-J. The increase in fluorescence intensity at the immune synapse was calculated as a ratio of the average fluorescence intensity along the effector-.174 interface compared with the average fluorescence intensity along a non-contact area of the effector cell plasma membrane, with both values corrected for background fluorescence as measured within an acellular region of the image. The percentage of conjugates with >1.4 fold increased fluorescence at the synapse was also calculated.
2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism, version 5 (GraphPad Software). A p value of less than 0.05 (p < 0.05) was deemed to be significant.
Chapter 3. MHC-class I binding peptides and NK cell inhibition

3.1. Peptide processing and specificity of MHC class I

MHC class I molecules are expressed by all nucleated cells, presenting peptides derived from nuclear and cytosolic proteases at the cell surface. Such peptides gain access to the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) molecules. In the absence of peptide, the MHC-encoded heavy chain and a β2-microglobulin light chain (β2m) are partially folded and stabilised in the endoplasmic reticulum by various chaperone proteins including tapasin, calreticulin and ERp57. Collectively, these components form the peptide loading complex (PLC). Tapasin interacts closely with TAP, coupling peptide delivery into the endoplasmic reticulum with loading onto MHC class I (Figure 3.1) (Neefjes et al., 2011). Within the ER, peptides may be trimmed by aminopeptidases producing a sequence of 8-10 amino acids. Following successful peptide loading on to MHC class I, the chaperone proteins are released while the stable MHC class I heterotrimer leaves the endoplasmic reticulum for presentation at the cell surface (Marsh et al., 2000; Schumacher et al., 1990; Townsend et al., 1989). MHC class I molecules and peptides that fail to associate in the endoplasmic reticulum are returned to the cytosol where they are degraded (Hughes et al., 1997; Koopmann et al., 2000).
In a healthy cell, degradation of cellular contents by cytosolic proteases, such as the multicatalytic proteasome complex, leads to the generation of a variety of 8-12 amino-acid self-peptides. These peptides are translocated from the cytosol into lumen of the endoplasmic reticulum (ER) by ATP-dependent transporter associated with antigen processing (TAP) molecules. The peptides are further trimmed by aminopeptidases to produce 8-10 amino-acid peptides. The peptide loading complex (PLC)- consisting of TAP, tapasin, calreticulin and ERp57- load peptide onto β2m and α-heavy chain to form a stable MHC class I molecule. The MHC class I heterotrimer is then transported to the golgi before exiting via the secretory pathway to reach the cell surface.

**Figure 3.1. Peptide loading onto MHC class I**
A variety of self peptides are thus displayed at the cell surface by MHC class I, providing information for CD8\(^+\) T cells surveying for the presence of non-self peptides using their \(\alpha/\beta\) TCR. MHC class I levels are sensitive to the amount of peptide available, which in turn relates to the rate of protein synthesis within the cell. Various stimuli such as ionizing radiation, IFN-\(\gamma\) and microRNAs can affect protein synthesis within a cell. Viral infection or tumorigenesis can result in alterations to intracellular protein production, leading to a change in the MHC class I bound peptide as these proteins are degraded in the cytosol (Hickman et al., 2003; Meiring et al., 2006). Such perturbation of peptide presentation by MHC class I enables CD8\(^+\) T cells to recognise unhealthy cells. Functional studies demonstrate that NK cells also demonstrate peptide specificity, in addition to recognising any overall reduction in MHC class I expression as a result of impaired protein synthesis or peptide processing within the cell.

Although MHC-Class Ia molecules can bind a diverse array of peptides, the various subclasses (HLA-A, -B and C) display a preference for particular amino-acids at certain positions in the peptide sequence. This is attributable to the high level of polymorphism in the heavy chains, resulting in a variety of peptide-binding grooves with distinct anchor positions to which peptides bind (Trowsdale, 2005). HLA-C is usually expressed at the cell surface at lower levels than HLA-A and HLA-B, despite similar intracellular levels of the heavy chains (Neefjes and Ploegh, 1988). This is partly explained by a more selective peptide repertoire for HLA-C; HLA-Cw\(^*\)04 and HLA-Cw\(^*\)02 require a 10 fold increase in peptide concentration to dissociate from TAP compared to HLA-A and –B alleles (Neisig et al., 1998). Consequently, 30-70% of HLA-C molecules may be retained in the ER bound to TAP awaiting an appropriate allele specific peptide (Neefjes et al., 2011). This may also make HLA-C more available for peptides that arise during viral or stress conditions.

Although HLA-C tends to be expressed at lower cell surface levels than HLA-A and HLA-B, it is perhaps more important in the regulation of NK cell function than these molecules. This is emphasised by recent immunogenetic studies showing the association of HLA-C polymorphisms and the outcome of HIV and HCV infection (Fellay et al., 2007; Khakoo et al., 2004; Romero et al., 2008). There is a dearth of data for peptide binding motifs of the various HLA-C alleles, but perhaps best characterised is HLA-Cw\(^*\)0102, a HLA-C1 molecule that functions as a ligand for KIR2D receptors.
Barber et al. eluted HLA-Cw*0102 peptides, determining their sequence using Edman degradation and tandem mass spectrometry. A motif for HLA-Cw*0102 binding was proposed comprising a leucine or alanine at amino acid position 2 (P2) and a leucine at P9, which are referred to as anchor residues (Figure 3.2) (Barber et al., 1996).
Figure 3.2. Peptide binding preference of HLA-Cw*0102

Dominant anchor residues for HLA-Cw*0102 are the C-terminus P9 residue and the P2 residue, favouring a leucine and alanine or leucine respectively. Predicted binding of amino acids at other residue positions are shown, decreasing in affinity from top to bottom. Adapted from Barber et al (1996).
The non classical MHC Class Ib molecule HLA-E is the oligomorphic ligand for the CD94:NKG2A receptor. In contrast to MHC class Ia molecules which bind a diverse range of peptides, HLA-E primarily binds a closely related set of nonameric peptides derived from other MHC class I molecules (Braud et al., 1997; Braud et al., 1998; Lee et al., 1998). HLA-A, -B, -C and HLA-G are expressed with a leader sequence targeting them to the secretory pathway. This leader sequence is thought to be cleaved from the ER membrane by a signal peptide peptidase. In the cytosol, the peptide is trimmed further by the proteasome to liberate a highly conserved nonameric signal peptide, corresponding to amino acids residues 3-11 of the leader sequence (Bland et al., 2003). The signal peptide enters the ER by a TAP dependent mechanism and is eventually presented at the cell surface by HLA-E (Lemberg et al., 2001). HLA-E is unable to bind its own signal peptide. Thus, HLA-E expression provides an indirect indicator of the production of the polymorphic MHC class I molecules, and the capacity of the cell to process and present antigen (Bland et al., 2003).

The principles governing the restricted peptide binding of HLA-E can be explained by its crystal structure (O’Callaghan et al., 1998). Five anchor positions have been described for HLA-E, compared to only two or three for MHC class Ia molecules (Sullivan et al., 2008). The dominant anchor residues are P2 and P9, however, further anchor positions exist at P3, P6 and P7. Folded HLA-E complexes have a strong preference for methionine and leucine at P2 and P9 respectively; with these residues present in all MHC Class I derived signal peptides that bind HLA-E. Leucine is also tolerated at P2; however, threonine is not favoured. This would explain the lower affinity of HLA-E for several of the HLA-B derived signal peptides containing threonine at P2 (Table 3.1.) (Brooks et al., 1999). Sequences predominating in hydrophobic amino acids could potentially bind strongly to HLA-E, therefore permitting binding of peptides that do not conform to predicted motifs (Miller et al., 2003; Stevens et al., 2001). This enables non-MHC class I derived peptides to bind to HLA-E, as has been demonstrated for the heat shock protein 60 signal peptide (hsp60,sp) QMRPVSRVL, and the HCV core35-44 peptide YLLPRRGPR (Michaelsson et al., 2002; Nattermann et al., 2005a).
3.1.1. Peptide preference of NK cell receptors

The MHC class I specificity of the various NK cell receptors is well characterised. KIR2DL receptors recognise HLA-C ligands but they discriminate between allotypes grouped according to the amino acid located at positions 77 and 80 on the alpha 1 helix of the HLA-C heavy chain. KIR2DL2 and KIR2DL3 receptors bind to HLA-C alleles with Serine at P77 and Asparagine at P80. Collectively, these molecules are called HLA-C1 allotypes. In contrast KIR2DL1 receptors recognise HLA-C2 allotypes with Asparagine at P77 and lysine at P80 (Vitale et al., 1995). KIR recognition of their cognate MHC class I ligands is sensitive to the nature of the bound peptide adding a further level of complexity to this system (Maenaka et al., 1999a; Malnati et al., 1995). KIR2D receptors display peptide selectivity for binding to HLA-C molecules and are most sensitive to substitutions at P7 and P8 (Rajagopalan and Long, 1997; Zappacosta et al., 1997). Rajagopalan and Long showed that HLA-Cw4 transfected RMA-S cells had a similar affinity for a range of peptides; however, those HLA-Cw4 bound peptides with a negatively charged P8 residue were not recognised by KIR2DL1 (Rajagopalan and Long, 1997). Analysis of the crystal structure of KIR2DL1 bound to HLA-Cw4 reveals that KIR2DL1 has a negatively charged polar surface around the P8 lysine side chain of the peptide that discourages acid residues at that position. Crystal structure studies of KIR2DL2 bound to the group 1 HLA-C molecule HLA-Cw3 reveal that the receptor makes direct contact with residues 7 and 8 of the peptide (Boyington et al., 2000a). Additionally, alterations to contact residues P7 and P8 of peptides bound to HLA-Cw*0102 can promote, abrogate and predict a hierarchy in KIR binding (Fadda et al., 2010).

The nature of the bound peptide can also markedly influence the affinity of the interaction between HLA-E and CD94:NKG2A. P5, P6 and P8 have been identified as the major contact residues with CD94:NKG2A. There is an absolute specificity for P5 arginine, as P5 lysine (representing a conserved substitution with positive charge) completely abrogates binding to CD94:NKG2A (Miller et al., 2003). The critical role of the P5 residue in HLA-E recognition by CD94:NKG2A is further exemplified by the observations of Michaelsson et al., using a peptide derived from the signal sequence of heat shock protein 60, a protein induced in response to cellular stress (Michaelsson et al., 2002). hsp60\textsubscript{sp} (QMRPVSRVL) has the preferred methionine at P2 and leucine at P9, but a valine residue at P5 interferes with recognition by CD94:NKG2A. Although P5 arginine to valine or lysine
substitution of HLA-E binding peptides abrogates inhibition at CD94:NKG2A, the reciprocal change in hsp60_sp (P5 valine to arginine) was insufficient to promote recognition at this receptor. This would suggest that other residue positions are important in CD94:NKG2A recognition, possibly changing the conformation of the peptide within the HLA-E binding groove. Llano et al. suggest P6 is also an important contact residue, as substitution of threonine with alanine, as seen naturally in the C7 leader peptide VMAPRALLL, results in loss of recognition by CD94:NKG2A (Llano et al., 1998). The residue at P8 may predict a hierarchy in binding to CD94:NKG2 receptors, with phenylalanine the strongest binder (Miller et al., 2003). This would explain the higher affinity of HLA-G signal peptide (sp) VMAPRTLFL for CD94:NKG2A compared to the MHC class Ia derived peptides (Table 3.1).

HLA-E can also bind to the activating C-type lectin receptor CD94:NKG2C. Interestingly, CD94:NKG2C has a six-fold lower affinity for HLA-E than both CD94:NKG2A and CD94:NKG2E (Kaiser et al., 2005; Vales-Gomez et al., 1999). Peptides bound to HLA-E can differentially affect binding to the inhibitory and activating receptors (Brooks et al., 1999; Llano et al., 1998; Vales-Gomez et al., 1999). The functional significance of this is not clear; however, HLA-E: HLA-G_sp triggers NKG2C activation very efficiently (Llano et al., 1998).

In this chapter, MHC class I signal peptide, virus derived peptides and hsp60_sp binding to HLA-E and HLA-Cw*0102 was compared using a TAP deficient .174 cell line pulsed with these various peptides. Recognition of peptide-MHC complexes by NK cell inhibitory receptors was ascertained by multi-parameter flow cytometry utilising CD107a expression as a marker of NK cell activation using PBMCs as effector cells against .174 cell targets.
<table>
<thead>
<tr>
<th>MHC class I</th>
<th>Signal peptide</th>
<th>Affinity for HLA-E</th>
<th>Complex affinity for CD94:NKG2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>VMAPRTLLL</td>
<td>High</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>VMAPRTLVL</td>
<td>High</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>IMAPRTLVL</td>
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<td>VMPPIRTLLL</td>
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<td>Unknown</td>
</tr>
<tr>
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<td>Low</td>
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<tr>
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</tr>
<tr>
<td>HLA-G</td>
<td>VMAPRTLFL</td>
<td>Intermediate-high</td>
<td>High</td>
</tr>
</tbody>
</table>

Table 3.1. MHC class I derived signal peptides and their affinities for HLA-E and CD94:NKG2A. Adapted from Sullivan et al, (2008).
3.2. Results

3.2.1. MHC Class I binding assays

HCV core<sub>35-44</sub> (YLLPRRGRL) and hsp60<sub>sp</sub> (QMRPVSRL) have been shown to bind to HLA-E. To confirm this, MHC deficient .174 cells were incubated overnight at with varying concentrations (0-100 μM) of these peptides, before staining for cell surface HLA-E (3D12) and HLA-Cw*0102 (VP6G3) (Figure 3.3). As a positive control, MHC class I stabilisation was assessed using the host derived MHC class I signal peptides HLA-G<sub>sp</sub> (VMAPRTLFL), HLA-A<sub>*</sub>02<sub>sp</sub> (VMAPRTLVL) and -B<sub>*</sub>07<sub>sp</sub> (VMAPRTVLL).

Flow cytometric analysis showed that although HLA-E and HLA-Cw*0102 expression was detectable on .174 cells incubated without peptide, cell surface levels increased when loaded with these specific peptides. In agreement with the previous observations, incubation with HCV core<sub>35-44</sub> led to enhanced HLA-E expression on .174 cells, saturating at ~80 μM (Nattermann et al., 2005a). HLA-G<sub>sp</sub>, HLA-A2<sub>sp</sub> and HLA-B7<sub>sp</sub> also bound to HLA-E, consistent with previous findings. HLA-B7<sub>sp</sub> and HLA-A2<sub>sp</sub> had a higher affinity for HLA-E, achieving a higher mean fluorescent intensity and saturating at ~10 μM and ~40 μM respectively, compared to HLA-G<sub>sp</sub> which reached saturating levels at ~80 μM (Figure 3.3. b and f).

hsp60<sub>sp</sub> enhanced HLA-E expression, but with a lower affinity than the other peptides (Figure 3.3 g). This is consistent with results from Michaelsson et al. who showed that HLA-E expression is maximal at 300 μM hsp60<sub>sp</sub> (Michaelsson et al., 2002). As a negative control, we used the HLA-A2 restricted influenza matrix peptide GILGFVFTL (GILG). This peptide binds strongly to HLA-A2 (detected using BB7.2), however at 300 μM GILG, there was no significant binding to either HLA-E or HLA-Cw*0102 (Figure 3.3 h & i). This would indicate that not all peptides can bind to HLA-E and HLA-Cw*0102.

Unexpectedly HCV core<sub>35-44</sub>, HLA-A2<sub>sp</sub>, HLA-B7<sub>sp</sub> and HLA-G<sub>sp</sub> also increased cell surface HLA-Cw*0102 (Figure 3.3 c & d).
Specific peptides induce MHC-Class I stabilisation on .174 cells

.174 cells were incubated for 16 hours at 26˚C either alone or with the specified peptide, before staining with an anti-HLA-E antibody (3D12), anti-HLA-Cw*0102 (VP6G3), anti-HLA-A2 (BB7.2) or an isotype control, as described in Materials and Methods. Data represent mean fluorescent intensity of 10,000 gated events, shown as histograms and plotted against peptide concentration. HLA-E upregulated on the surface of .174 cells by (a & b) HCVcore35-44, (e & f) A2_sp and B7_sp. HLA-Cw*0102 upregulated by (c & d) HCV core35-44 and HLA-G_sp, hsp60_sp also binds to HLA-E (g); however the irrelevant HLA-A2 restricted peptide GILGFVFTL (GILG) binds neither HLA-Cw*0102 or HLA-E (h). (i) GILG increases cell surface HLA-A2.

Figure 3.3. Specific peptides induce MHC-Class I stabilisation on .174 cells
3.2.1.1. Effect of incubation temperature on MHC Class I stabilisation

“Empty” MHC class I molecules, consisting of α-heavy chain and β2 microglobulin, can be assembled and expressed at the cell surface in the absence of endogenous peptide provided the cells are cultured at temperatures below 37 °C (Karre et al., 1986; Ljunggren et al., 1990). TAP deficient .174 cells can be loaded with exogenous peptide to enhance this cell surface MHC class I but are known to be sensitive to incubation temperature (Levy et al., 1991). To confirm this, peptide induced MHC class I stabilisation on .174 cells was compared following overnight incubation at 26 °C and 37 °C (Figure 3.4 a & b). Incubation at 26 °C with 100 µM HLA-Gsp increased cell surface levels of both HLA-E and HLA-Cw*0102 compared to .174 cells incubated in the absence of peptide, whereas incubation at 37°C did not. Additionally, in the absence of exogenous peptide, at 26 °C there was a higher constitutive level of both of these class I molecules compared to overnight incubation at 37 °C.

To determine the stability of MHC class I complexes a similar assay was performed, incubating .174 cells alone or loaded with 100 µM HLA-Gsp. Following overnight incubation at 26°C, peptide loaded .174 cells were exposed to a 37 °C water bath for 0-5 hours, before placing on ice and staining for cell surface HLA-E and HLA-Cw*0102 (Figure 3.4 c & d). Peptide loaded .174 cells not exposed to 37 °C temperature had increased levels of both HLA-E and HLA-Cw*0102 compared to unpulsed .174 cells, consistent with previous experiments. However, following incubation at 37 °C there was a time dependent reduction in mean fluorescent intensity (MFI) of the stained samples indicating dissociation of MHC class I complexes. After 3 hours at 37 °C, MFI for HLA-E and HLA-Cw*0102 staining was comparable to that for unpulsed .174 cells. Therefore although CD107a degranulation is greater at 37 °C than at 26 °C, there is rapid decay in cell surface stabilised MHC class I at the higher temperature. Therefore, 26 °C was selected as the temperature for the .174 cell/ PBMC co-incubation step in degranulation assays.
Figure 3.4. Effect of temperature on MHC class I stabilisation

174 cells unloaded (np) or pulsed with 100 μM HLA-Gsp were incubated overnight either at 26°C or at 37°C before staining for (a) HLA-E (3D12) and (b) HLA-Cw*0102 (VP6G3), or the appropriate isotype control (clg). (c) & (d) 174 cells incubated overnight at 26°C either alone (np) or with 100 μM HLA-Gsp. Cells were then transferred to 37°C water bath for indicated time in hours (hr), before staining for (c) 3D12, (d) HLA-Cw*0102 or isotype control as previously. Data represent mean fluorescent intensity (MFI) of 10,000 gated events.
3.2.3. CD107a degranulation assays assessing recognition of Peptide:MHC class I by NK cell inhibitory receptors

Lysosomal associated membrane protein-1 (LAMP-1 or CD107a) is expressed on the membrane of cytolytic granules and can be used as a sensitive marker of NK cell activation, correlating with both cytokine secretion and target cell lysis (Alter et al., 2004). To determine the effects of cell surface stabilised MHC class I on NK cell activity a CD107a degranulation assay was performed. IL-15 treated PBMCs were incubated at 26°C with 721.174 targets in the presence of monensin and an anti-CD107a antibody or isotype control antibody. Five colour flow cytometric analysis enabled identification of the lymphocyte population (forward and side scatter), total NK cell population (CD3−, CD56+), KIR2DL2/2DL3 (CD158b+) and CD94:NKG2A (NKG2A+) subsets (Figure 3.5 a). The percentage of CD107a+ NK cells was determined in PBMCs stimulated with IL-15 alone, or PBMCs stimulated with IL-15 and .174 target cells (data from one donor shown in Figure 3.5 b). NK cell CD107a expression increased in the presence of unloaded .174 target cells (36.8%) compared those stimulated with IL-15 alone (2.4%).
Figure 3.5. NK cell CD107a degranulation assay - data from one representative experiment

Example of gating strategy used for FACS analysis (a). Top row (L to R). The lymphocyte population is identified on forward and side scatter. The NK cell population is identified as CD3−CD56+. CD3−CD56+ NK cells can be subdivided into NKG2A+ and NKG2A−. Bottom row (L to R). CD3−CD56+ NK cells sub-classified for into CD158b+ and CD158b−. CD3−CD56− separated into 4 populations based on expression of NKG2A and CD158b: NKG2A+CD158b+, NKG2A−CD158b+, NKG2A+CD158b− (double positive) and NKG2A−CD158b− (double negative). (b) histograms illustrating the percentage of CD3−CD56+ NK cells expressing CD107a following incubation with .174 target cells at E:T ratio 5:1 in the presence of CD107a antibody or an isotype control.
3.2.4. Optimisation of CD107a assay

The optimal incubation time, effector to target cell (E:T) ratio and IL-15 concentration was determined for the CD107a assay using a series of experiments.

3.2.4.1. Incubation time

To determine whether a 4 hour co-incubation time is adequate to observe maximal CD107a expression, IL-15 stimulated PBMCs were incubated alone or with unloaded .174 target cells for between 1-6 hours at 26˚C. CD107a was detectable on NK cells after 1 hour of co-incubation with .174 cells, reaching saturating levels after 4 hours (Figure 3.6), confirming this was a sufficient incubation time.

3.2.4.2. E:T ratio

To assess the optimal E:T ratio, IL-15 stimulated PBMCs were incubated with .174 cells (unloaded or pulsed with 100 µM HLA-Gsp) at E:T ratios ranging from 40:1 to 1:1 for 4 hours (Figure 3.7). The maximal difference in CD107a expression induced by unloaded .174 cells compared to HLA-Gsp pulsed .174 was observed at an E:T of 5:1, therefore this ratio was used for all further experiments.

3.2.4.3. IL-15 concentration

IL-15 stimulation increases NK cell CD107a expression in the presence of MHC deficient target cells. PBMCs were incubated overnight at a range of IL-15 concentrations (0-3 ng/ml), before co-incubation with .174 target cells (unpulsed or loaded with 100 µM HLA-Gsp) at an E:T ratio of 5:1 for 4 hours at 26˚C (Figure 3.8). 1 ng /ml IL-15 produced almost saturating levels of CD107a expression when stimulated with unloaded .174 cell targets. Although higher IL-15 concentrations produced a small increase in CD107a expression, there was also an increase in CD107a expression in the presence of peptide loaded .174 cells. Thus, the optimal IL-15 concentration required to observe the inhibitory effect of MHC class I binding peptides is 1ng/ ml.
Figure 3.6. Kinetics of CD107a expression on NK cells following stimulation with .174 cells

CD107a assay protocol as described in Materials and Methods. IL-15 treated PBMCs were incubated alone or with unloaded .174 cells at an E:T ratio of 5:1 at 26°C for between 1-6 hours. NK cell CD107a expression was determined using a FACS Canto flow cytometer.
Figure 3.7. Optimal E:T ratio for CD107a assay

CD107a assay performed as described in Material and Methods. 3 x 10^5 PBMCs co-incubated with different concentrations of .174 target cells (unpulsed or loaded with 100 µM HLA-Gsp) to determine the optimal E:T ratio for CD107a expression.
Figure 3.8. Determining the optimal IL-15 concentration required to stimulate PBMCs for CD107a assay

CD107a assay performed as described in Material and Methods. PBMCs stimulated overnight with different concentrations of IL-15 (0 - 3ng/ml), before co-incubating with .174 cells (unloaded or pulsed with 100 µM HLA-Gsp) at an E:T ratio of 5:1 for 4 hours at 26°C.
3.2.5. Peptide specific NK cell inhibition by HLA-E and HLA-Cw*0102

MHC class I stabilisation assays revealed that HCV core$_{35-44}$ and HLA-Gsp both increased cell surface HLA-E and HLA-Cw*0102 on .174 cells. To determine the effects of peptide:MHC class I complexes on NK cell inhibition a CD107a degranulation assay was performed. .174 cells were incubated overnight at 26˚C either alone, or in the presence of 100 µM HCV core$_{35-44}$ or HLA-Gsp, before co-incubating with IL-15 stimulated PBMCs for 4 hours at 26˚C. Multiparameter flow cytometry was performed to determine the frequency of CD107a$^+$ cells in each of the defined NK cell populations, including NKG2A (receptor for HLA-E) and CD158b (receptor for HLA-Cw102).

In the absence of peptide, MHC deficient .174 cells stimulated NK cell activation, with increased CD107a frequency in the total NK (CD3$^-$ CD56$^+$), NKG2A$^-$ CD158b$^+$ and NKG2A$^+$ CD158b$^-$ subpopulations (Figure 3.9). In the presence of 100 µM HCV core$_{35-44}$, there was a reduction in total CD107a expression, from 19.4% to 13.2%. Looking at the subpopulations, no inhibition was observed in the NKG2A$^-$ CD158b$^-$ subpopulation (27.9% no peptide v 27.4% 100µM HCV core$_{35-44}$). However, HCV core$_{35-44}$ induced strong inhibition at NKG2A$^-$ CD158b$^+$, with a 65% reduction in CD107a expression (14.6% no peptide v 5% 100 µM HCV core$_{35-44}$).

100 µM HLA-Gsp also inhibited CD107a expression on the total NK cell population (19.4% no peptide v 8.1% 100 µM HLA-Gsp). In contrast to HCV core$_{35-44}$, the inhibitory effect HLA-Gsp was mediated by NKG2A$^+$ subset (27.9% no peptide v 5.2% 100 µM HLA-Gsp), while no inhibition was seen at NKG2A$^-$ CD158b$^+$ subpopulation (14.6% no peptide v 17.7% 100 µM HLA-Gsp).

As a control, MHC class I negative K562 cells were incubated alone or with peptide. Addition of peptide to K562 cells did not confer protection against PBMC degranulation, implying the effect of these peptides is mediated by the surface stabilisation of MHC class I as opposed to a direct effect of the peptide at inhibitory NK cell receptors.
Figure 3.9. Peptide specific recognition of MHC class I by NK cells

CD107a (LAMP-1) was used as a marker of NK cell activation and degranulation. A summary of the results from one representative assay are presented here. Briefly, .174 target cells were incubated overnight at 26°C either alone (no peptide) or with 100 µM of the indicated peptide. The following day the .174 cells were used to stimulate IL-15 treated PBMCs for 4 hours at 26°C in the presence of CD107a antibody. Histogram plots for CD107a expression by the total NK (CD3− CD56+), NKG2A− CD158b+ and NKG2A+ CD158b− subsets are shown.
To confirm the differing effects of HCV core$_{35-44}$ and HLA-G$_{sp}$ on NK cell degranulation, the experiment was repeated including a peptide titration 0-100 µM. (Figure 3.10). This confirmed that both HCV core$_{35-44}$ and HLA-G$_{sp}$ inhibit NK cell degranulation in a dose dependent manner (one way ANOVA $p<0.0001$). However, they appear to mediate their inhibitory effects by different mechanisms. HCV core$_{35-44}$ inhibits the KIR2DL2/KIR2DL3$^+$ (CD158b$^+$) subset ($p<0.0001$) but has no effect on the CD94:NKG2A$^+$ NK cells ($p=0.94$). In contrast, HLA-G$_{sp}$ inhibits via CD94:NKG2A$^+$ NK cells ($p<0.0001$) and has no effect on KIR2LD2/2DL3$^+$ NK cells ($p = 0.99$). Thus, although both these peptides upregulate HLA-Cw*0102 and HLA-E expression on .174 cells, there appears to be a difference in recognition of these peptide complexes when bound to MHC class I. HLA-E:HLA-G$_{sp}$ complex is recognised by CD94:NKG2A$^+$ NK cells, leading to inhibition of degranulation, while HLA-E:HCV core$_{35-44}$ complex does not appear to be recognised by this NK cell subset. In contrast, HLA-C:HCV core$_{35-44}$ complex interacts with KIR2DL2/2DL3$^+$ NK cells leading to inhibition of this subset, while HLA-C:HLA-G$_{sp}$ complex is not recognised by this receptor.
CD107a assay was repeated on single donor to include varying concentrations 0-100 µM of indicated peptide. (a) HCV core$_{35-44}$ (n=9), (b) HLA-G$_{sp}$ (n=4) CD107a expression presented for CD3$^-$ CD56$^+$ (total NK), NKG2A$^-$ CD158b$^+$ and NKG2A$^+$ CD158b$^-$ subsets. CD107a measured as percentage of specified population expressing CD107a. The means and SEM are shown. $p$ values calculated using one-way ANOVA.
To confirm the differential effects of HCV core\textsubscript{35-44} and HLA-G\textsubscript{sp} pulsed .174 cells on NK cell degranulation, the CD107a assay was performed using blocking antibodies (Figure 3.11). Antibodies to KIR2DL2/ KIR2DL3 (GL183), CD94 subunit of CD94:NKG2A (DX22) or an isotype control IgG1 antibody, were added to PBMCs 30 minutes before the addition of target cells. In the presence of a IgG1 control antibody, degranulation of the total NK population fell from a mean of 14.9% in the absence of peptide, to 8.6% in the presence of 100 µM HCV core\textsubscript{35-44}. However, incubation of PMBCs with GL183 for 30 minutes prior to adding target cells completely abrogated the inhibitory effect of HCV core\textsubscript{35-44} on NK cell degranulation (14.9%), whilst DX22 had no effect on inhibition (8.5%). 100 µM HLA-G\textsubscript{sp} produced a reduction (4.8%) in CD107a expression after pre-incubation with the IgG1 control antibody compared to no peptide (14.9%). DX22 negated the inhibitory effect of HLA-G\textsubscript{sp} (16.2%) while GL183 did not interfere with inhibition (4.3%).

The results of the blocking assay support the observation that HLA-E:CD94:NKG2A interaction is not responsible for the inhibitory effect of HCV core\textsubscript{35-44}. Instead HCV core\textsubscript{35-44} directly inhibits NK cell degranulation via enhanced expression of HLA-Cw\textsuperscript{*0102} interacting with KIR2DL2/2DL3 NK cell receptors. In contrast, HLA-G\textsubscript{sp} inhibits NK cell degranulation via CD94:NKG2A\textsuperscript{*} and not KIR2DL2/2DL3\textsuperscript{*} NK cells.
PBMCs were pre-incubated with blocking antibodies to KIR2DL2/2DL3 (GL183), CD94:NKG2A (DX22) or an isotype matched IgG1 control antibody. After 30 minutes, 174 target cells were added, either unpulsed (np) or loaded with 100 µM (a) HCV core<sub>35-44</sub> or (b) HLA-G<sub>sp</sub>. Degranulation of CD3<sup>+</sup> CD56<sup>+</sup> NK cells was assessed using the previously described protocol. Mean +/- SEM of 2 experiments is shown.
3.2.6. Peptide mix experiments

It has recently been demonstrated that HLA-C bound peptides that induce weak KIR binding can depress the inhibitory effect of a strong KIR binding peptide, thus acting as antagonistic peptide ligands (Fadda et al., 2010). Having observed that HCV core\textsubscript{35-44}:HLA-E complex does not appear to be recognised by CD94:NKG2A, a peptide mix experiment was performed to determine whether HCV core\textsubscript{35-44} could interfere with the inhibitory effects of HLA-G\textsubscript{sp} at CD94:NKG2A (Figure 3.12). 1 µM of HLA-G\textsubscript{sp} was sufficient to inhibit degranulation of approximately 35% of the .174 reactive NKG2A\textsuperscript{+} NK cells (23.2% no peptide v 15.1% 1 µM of HLA-G\textsubscript{sp}), whereas 100 µM of HCV core\textsubscript{35-44} had no effect (22.9%). However, when 100 µM of HCV core\textsubscript{35-44} was mixed with 1 µM of HLA-G\textsubscript{sp}, rather than interfering with the inhibitory effect of HLA-G\textsubscript{sp} there was a two fold increase in NK cell inhibition (6.4%).
Figure 3.12. HCV core\textsubscript{35-44} and HLA-G\textsubscript{sp} peptide mix assay

CD107a assay of NKG2A\textsuperscript{+} NK cells in response to 0-100 µM HCV core\textsubscript{35-44} or HLA-G\textsubscript{sp} alone or in combination. Dot plots are gated on CD3\textsuperscript{−} CD56\textsuperscript{−} CD158b\textsuperscript{−} lymphocytes. Percentage indicates frequency of CD107a positive events.
NK cell degranulation in response to increasing concentrations of HCV core$^{35-44}$ in the presence or absence of 1 µM HLA-G$^{sp}$

CD3$^+$CD56$^+$ NKG2A$^+$CD158b$^+$ NK cells are shown in the panel (a) and CD3$^+$CD56$^-$ NKG2A$^-$CD158b$^+$ in panel (b). The mean +/- SEM of 3 independent experiments using one donor are shown. Slope difference $p$ values were calculated using linear regression comparing difference between the two lines. All other $p$ values calculated using one-way ANOVA.
Further assays were performed using different concentrations (0-100 µM) of HCV core35-44 alone or in addition to 1µM HLA-Gsp (Figure 3.13a). HCV core35-44 in isolation did not inhibit degranulation of CD94:NKG2A+ NK cells (p= 0.49), as demonstrated in previous experiments. However, in the presence of 1 µM HLA-Gsp, the addition of HCV core35-44 had a synergistic inhibitory effect at CD94:NKG2A (one way ANOVA p <0.0001). This synergy was specific to the NKG2A+ subset, and was not observed for CD158b+ cells (p=0.51) (Figure 3.13b).

3.2.6.1. Effect of HLA-Gsp concentration on HCV core35-44 synergy

HLA-Gsp mediated inhibition at CD94:NKG2A saturates at approximately 50 µM (Figure 3.10 b). The synergistic effect of HCV core35-44 at NKG2A was determined at saturating levels of inhibition of HLA-Gsp (Figure 3.14. a) to determine whether any additional inhibition occurs. At 100 µM HLA-Gsp, the presence of HCV core35-44 produced no additional inhibitory effect at NKG2A compared with 100 µM HLA-Gsp alone. A titration of HLA-Gsp was performed to observe what effect changes in sub-saturating levels of HLA-Gsp would have on HCV core35-44 mediated synergy. In the absence of HCV core35-44, increasing HLA-Gsp from 1-4 µM produced an increase in NK cell inhibition. However, there was no significant increase in the maximum HCV core35-44 mediated synergistic inhibition at CD94:NKG2A (Figure 3.14 b). Thus the synergistic effect of HCV core35-44 does not exceed the inhibitory effect of saturating levels of HLA-Gsp.
Figure 3.14. The effect of saturating levels of HLA-Gsp inhibition at NKG2A on HCVcore35-44 mediated synergy

(a) CD107a assay performed using .174 target cells incubated alone (no peptide), with 100 µM HCV core35-44 (HCV), 100 µM HLA-Gsp (Gsp), or 100µM of both peptides (Gsp + HCV). Mean and SEM CD107a+NKG2A+CD158b− NK cell events are shown from 3 experiments using one donor. Statistics calculated using one way ANOVA; ns = p > 0.05, *** = p<0.0001. (b) Titration of HLA-Gsp and HCV core35-44 synergy. Percentage of CD107a+NKG2A+CD158b− NK cells are shown at varying concentrations of HCV core35-44 and HLA-Gsp. Mean and SEM of 3 experiments using one donor.
3.2.6.2. HCV core$_{35-44}$ synergy in the presence of other MHC class I leader peptides

As HLA-G is primarily expressed in the foetal trophoblast, HCV core$_{35-44}$ mix experiments were repeated using leader peptides derived from more widely expressed classical MHC class I molecules HLA-A2 (HLA-A2$_{sp}$ VMAPRTLVL) and HLA-B7 (HLA-B7$_{sp}$ VMAPRTVLL). As was observed with HLA-G$_{sp}$, the presence of HCV core$_{35-44}$ peptide significantly enhanced NKG2A-mediated inhibition of NK cells by HLA-A2$_{sp}$ or HLA-B7$_{sp}$ (both p <0.0001), (Figure 3.15). The synergy of HCV core$_{35-44}$ with HLA-A2$_{sp}$ was confirmed in PBMCs from three additional healthy donors (Figure 3.16).

HCV core$_{35-44}$ induced a lower percentage synergistic effect in the presence of 1 µM HLA-B7$_{sp}$. This may be because HLA-B7$_{sp}$ was a more potent inhibitor via CD94:NKG2A, thus inhibition was already approaching the maximal level induced by this peptide.
Figure 3.15. NK cell degranulation to .174 cells with HCV core35-44 in the presence or absence of HLA-A2_{sp} and HLA-B7_{sp}

Data are normalised to CD107a expression by CD3^+ CD56^+ NGK2A^+ CD158b^+ NK cells incubated with unloaded .174 cells (HCV core35-44 alone), or to the level of inhibition at 1 µM HLA-A2_{sp} or 1 µM HLA-B7_{sp}. Mean ± SEM of 6 independent experiments are shown. P values calculated using one way ANOVA.
Figur 3.16. Synergy at NKG2A observed for three other donors

Degranulation of CD3⁺ CD56⁺ NKG2A⁺ CD158b⁺ NK cells in response to 174 cells incubated with 0-100 HCV core₃₅-₄₄ alone (closed symbols) or in the presence of 1 µM HLA-A₂₃p (open symbols). Data from three independent experiments showing mean and SEM; p values calculated using one-way ANOVA.
3.2.6.3. Other viral and host derived peptides inducing synergy at NGK2A

Peptides derived from a variety of viruses, including EBV, HIV and CMV, are known to bind HLA-E (Nattermann et al., 2005b; Tomasec et al., 2000; Ulbrecht et al., 1998). The HIV p24 peptide residues 14-22 (HIVp2414-22; AISPRTLNA) was identified by a motif-based approach (Nattermann et al., 2005b). In chronic HIV infection there is up-regulation of HLA-E on CD4+ T cells resulting in increased inhibition of NKG2A+ NK cells (Martini et al., 2005; Nattermann et al., 2005b). The peptide SQAPLPCVL from EBV BZLF-1 protein residues 39-47 (EBVbzlf39-47) has also been shown to bind to HLA-E (Ulbrecht et al., 1998). Our data showed that although both these viral peptides stabilize cell surface HLA-E on the .174 cell line (Figure 3.17), in isolation they do not inhibit NKG2A+ NK cells. However in the presence of 1µM HLA-A2sp, these peptides had a dose dependent synergistic inhibitory effect (Figure 3.18). Similarly hsp60sp, the host derived stress induced peptide that is known to bind to HLA-E but does not inhibit at CD94:NKG2A, had a synergistic effect on NKG2A+ cell inhibition mediated by HLA-A2sp. Thus, synergistic inhibition at CD94:NKG2A can be induced by both host and viral peptides.
Figure 3.17. HLA-E stabilisation by other viral and host derived peptides

HLA-E upregulated on the surface of .174 cells by (a) EBV b2lf_{39-47}, (b) HIV p24_{14-22} and (c) hsp60_{sp}. .174 cells were incubated for 16 hours at 26°C either alone or with the specified peptide, before staining with an anti-HLA-E antibody, as described in Materials and Methods. Data represent mean fluorescent intensity of 10,000 gated events shown as histograms.
Figure 3.18. Synergy at NKG2A is a shared mechanism for other viral and host derived peptides

CD3^+ CD56^+ CD158b^+ NK cell degranulation in response to increasing concentrations of indicated peptide in the presence or absence of 1µM HLA-A2sp. Data are normalised to NK cell degranulation in response to unpulsed .174 cells (in the absence of HLA-A2sp) or to 1 µM HLA-A2sp. (a) EBV BZLF peptide (residues 39-47). (b) HIVp24 peptide (residues 14-22). (c) hsp60 signal peptide. Mean +/- SEM of 3 independent experiments using one donor are shown, p values calculated using one-way ANOVA.
3.2.6.4. Peptide synergy is specific to NKG2A

The effect of synergistic peptides at NKG2A and KIR2DL2/2DL3 (CD158b) was further characterised by comparing CD107a expression by different NK cell subsets: NKG2A\(^+\) CD158b\(^-\), NKG2A\(^-\) CD158b\(^+\), NKG2A\(^+\) CD158b\(^+\) and NKG2A\(^-\) CD158b\(^-\). At CD158b, HCV core\(_{35-44}\) and HLA-A2\(_{sp}\) independently produce direct inhibition; however, no synergistic inhibitory effect was observed at this receptor when these peptides were mixed (Figure 3.19). Synergy was specific to the NKG2A\(^+\) CD158b\(^-\) population, and was not observed in the NKG2A\(^-\) CD158b\(^-\) population.

In contrast to HCV core\(_{35-44}\), EBV bzlf\(_{39-47}\) alone did not inhibit NKG2A\(^-\)CD158b\(^+\) NK cells (Figure 3.20). Additionally, in the presence of HLA-A2\(_{sp}\), EBV bzlf\(_{39-47}\) did not synergise to inhibit NKG2A\(^-\) CD158b\(^+\), or the NKG2A\(^-\) CD158b\(^-\) subset. A synergistic effect was observed for NKG2A\(^+\) CD158b\(^-\) NK cells, with a similar level of inhibition seen for the NKG2A\(^+\) CD158b\(^+\) subset.
Figure 3.19. HCV core\textsubscript{35-44} and HLA-A2\textsubscript{sp} peptide mix experiment comparing different NK cell populations

CD3\textsuperscript{+} CD56\textsuperscript{+} NK cell degranulation in response to increasing concentrations of HCV core\textsubscript{35-44} alone (black symbols/ line) or mixed with 1\mu M HLA-A2\textsubscript{sp} (blue symbols/ line). Top left panel gated on NKG2A\textsuperscript{−} CD158b\textsuperscript{−}, top right panel NKG2A\textsuperscript{+} CD158b\textsuperscript{−}, bottom left panel NKG2A\textsuperscript{+} CD158b\textsuperscript{+}, bottom right panel NKG2A\textsuperscript{−} CD158b\textsuperscript{+} subset. Mean +/- SEM of 3 independent experiments using one donor are shown. Slope difference \( p \) values were calculated using linear regression comparing difference between the two lines.
Figure 3.20. EBV bzlf\textsubscript{39-47} and HLA-A\textsubscript{2}\textsubscript{sp} peptide mix experiment

CD\textsuperscript{3} CD56\textsuperscript{+} NK cell degranulation in response to increasing concentrations of EBV bzlf\textsubscript{39-47} alone (black symbols/ line) or mixed with 1\mu M HLA-A\textsubscript{2}\textsubscript{sp} (blue symbols/ line). Top left panel gated on NKG2A\textsuperscript{−} CD158b\textsuperscript{−}, top right panel NKG2A\textsuperscript{+} CD158b\textsuperscript{−}, bottom left panel NKG2A\textsuperscript{−} CD158b\textsuperscript{+}, bottom right panel NKG2A\textsuperscript{−} CD158b\textsuperscript{+} subset. Mean +/- SEM of 3 independent experiments using one donor are shown. Slope difference \textit{p values} were calculated using linear regression comparing difference between the two lines.
3.2.6.5. Variant of HCV core$^{35-44}$ peptide

HCV sequence screening from a cohort of individuals infected with a single source exposure to HCV demonstrated only one variant of the HCV core$^{35-44}$ peptide, that has a lysine for arginine substitution at position 9 (HCV core$^{R9K}$ YLLPRRGPKL). This epitope has previously been shown to be a CTL escape variant at HLA-A2, but in general is well conserved (Nattermann et al., 2005a). HCV core$^{R9K}$ stabilised HLA-Cw*0102 and HLA-E to similar levels as HCVcore$^{35-44}$ (Figure 3.21). In degranulation assays, HCV core$^{R9K}$ did not inhibit CD158b$^+$ NK cells but had a similar synergistic inhibitory effect to the wild-type peptide on NKG2A$^+$ NK cells (Figure 3.22). Thus although this mutation retains the ability of the wild type epitope to inhibit NK cells through NKG2A, it loses the ability to inhibit through engagement of KIR.
Figure 3.21. HCV core<sub>R9K</sub> induces stabilisation of HLA-E and HLA-Cw*0102

174 cells were incubated for 16 hours at 26˚C either alone or with the specified peptide, before staining with (a) anti-HLA-E antibody (3D12) or (b) anti-HLA-Cw*0102 (VP6G3), or an isotype control as described in Materials and Methods. Data represent mean fluorescent intensity of 10,000 gated events shown as histograms.
Figure 3.22. NK cell degranulation (CD107a expression) in response to target cells incubated with 0-100 µM HCVcoreR9K

Gated on (a) Total NK (CD3- CD56+), (b) NKG2A+ CD158b+, (c & d) NKG2A+ CD158b-, comparing effect in the presence or absence of 1 µM HLA-A2sp. Mean +/- SEM of 3 independent experiments using one donor are shown, p values calculated using one-way ANOVA.
3.3. Discussion

The non-classical MHC class Ib molecule HLA-E is known to bind a closely related set of highly conserved nonameric peptides derived from the leader sequences of other MHC class I molecules (Miller et al., 2003). However, it is also capable of binding viral peptides and peptides composed of hydrophobic residues, which may not conform to predicted binding motifs (Brooks et al., 1999; Michaelsson et al., 2002; Nattermann et al., 2005a; Nattermann et al., 2005b). Flow cytometry assays confirmed that the classical MHC class I signal peptides HLA-A2_sp, HLA-B7_sp and HLA-G_sp all enhanced cell surface HLA-E on an MHC deficient .174 cell line. HLA-E binding was also demonstrated for virus derived peptides HCVcore35-44, EBVbzlf39-47, HIVp2414-22 and the leader sequence of the heat shock protein 60 peptide, hsp60_sp, consistent with published observations. CD107a degranulation assays were used to investigate the effects of host and virus derived peptides on NK cell activity. HLA-E bound HLA-A2_sp, HLA-B7_sp and HLA-G_sp all inhibited degranulation of the NKG2A+ NK cell subset, whereas HCV core35-44, EBV bzlf39-47, HIV p2414-22 and hsp60_sp did not.

It has recently been demonstrated that HLA-C bound peptides that induce weak KIR binding can interfere with the inhibitory effect of a strong KIR binding peptide (Fadda et al., 2010). This study used two peptides, VAP-FA (VAPWNSFAL) and VAP-DA (VAPWNSDAL), that had similar affinity for HLA-Cw*0102. VAP-FA:HLA-C was recognised by CD158b+ NK cells producing inhibition of this NK cell subset, whereas VAP-DA:HLA-C was not. However, when these two peptides were mixed, VAP-DA interfered with the inhibitory effect of the VAP-FA peptide, a phenomenon termed peptide antagonism. To determine whether comparable antagonism occurred at CD94:NKG2A, peptide mix experiments were performed using HLA-G_sp and HCV core35-44. In the absence of MHC class I leader peptides, HCV core35-44 pulsed .174 cells did not inhibit degranulation of NKG2A+ NK cells. However, .174 cells pulsed with HCV core35-44 and 1 µM HLA-G_sp led to twice as much inhibition of the NKG2A+ subset compared to 1 µM HLA-G_sp alone. This enhanced inhibition in the presence of HCV core35-44, termed peptide synergy, was also observed in the presence of 1 µM HLA-A2_sp and 1 µM HLA-B7_sp and was confirmed in three independent PBMC donors.
HCV core\textsubscript{35-44}, EBV bzlf\textsubscript{39-47}, HIV p24\textsubscript{14-22} and hsp60\textsubscript{sp} all induce dose related synergistic inhibition at CD94:NKG2A when mixed with 1 \(\mu\)M MHC class I leader peptide, without any direct inhibitory effect at this receptor in single peptide experiments. This would suggest that synergy at CD94:NKG2A is a shared mechanism for viral and certain host peptides that bind to HLA-E. Additionally, the observation of peptide synergy at CD94:NKG2A contrasts peptide antagonism seen at KIR.

hsp60\textsubscript{sp} (QMRSVPQRST) and EBV bzlf\textsubscript{39-47} (SQAPLPCVL) have previously been shown to bind to HLA-E but not CD94:NKG2A (Brooks et al., 1999; Michaelsson et al., 2002). hsp60\textsubscript{sp} contains P2 methionine and P9 leucine, consistent with HLA-E binding MHC class I derived leader peptides, but the presence of P5 valine is thought to interfere with recognition by CD94:NKG2A. EBV bzlf\textsubscript{39-47} has glutamine at P2, which is reported to be second only to methionine in predicted affinity for HLA-E, while P9 leucine would favour binding to HLA-E (Miller et al., 2003). However, P5 leucine does not permit recognition by CD94:NKG2A. HCV core\textsubscript{35-44} (YLLPRRGPRL) and HIVp24\textsubscript{14-22} (AISPRTLNA) also bound to HLA-E, consistent with previous studies (Nattermann et al., 2005a; Nattermann et al., 2005b). HCV core\textsubscript{35-44} does not conform to the predicted binding motif of HLA-E, but has an abundance of hydrophobic residues which favour binding. HIVp24\textsubscript{14-22} has P2 isoleucine, has been predicted to permit HLA-E binding, while the residues at lesser anchor positions of P4, P6 and P7 are shared with host MHC class I signal peptides that bind to HLA-E (Miller et al., 2003). Both HCV core\textsubscript{35-44} and HIV p24\textsubscript{14-22} contain P5 arginine, which is known to be dominant contact residue for binding to CD94:NKG2A; however, in our single peptide experiments neither of these peptides were capable of inhibiting NKG2A\textsuperscript{+} cells. It is possible that with HCV core\textsubscript{35-44} being a 10 amino-acid peptide, there is an alteration in the conformation of this peptide within the binding groove of HLA-E.

In 9mer peptides, the P5 and P6 side chains align toward the \(\alpha_2\) and \(\alpha_1\) helices, respectively, but for the 10mer the orientation changes so that P5 points toward the \(\alpha_1\) helix and P6 toward the \(\alpha_2\) helix (Madden et al., 1993; O'Callaghan et al., 1998). In the case of HIV p24\textsubscript{14-22} residues P8 (asparagine) and P9 (alanine) may be relevant, as a standard conformation of P8 and P9 residues is imposed by conserved network of hydrogen bonds. Alterations at P8 and P9 can affect side chain positions at the centre (P5, P6, or P7) of the peptide (Madden et al., 1993). Thus, although both HCV core\textsubscript{35-44} and HIV p24\textsubscript{14-22} contain P5 arginine, our experiments suggest this is not sufficient to enable inhibition at CD94:NKG2A. This may be explained by crystal structure analyses showing that binding affinities of
peptides to HLA-E are complex and amino acid residues interact within the peptide to produce conformational structures that may conflict with current binding prediction motifs.

Peptide stabilisation assays showed that HLA-A2<sub>sp</sub>, HLA-B7<sub>sp</sub>, HLA-C<sub>sp</sub>, hsp60<sub>sp</sub>, HCV core<sub>35-44</sub> and EBV bzlf<sub>39-47</sub> also increased cell surface HLA-Cw*0102, the ligand for KIR2DL2/2DL3 (CD158b) receptor. This dual affinity of peptides for HLA-C and HLA-E may be consistent with published data. It has been reported that HLA-C and HLA-A2 bind similar peptides, as do HLA-E and HLA-A2 (Andersen et al., 1999; Lampen et al., 2013). Therefore, it could be predicted that HLA-C and HLA-E may have similar peptide binding affinities. These peptides also conform to predicted HLA-Cw<sup>0102</sup> binding motifs. MHC class I derived signal peptides, hsp60<sub>sp</sub>, and EBV bzlf<sub>39-47</sub> are all nonameric peptides possessing P9 leucine, which is a key anchor residue for HLA-Cw*0102. The decamer HCV core<sub>35-44</sub> has P2 and P3 leucine, which would predict binding, while P10 leucine may also be relevant in the conformational structure of the peptide.

174 target cells pulsed with HCV core<sub>35-44</sub> induce direct inhibition of the CD158b<sup>+</sup> subset, whereas EBV bzlf<sub>39-47</sub> had no inhibitory effect. Residues P7 and P8 of HLA-Cw*0102 bound nonameric peptides can predict recognition by KIR2DL2/2DL3<sup>+</sup> NK cells, as demonstrated by Fadda et al, 2010. Using this predicted binding tool, EBV bzlf<sub>39-47</sub> (SQAPLPCVL) would be predicted to have low affinity for HLA-Cw*0102, consistent with our findings. As HCV core<sub>35-44</sub> (YLLPRRGPRL) is a decamer, the predicted binding tools could be less accurate as the exact conformation of the peptide may vary compared to nonamer sequences. Glycine, proline and arginine are favourable residues at P7 or P8, once again consistent our findings. In peptide mix experiments using MHC class I signal peptides and either HCV core<sub>35-44</sub> or EBV bzlf<sub>39-47</sub>, analysis of NK cell subsets showed no synergistic effect observed for either peptide at CD158b, suggesting that peptide synergy is specific to the NKG2A<sup>+</sup> cells.

HCV sequence screening from a cohort of individuals infected with a single source exposure to HCV demonstrated just one variant of HCV core<sub>35-44</sub> with a P9 lysine for arginine substitution (HCV
In peptide stabilization assays, HCV core_{R9K} enhanced cell surface HLA-Cw*0102 and HLA-E to similar levels as HCV_{core,35-44}. CD107a assays revealed HCV core_{R9K} did not inhibit CD158b^+ NK cells but induced synergy similar to HCV_{core,35-44} on NKG2A^+ NK cells. Thus although this mutation retains the ability of the wild type peptide to inhibit NK cells by engaging NKG2A, it has lost the ability to inhibit through KIR.

The next chapter will address possible mechanisms for peptide synergy observed at CD94:NGK2A.
Chapter 4. CD94 homodimers and peptide synergy

4.1. Introduction

CD94:NKG2A is a heterodimeric inhibitory receptor belonging to the C-type lectin-like receptor family. This family of transmembrane glycoproteins additionally includes the activating molecules NKG2C, NKG2E and NKG2D. NKG2C and NKG2E also dimerise with CD94, displaying over 90% sequence homology to NKG2A in their ectodomains (Lazetic et al., 1996). In contrast, NKG2D appears a somewhat different receptor sharing only 21% homology with other NKG2 molecules (Ho et al., 1998). NKG2D does not pair with CD94, instead operating as a homodimer (McFarland and Strong, 2003).

CD94:NKG2 receptors are expressed in both man and mouse. In humans, all CD94:NKG2 members share HLA-E as their ligand with the CD94 moiety dominating (80%) interaction with this MHC class Ia molecule (Braud et al., 1998). The main cell types expressing CD94:NKG2 receptors are NK cells and CD8+ T cells. Following successful ligation of CD94:NKG2A, an inhibitory signal is mediated through two ITIM sequences attached to the cytoplasmic tail of the NKG2A moiety. Though the activating molecules NKG2C and NKG2E do not possess a cytoplasmic signalling domain, they have a positively charged residue within their transmembrane region which forms an ionic bond to the ITAM-containing adaptor protein DAP-12 (Lanier et al., 1998). In contrast, CD94 does not possess its own signalling motif and therefore cannot transduce a signal in the absence of its NKG2 partners.

Crystal structure analysis of CD94:NKG2A bound to HLA-E demonstrates CD94 and NKG2A interacting mostly with the α1 and α2 domains of HLA-E respectively (Li et al., 2001; Petrie et al., 2008; Sullivan et al., 2007). The CD94 footprint on HLA-E is broad, with residues within loops 2, 3, and 5, and β strands 6 and 7 from CD94 interacting with a region spanning residues 65-89 of the α1 helix of HLA-E (Petrie et al., 2008). The interaction matches a hydrophobic region on HLA-E (Ile73, Val76) and a similar region on CD94 (Phe114, Leu162) (Wada et al., 2004). This hydrophobic region is associated with a cluster of complementary charged residues: a basic cluster in HLA-E (Arg75, Arg79) and an acidic cluster in CD94 (Asp163, Glu164 and Asp168). The footprint of NKG2A on HLA-E is much smaller compared to CD94, with loop 3 and β strands 2, 5, and 6 interacting with residues 151–162 of the α2 helix of HLA-E. Wada et al. used individual alanine-substitutions to
demonstrate that mutation of Arg75 and Arg79 impairs HLA-E binding to CD94:NKG2 molecules. A distinction between NKG2A and NKG2C recognition of HLA-E was also shown in this study. D69A and H155A mutations on HLA-E selectively impair HLA-E binding to CD94:NKG2A, but generally do not affect HLA-E binding to CD94:NKG2C. Asp69 and His155 are situated at the centre of the α1 and α2 helices, respectively, which accommodate the HLA-E-bound peptide. This raises the possibility that HLA-E loaded with certain peptides may preferentially bind CD94:NKG2C but not CD94:NKG2A. Furthermore, it has been shown that there is a 6-fold greater affinity in the interaction between CD94-NKG2A/HLA-E compared to CD94-NKG2C/HLA-E (Kaiser et al., 2005).

Petrie et al. have shown that the CD94 subunit has a much more dominant role than NKG2A in interacting with the peptide bound by HLA-E, forming 80% of the contact mainly over the C terminus of the peptide (Petrie et al., 2008). In agreement with this, it has been observed that substitutions at the C-terminus of the HLA-E bound peptide affect the interaction with of all CD94:NKG2 receptors in a similar manner (Kaiser et al., 2005). CD94 interacts with P5 arginine, P6 threonine and P8 phenylalanine. In contrast, NKG2A interaction with the peptide is less prominent, with the subunit lying adjacent to the P5 arginine, forming van-der-Waals interaction using residue Pro171 (Petrie et al., 2008).

In the previous chapter we observed that P5 arginine to lysine impaired recognition by CD94:NKG2A but had no effect on HLA-E binding. The P5 arginine protrudes into a cavity between NKG2A and CD94 subunits, with its aliphatic side chain flanked in an anti-parallel manner with Gln112 of CD94 (O’Callaghan et al., 1998). The P5 arginine is not well complemented by any charged interactions with CD94:NKG2A, which may be attributable to it salt bridging to Glu152 on the top surface of the α2 helix of HLA-E (Figure 4.1). Petrie et al. also assessed the effect of P5 arginine to lysine substitution. This abrogates recognition by CD94:NGK2A, suggesting either that the P5 lysine to Glu152 salt bridge is lost, thereby introducing a buried positive charge at the interface, or highlighting the importance of the P5 residue interacting with Ser110 of CD94 (Petrie et al., 2008).

In this chapter we assess in greater detail the phenomenon of peptide synergy observed at CD94:NKG2A. We determine whether synergy is specific to HLA-E binding peptides or whether peptides binding to other MHC class I molecules behave similarly. Peptide specificity is also
assessed by lysine substitution at certain positions in the peptide sequence. Finally, using confocal microscopy and blocking antibody CD107a assays we determine the respective significance of the CD94 and NKG2A subunits in peptide synergy.

**Figure 4.1. The CD94:NKG2A-HLA-E-HLA-G\textsubscript{sp} complex**

NKG2A and CD94 represented as blue and pale green ribbon structures respectively. The HLA-E heavy chain and β2m are shown as violet and cyan ribbons respectively, with HLA-G\textsubscript{sp} in orange sticks. (A) side view of CD94:NKG2A docking onto HLA-E:HLA-G\textsubscript{sp}. (B) Top view of CD94:NKG2A docking onto the surface of HLA-E:HLA-G\textsubscript{sp}. Figure from Petrie et al. (2008).
4.2. Results

4.2.1. Is synergistic inhibition at CD94:NKG2A induced by generalised upregulation of MHC class I?

In the previous chapter, we showed that certain virus and host derived peptides (HCV core\textsubscript{35-44}, HCV core\textsubscript{R9K}, EBV bzl\textsubscript{39-47}, HIV p24\textsubscript{14-22} and hsp60\textsubscript{sp}) alone do not inhibit at CD94:NKG2A but promoted synergistic inhibition at this receptor when mixed with 1 µM MHC class I leader peptide. These synergistic peptides all bind to HLA-E in MHC class I binding assays. To determine whether this effect is specific to HLA-E, peptide mix experiments were performed using the HLA-A2 specific influenza matrix-derived peptide GILGFVFTL (GILG) (Shimojo et al., 1989). MHC class I stabilization assays using the HLA-A2 specific BB7.2 antibody showed increased cell surface HLA-A2 on .174 cells pulsed with the GILG peptide, with no HLA-E upregulation detected using the HLA-E specific 3D12 antibody (Figure 4.2 a & b). Consistent with the absence of HLA-E binding, CD107a degranulation assays revealed no inhibitory effect of GILG at CD94:NKG2A. However, in contrast to the HLA-E binding peptides, GILG did not induce synergy at NKG2A when mixed with 1 µM HLA-A2\textsubscript{sp} (Figure 4.3).

VAPWNSDAL (VAP-DA) is a model peptide that has been shown to bind HLA-Cw\*0102 but it does not inhibit via KIR2DL2/2DL3 (Fadda et al., 2010). It was defined following screening of a library of variants of the tissue inhibitor of metalloproteinase (TIMP)-1 peptide VAPWNSLSL. VAP-DA binding to HLA-Cw\*0102 on .174 cells was confirmed using the VP6G3 antibody, in the absence of HLA-E binding. Once again CD107a assays revealed no inhibition of either KIR2DL2/2DL3\textsuperscript{+} (CD158b\textsuperscript{+}) or NKG2A\textsuperscript{+} NK cells. Additionally, peptide mix experiments with 1 µM HLA-A2\textsubscript{sp} did not promote synergy at either NKG2A or CD158b (Figure 4.4).
Figure 4.2. MHC class I stabilisation assay for GILG and VAP-DA peptides

174 cells were incubated for 16 hours at 26°C either alone or with the specified peptide, before staining with (a & c) anti-HLA-E antibody (3D12), (b) anti HLA-A2 (BB7.2) or (d) anti-HLA-Cw*0102 (VP6G3), as described in Materials and Methods. Data represent mean fluorescent intensity of 10,000 gated events shown as histograms.
Figure 4.3. NK cell degranulation in response to increasing concentrations of GILG peptide in the presence or absence of 1 µM HLA-A2\textsubscript{sp}.

The percentage of CD\textsuperscript{3−}CD\textsubscript{56\textsuperscript{+}}NK\textsubscript{G2A\textsuperscript{+}}CD\textsubscript{158b\textsuperscript{−}} NK cells expressing CD107a is shown. Mean +/- SEM of 3 independent experiments using one donor are shown. Slope difference \( p \) values were calculated using linear regression comparing difference between the two lines. All other \( p \) values calculated using one-way ANOVA.
Figure 4.4. NK cell degranulation in response to increasing concentrations of VAP-DA peptide in the presence or absence of 1 µM HLA-A2<sub>sp</sub>

Showing percentage of CD107a<sup>+</sup> NK cells gated on (a) NKG2A<sup>+</sup> CD158b<sup>-</sup> and (b) NKG2A<sup>-</sup> CD158b<sup>+</sup> subsets. Mean +/- SEM of 3 independent experiments using one donor are shown. Slope difference p values were calculated using linear regression comparing difference between the two lines. All other p values calculated using one-way ANOVA.
4.2.2. Is synergistic inhibition at CD94:NKG2A dependent on the presence of a host MHC class I derived leader peptide?

We have shown that synergy at CD94:NKG2A occurs in the presence of 1 μM of HLA-Gsp, HLA-A2sp, or HLA-B7sp. These host MHC class I derived leader peptides all bind to HLA-E producing inhibition at CD94:NKG2A. To determine if a synergistic peptide requires the presence of a cognate ligand for HLA-E/CD94:NKG2A we repeated HCV core35-44 peptide mix CD107a degranulation assays in the presence or absence of either 1 μM VAP-DA or 1 μM GILG peptide (Figure 4.5). HCV core35-44 failed to produce synergistic inhibition at CD94:NKG2A in the presence of either the HLA-Cw*0102 specific VAP-DA peptide or the HLA-A2 specific GILG peptide. Thus, to exert their inhibitory effect, synergistic peptides require the presence of a HLA-E/CD94:NKG2A binding peptide.
Figure 4.5. NK cell degranulation in response to increasing concentrations of HCV core\textsubscript{35-44} peptide in the presence or absence of (a) 1 µM VAP-DA and (b) 1 µM GILG peptide.

Percentage of CD107\textsuperscript{a} events are shown gated on CD3\textsuperscript{−}CD56\textsuperscript{−}NKG2A\textsuperscript{+}CD158b\textsuperscript{−}. Mean +/- SEM of 3 independent experiments using one donor are shown. Slope difference *p* values were calculated using linear regression comparing difference between the two lines. All other *p* values calculated using one-way ANOVA.
4.2.3. Do all HLA-E binding peptides induce synergy? Effect of Position 5 Lysine substitution

The P5 residue of HLA-E bound peptide is in direct contact with CD94 and NKG2A (Petrie et al., 2008). P5 arginine to lysine (R5K) substitution of HLA-Gsp reduces the affinity of the peptide/HLA-E complex for CD94-NKG2A by more than ten-fold (Kaiser et al., 2008). Host and viral peptides that mediate synergy share the common feature of HLA-E binding in the absence of direct inhibition at the CD94:NKG2A receptor. To determine whether interaction with CD94:NKG2A was essential for a synergistic peptide, or if HLA-E binding alone was sufficient, peptide mix experiments were performed using HLA-G_{R5K} (VMAPKTLFL).

HLA-G_{R5K} stabilized HLA-E to a similar extent as the wild-type peptide, but did not induce inhibition of NKG2A^+ NK cells (Figure 4.6). Furthermore, the addition of HLA-G_{R5K} to HLA-A2_{sp} did not result in synergy. Instead, as the concentration of HLA-G_{R5K} was increased, there was a dose related reversal of NK cell inhibition mediated by HLA-A2_{sp}. This is most likely due HLA-E becoming progressively more occupied with HLA-G_{R5K} rather than the CD94:NKG2A agonist HLA-A2_{sp}.
Figure 4.6. HLA-G<sub>R5K</sub> binding to HLA-E and effect on NK cell degranulation

(a & b) .174 cells were incubated for 16 hours at 26˚C either alone or with the specified peptide, before staining with an anti-HLA-E antibody (3D12), as described in Materials and Methods. Data represent mean fluorescent intensity of 10,000 gated events shown as (a) histograms. (b) plotted against peptide concentration. (c) NK cell degranulation in response to .174 cells loaded with increasing concentrations of HLA-G<sub>R5K</sub> or HLA-G<sub>sp</sub> peptide. (d) NK cell degranulation in response to .174 cells loaded with HLA-G<sub>R5K</sub> in the presence or absence of 1 µM HLA-A2<sub>sp</sub> (mean ± SEM of 3 independent experiments). p values calculated using one-way ANOVA. For (c) & (d) Percentage of CD107a<sup>+</sup> NKG2A<sup>+</sup> CD158b<sup>+</sup> cells shown.
The observation that HLA-G_{R5K} binds to HLA-E but does not synergise with MHC class I derived peptides to inhibit NK cells led us to investigate the effect of lysine substitution on the synergistic effect of HCV core_{35-44} and EBV bzl_{39-47}. As HCV core_{35-44} is a 10mer, substitutions were performed at either position 5 (HCV core_{R5K}) or position 6 (HCV core_{R6K}), in both cases replacing an arginine residue with lysine. A position 5 substitution was also performed for the EBV bzl_{39-47}, in this instance replacing leucine for lysine (EBV bzl_{L5K}).

The effect of this lysine substitution on HLA-E affinity was determined in an HLA-E stabilization assay, in each case comparing the lysine substituted peptide with the wild type peptide (Figure 4.7). EBV bzl_{L5K}, HCV core_{R5K} and HCV core_{R6K} all had similar affinity for HLA-E as their respective wild type peptides. This is consistent with previous observation showing that P5 lysine substitution has a relatively small effect on affinity for HLA-E (Miller et al., 2003).

The effect of lysine substitution on the synergistic inhibition of NK cell degranulation was investigated. In the presence of 1 µM HLA-A2_{sp}, HCV core_{35-44} and EBV bzl_{39-44} inhibit CD107a expression of NKG2A^{+} in a dose dependent manner, but do not induce inhibition in the absence HLA-A2_{sp} consistent with our observations presented in the previous chapter. In contrast, EBV bzl_{L5K}, HCV core_{R5K} and HCV core_{R6K} failed to induce any inhibitory effect on NKG2A^{+} NK cell activity, regardless of the presence of HLA-A2_{sp} (Figure 4.8).

Thus lysine substitution at position 5 of EBV bzl_{39-47} and position 5 or position 6 of HCV core_{35-44} annuls the synergistic inhibitory effect of these peptides on NKG2A^{+} NK cells, without affecting their binding to HLA-E. This would support the model that not all HLA-E binding peptides promote synergistic inhibition at CD94:NKG2A.
Figure 4.7. Effect of lysine substitution on peptide mediated HLA-E stabilisation

174 cells were incubated for 16 hours at 26°C either alone or with 100 µM of the specified peptide, before staining with an anti-HLA-E antibody (3D12), as described in *Materials and Methods*. Data represent mean fluorescent intensity of 10,000 gated events shown as histograms.
Figure 4.8. Effect of lysine substitution on peptide synergy at CD94:NKG2A

NK cell degranulation (CD107a expression) in response to increasing concentrations of the specified peptide, in the presence or absence of 1 µM HLA-A2sp. (a) left panel HCV core35-44, right panel HCV coreR5K, (b) left panel HCV core35-44, right panel HCV coreR6K, (c) left panel EBV bzl39-47, right panel EBV bzlL5K. Percentage of CD107a+ NKG2A+CD158b+ NK cells shown for one donor (mean +/- SEM of 3 independent experiments). Slope difference p values were calculated using linear regression comparing difference between the two lines. All other p values calculated using one-way ANOVA.
4.2.4. Peptides that stabilise HLA-E but do not inhibit NK cells induce clustering of CD94 but not NKG2A at the Immune Synapse

The signalling component of the CD94:NKG2A heterodimer is NKG2A which possesses two ITIMs within its cytoplasmic tail, whereas CD94 contains no signaling motifs. Confocal microscopy was used to examine the effect of peptides presented by HLA-E on CD94 and NKG2A clustering at the contact surface formed between effector and target cell membranes, termed the immune synapse. NKL cells were co-incubated with .174 cells that were loaded with peptide (HLA-Gsp, HLA-A2sp, HCVcore35-44, HLA-A2sp + HCV core35-44, or HLA-GR5K). The target and effector cell complexes were stained for CD94 (mAb DX22) or NKG2A (mAb Z199). Quantification of staining intensity at the immune synapse showed that the MHC class I inhibitory peptides HLA-Gsp and HLA-A2sp promoted aggregation of both CD94 and NKG2A (Figure 4.9). However, HCV core35-44 induced clustering of CD94 (one way ANOVA p<0.0001), but not NKG2A at the immune synapse (p>0.05). The combination of HCV core35-44 and HLA-A2sp resulted in greater CD94 aggregation (both fold increase intensity and as percentage of total aggregates, p<0.05) compared to HLA-A2sp alone, implying recruitment of additional CD94-associated complexes. In contrast, HLA-GR5K did not promote aggregation of either CD94 or NKG2A.

Clustering of CD94 but not NKG2A was also demonstrated for target cells loaded with other HLA-E binding synergistic peptides, EBV bzlF39-47, HIVp2414-22 and hsp60sp (Figure 4.10). Therefore synergistic peptides appear to induce aggregation of the non-signalling molecule CD94 in the absence of the signalling moiety NKG2A.

Imaging of the immune synapse was also performed using untouched primary NK effector cells isolated from PBMCs by negative selection. In contrast to the NKL cell line where CD94:NKG2A is expressed on almost all cells (>90% NKL cells are NKG2A+ on flow cytometry analysis), NKG2A expression on primary NK cell varies from 30-60%. In the selected donor 45% of NK cells were NKG2A+ on flow cytometry analysis. Confocal microscopy revealed a lower fluorescent intensity of both CD94 and NKG2A staining compared to NKL imaging, with a proportion of NK cells staining...
negative for both receptor subunits, consistent with the flow cytometry data for this donor. HLA-A2sp promoted aggregation of both CD94 and NKG2A, while HCV core35-44 induced clustering of CD94 but not NKG2A, consistent with experiments performed using the NKL cell line (Figure 4.11). Similar to the HLA-GRSK peptide, imaging showed that HCV coreRSK did not promote clustering of either CD94 or NKG2A. Therefore the loss of synergy observed following lysine substitution of HLA-E binding peptides is accompanied by an abrogation of CD94 clustering.

Double staining of immune synapses with both CD94 and NKG2A to assess localisation of these subunits at the same synapses was attempted but was unsuccessful. None of the antibody combinations suitable for double staining produced sufficient fluorescent intensity on confocal microscopy to enable an assessment of clustering. This may have been due to steric hindrance since flow cytometry experiments demonstrated NKG2A (Z199) binding was markedly impaired in the presence of anti-CD94 (DX22 clone), despite strong single staining (Figure 4.12). An alternative CD94 clone (3D9) pre-labelled FITC antibody was tried but was only weakly fluorescent on confocal microscopy and also seemed to reduce NKG2A (Z199) binding. An antibody labelling kit was used to attach a fluorophore label to unconjugated Z199. The resulting antibody worked correctly for FACS experiments but did not fluoresce adequately on confocal microscopy. In future, the development of new clones and fluorochromes may enable double staining.
Figure 4.9. HCV core$_{35-44}$ induces aggregation of CD94 but not NKG2A at the interface between NK and target cells

(a) Comparison of CD94 (left panel) and NKG2A (right panel) clustering at the interface between NKL effector and .174 target cells, loaded with the indicated peptides. Pseudocolour scales indicates the fluorescent intensity from low (black/ violet) to high (white/ orange). (b) Fold increase of fluorescence intensity at the interface between NKL and .174 target compared with a non-contact area of the NKL plasma membrane, CD94 (left panel) and NKG2A (right panel). Data from three independent experiments including the mean +/- SEM are shown. (c) The percentage of conjugates with >1.4 fold increased fluorescence at the immune synapse was also calculated (mean +/- SEM from three independent experiments are shown). For (b) and (c) statistics were performed using ANOVA with Dunnetts post test to compare individual pairs of conditions; * = p < 0.05, *** = p < 0.001, ns = non-significant.
Figure 4.10. Selective aggregation of CD94, but not NKG2A, at the immune synapse is a generic feature of the known HLA-E binding synergistic peptides

(a) Fold increase of fluorescence intensity at the interface between NKL and .174 target compared with a non-contact area of the NKL plasma membrane, CD94 (left panel) and NKG2A (right panel). Data from three independent experiments including the mean ± SEM are shown. (b) The percentage of conjugates with >1.4 fold increased fluorescence at the immune synapse was also calculated (mean ±/− SEM from three independent experiments are shown). *p values calculated using ANOVA with Dunnett’s post test to compare individual pairs of conditions; **∗ = p < 0.001, ns = non-significant.
Figure 4.11. Primary NK cell imaging: effect of P5K substitution on HCV core_{35-44} mediated CD94 clustering

(a) Comparison of CD94 (left panel) and NKG2A (right panel) clustering at the interface (immune synapse) between primary NK cells and .174 target cells, loaded with the indicated peptides. Pseudocolour scales indicates the fluorescent intensity from low (black/ violet) to high (white/ orange).

(b) Fold increase of fluorescence intensity at the immune synapse compared with a non-contact area of the NK cell plasma membrane, CD94 (left panel) and NKG2A (right panel). Data from three independent experiments including the mean +/- SEM are shown. (c) The percentage of conjugates with >1.4 fold increased fluorescence at the immune synapse was also calculated (mean +/- SEM from three independent experiments are shown). p values calculated using ANOVA with Dunnett's post test to compare individual pairs of conditions; * = p < 0.05, ** = p < 0.01, *** = p < 0.001, ns = non-significant.
Figure 4.12. Impaired NKG2A staining in the presence of anti-CD94

NKG2A (Z199)-PE staining was assessed in the absence (left panel) or presence (middle panel) of anti-CD94 (DX22). Number, percentage and mean fluorescent intensity of NKG2A events are shown. Right panel- Overlay of NKG2A-PE staining in the presence or absence of anti-CD94 (DX22). Dot plots gated on CD3⁺ CD56⁺ NK cells.
4.2.5. Clustering of CD94 in the absence of NKG2A is not due to recruitment of NKG2C

Having demonstrated that certain HLA-E binding peptides that do not inhibit NK cells can promote clustering of CD94 but not NKG2A at the immune synapse, we wanted to exclude an effect mediated by NKG2C. Firstly, we screened our donors in whom peptide synergy at CD94:NKG2A had been demonstrated for NKG2C expression. NKG2C expression varied between donors, with the majority of donors tested expressing a relatively small percentage of NKG2C positive cells (Table 4.1). This was not due to the fluorochrome as we tested both PerCP and PE conjugated NKG2C clones. One donor, Donor 5, had a higher percentage (11.1%) of NKG2C+ NK cells. We repeated the CD107a degranulation assay using PBMCs from Donor 5, adding an additional channel for NKG2C-PerCP.

<table>
<thead>
<tr>
<th>Donor</th>
<th>NKG2C+ cells (as a % of CD3-CD56+ events)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.5%</td>
</tr>
<tr>
<td>2</td>
<td>0.1%</td>
</tr>
<tr>
<td>3</td>
<td>0.9%</td>
</tr>
<tr>
<td>4</td>
<td>1.2%</td>
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<tr>
<td>5</td>
<td>11.1%</td>
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Table 4.1. NKG2C expression on CD3-CD56+ NK cells, comparison between different donors

Donors in whom peptide synergy was observed at CD94:NKG2A were screened for NK cell NKG2C expression. The percentage of NKG2C+ NK cells for each donor is shown.

Six colour flow cytometry revealed that the overwhelming majority of NKG2A+ cells were NKG2C- (Figure 4.13 a). Gating on NKG2A+ NKG2C- CD158b- population showed that NKG2C is not necessary for synergy between HCV core35-44 and HLA-A2sp. NKG2C does not appear to contribute to synergy as NKG2A+ NKG2C- NK cells had very similar degranulation results to NKG2A+ NKG2C- NK cells (Figure 4.13 b). Furthermore, neither HCV core35-44 or HLA-A2sp increased CD107a expression by NKG2A+ NKG2C+ cells, suggesting that these peptide:HLA-E complexes do not activate the NKG2C+ population.
Figure 4.13. Effect of NKG2C on synergy at CD94:NKG2A

Six colour flow cytometry was performed to include anti-NKG2C staining. (a) Four quadrant plot of NKG2A expression versus NKG2C gated on CD3+ CD56+ NK cells, showing the percentage of NKG2A+ NKG2C− and NKG2A− NKG2C+ events. NKG2A+ NKG2C− CD158b+ NK cell degranulation (CD107a expression) in response to .174 cells either unloaded (no peptide) or loaded with the specified peptide(s), shown as histograms from one representative experiment. (b) Data comparing CD3+ CD56+ CD158b− NK cell CD107a expression in the presence or absence of 100 µM HCV core35-44 and 1 µM HLA-A2sp, gated on NGK2A+ NKG2C− (top left), NKG2A− NGK2C+ (top right), NGK2A+ NKG2C+ (bottom left) and NKG2A− NKG2C+ (bottom right). Means and SEMs from three independent experiments are shown.
NKL cells were stained for cell expression of CD94, NKG2A, NKG2C and CD158b (Figure 4.14). NKL cells express both CD94 and NKG2A, but are negative for NKG2C and CD158b consistent with previous observations (Fadda et al., 2010; Michaelsson et al., 2002). Thus any clustering of CD94 in the absence of NKG2A on the surface of NKLs is not due to recruitment of NKG2C.

Finally, we investigated the effect of synergistic peptides on CD94 aggregation on a cell line that does not express NKG2A. The Jurkat cell line is a leukaemic T cell line first isolated from the peripheral blood of a patient with T cell leukaemia (Schneider et al., 1977). Wild type Jurkat T cells do not express the CD94:NKG2A receptor. Perez-Villar et al. successfully managed to stably transfect Jurkat cells with non-fluorescent labeled CD94 and kindly donated us this cell line (Perez-Villar et al., 1996). We stained the CD94-Jurkats for cell surface CD94, NKG2A and NKG2C expression, confirming expression of CD94 but not the NKG2 molecules (Figure 4.15). As a control, we also stained non-transfected Jurkats, which failed to express CD94, NKG2A or NGK2C.

Confocal imaging was performed on CD94 Jurkat cells incubated with .174 cells in the absence or presence of peptide (Figure 4.16). Microscopy revealed contacts between CD94 Jurkat cells and .174 cell targets. HLA-A2 SP and HCV core35-44 loaded .174 cells induced aggregation of CD94, whereas HCV coreR5K and HLA-GR5K did not. These findings suggest that cells expressing CD94 have the ability to interact with HLA-E in the absence of NKG2 partners. However, HLA-E/CD94 interaction can be abrogated with R5K lysine substitution of the HLA-E bound peptide. These observations are consistent with our flow cytometry data presented in the previous chapter, showing that peptide synergy is abrogated by lysine substitution at position 5 of the synergistic peptide.
Figure 4.14. NKL staining for CD94, NKG2A, NKG2C and CD158b

NKL cells were stained for cell surface (a) CD94 (clone DX22), (b) NKG2A (clone Z199) or (c) NKG2C (clone 134591), (d) CD158b (clone CH-L) or the corresponding isotype control antibody, as described in Materials and Methods. Data represent mean fluorescent intensity of 10,000 gated events shown as histograms.
Figure 4.15. Comparison between cell surface expression of CD94, NKG2A and NKG2C on CD94 transfected Jurkat cells (CD94-Jurkat) compared with wild-type Jurkat cells

CD94-Jurkat cells (left panels), were stained for cell surface (a) CD94 (clone DX22), (b) NKG2A (clone Z199) or (c) NKG2C (clone 134591), or the corresponding isotype control antibody, as described in Materials and Methods. Comparison was made with wild-type Jurkat cells (right panels). Data represent mean fluorescent intensity of 10,000 gated events shown as histograms.
Figure 4.16. CD94 aggregation at the immune synapse between CD94-Jurkat cells and .174 cell targets

(a) CD94 clustering at the interface between CD94 transfected Jurkat cells and .174 target cells, loaded with the indicated peptides. Pseudocolour scales indicates the fluorescent intensity from low (black/ violet) to high (white/ orange). (b) Fold increase of CD94 fluorescence intensity at the interface (immune synapse) between CD94 Jurkat cell and .174 target compared with a non-contact area of the NK cell plasma membrane. Data from three independent experiments including the mean +/- SEM are shown. (c) The percentage of conjugates with >1.4 fold increased fluorescence at the immune synapse was also calculated (means +/- SEM from three independent experiments are shown). p values calculated using ANOVA with Dunnett's post test to compare individual pairs of conditions; *** = p < 0.001, ** = p 0.001- 0.01, ns = non-significant.
4.2.6. Blocking assays showing that CD94 but not LILRB1 is essential for synergy

It has been shown using a human NK cell line that ITIM deficient KIR can mediate an inhibitory signal through interaction with LILRB1 receptors (Kirwan and Burshtyn, 2005). In their model, Kirwan and Burshtyn showed that LILRB1 expression alone was insufficient to respond to HLA-C expressed on target cells. However, LILRB1 was able to signal in a KIR-dependent manner, revealing a cooperation between these two receptor families. Mutant KIR signalling could be abrogated by antibodies which block the interaction between KIR and HLA-C, or between LILRB1 and the α3-domain of MHC class I molecules. LILRB1 binds a broad range of MHC class I ligands, including HLA-A, -B, C and HLA-G (Chapman et al., 1999; Cosman et al., 1997; Fanger et al., 1998; Shiroishi et al., 2003). HCV core35-44 is an HLA-A2 binding epitope that also binds to HLA-Cw*0102, therefore could potentially interact with LILRB1. Therefore, to address whether CD94 or LILRB1 was required for CD94:NKG2A synergy, we performed CD107a degranulation assays using blocking antibodies. Pre-incubation of PBMCs with anti-CD94 completely reversed the synergistic inhibitory effect of HCV core35-44 in the presence of 1 µM HLA-A2sp, but there was no effect observed in the presence of anti-LILRB1 specific blocking Ab HP-F1 (Figure 4.17). Thus, overall, the synergistic effect of HLA-E binding peptides is critically dependent on CD94.
Figure 4.17. CD107a degranulation assay using blocking antibodies

Degranulation assay of CD3- CD56+ CD158b- NK cells incubated with .174 cells in the presence or absence of the indicated peptides and blocking antibodies to CD94 (DX22), LILRB-1 (HP-F1) or an isotype control antibody (clg). A2sp = 1 µM HLA-A2sp; HCV_35-44 = 100 µM HCV core_35-44. The mean +/- SEM of 2 experiments is shown.
4.3. Discussion

The sensitivity of NK cell inhibitory receptors to the peptide bound to MHC class I is well recognised (Brooks et al., 1999; Fadda et al., 2010; Llano et al., 1998; Miller et al., 2003; Sullivan et al., 2008). We have demonstrated that certain peptides that bind HLA-E but alone do not inhibit at CD94:NKG2A are able to synergistically inhibit at this receptor in the presence of a host MHC class I derived leader peptide. We sought to determine whether peptides that bind to MHC class I molecules other than HLA-E also result in synergy. Peptide stabilisation assays showed that the HLA-A2 specific influenza matrix peptide GILGFVFTL (GILG) and the HLA-Cw*0102 binding TIMP1-derived peptide VAPWNSDAL (VAP-DA) did not bind to HLA-E. CD107a assays showed that the neither of these peptides induced synergistic inhibition at CD94:NKG2A in the presence of HLA-A2sp. Thus, it would appear that the ability to bind to HLA-E is a pre-requisite for a synergistic peptide. VAP-DA:HLA-Cw*0102 complexes do not inhibit at KIR2DL2/2DL3, antagonising the inhibitory effect of recognised peptides at this receptor (Fadda et al., 2010). We have shown that HLA-A2sp is a weak agonist at KIR2DL2/2DL3; however, peptide mix experiments did not demonstrate any synergistic inhibition at KIR2DL2/2DL3 when VAP-DA was added to HLA-A2sp. This would imply that peptide synergy is a specific feature of HLA-E binding peptides and CD94:NKG2A.

With the knowledge that synergistic peptides bind to HLA-E without inhibiting at CD94:NKG2A, we wanted to determine whether interaction with the CD94:NKG2A was essential for synergy. Position 5 of HLA-E bound nonameric peptides is known to be a key contact residue with CD94:NKG2A receptor, with a lysine residue poorly tolerated at this position (Michaelsson et al., 2002; Miller et al., 2003; Petrie et al., 2008; Sullivan et al., 2008). Using the HLA-Gsp sequence, we mutated position 5 substituting the P5 arginine for a lysine residue (HLA-GR5K). This mutation did not affect the key anchor residue positions P2, 6, 7 or 9, as demonstrated by a similar affinity for HLA-E compared to the wild type peptide. Consistent with expectations, HLA-E:HLA-GR5K had no inhibitory effect at CD94:NKG2A, in contrast to HLA-E:HLA-Gsp which is a potent inhibitor at this receptor. Thus HLA-GR5K shares some characteristics with the synergistic peptides HCV core35-44, HIVp14-22, EBV bzlfr39-47 and hsp60sp by binding to HLA-E in the absence of inhibition at CD94:NKG2A. However, in peptide mix CD107a assays HLA-GR5K failed to synergise with HLA-A2sp. Mutation of P5 of EBV bzlfr39-47 (EBV bzlfl5K) and P5 or P6 of the 10mer peptide HCV core35-44 (HCV coreR5K and HCV coreR6K,
respectively) annulled the synergistic inhibitory effect of these peptides without affecting their binding to HLA-E. Thus, lysine substitution at position 5 or 6 of synergistic peptides abrogates their synergistic effect, suggesting that interaction with CD94:NKG2A is a pre-requisite for this group of peptides.

Confocal microscopy was used to assess peptide promoted clustering of CD94 and NKG2A subunits at the immune synapse formed between effector cells and .174 targets. Target cells pulsed with a synergistic peptide induce aggregation of the non-signalling molecule CD94 in the absence of the signalling moiety NKG2A, in contrast to the MHC class I derived host peptides HLA-A2sp and HLA-Gsp which promote clustering of both subunits. This is not due to recruitment of NKG2C to the immune synapse, as this disparity in CD94 and NKG2A recruitment by synergistic peptide was observed using the NKG2C‘ NKL cell line (Michaelsson et al., 2002). Furthermore, CD107a assays incorporating NKG2C staining showed that NKG2C neither contributed nor was necessary for peptide synergy. Imaging of the NKG2A‘ NKG2C‘ CD94 transfected Jurkat T cell line confirmed that .174 cells loaded with a synergistic peptide induces accumulation of CD94 at the interface between effector and target cell. Thus, synergistic peptides induce the recruitment of CD94 to the immune synapse but not NKG2A and NGK2C signalling partners. CD94 aggregation in the absence of NKG2 molecules could represent the presence of monomeric CD94 or CD94 homodimers. CD94 monomers are unable to bind to HLA-E (Braud et al., 1998). CD94 homodimers have been observed although the functional significance of their binding to HLA-E is unclear due to the absence of an intracellular signalling domain (Boyington et al., 2000b; Carretero et al., 1997).

The formation of homodimers is not usual for C-type lectin molecules. Several C-type lectin molecules are encoded by the NKC complex in rodents and humans, including CD94, NKG2D, CD69 and Ly49. CD69, Ly49 family members and NKG2D exist as homodimeric receptors, whereas the other NKG2 molecules form heterodimers with CD94 at the cell surface (Brooks et al., 1997; Lazetic et al., 1996; Li et al., 2001; Natarajan et al., 2000; Tormo et al., 1999). Heterodimer formation is driven by the attraction of the CD94 and NKG2 C-type lectin domains as evidenced by the extensive hydrophobic interface between these domains, the formation of an intermolecular disulphide bond and also by asymmetric interactions due the a2 helix of NKG2A interacting with the corresponding extended loop region of CD94 (Sullivan et al., 2007).
In transfected cell lines lacking NKG2 signalling partners, homodimeric CD94 has been demonstrated (Carretero et al., 1997). Orr et al. used CD94 transgenic mice to demonstrate CD94 expression in the absence of NKG2 molecules (Orr et al., 2010). Following transfection of the CD94 gene into CD94 deficient mice they observed low level CD94 expression in all cells, including non-immune cells that would not express NKG2 molecules, supporting the formation of CD94 homodimers. Boyington et al. assessed the crystal structure of CD94, showing that the extracellular portion of CD94 forms non-covalent dimers (Figure 4.18) (Boyington et al., 1999). The interface of the dimer contains a central hydrophobic region surrounded by hydrophilic residues. Hydrogen bonds formed between the first β-strand of each monomer creates an extended six stranded anti-parallel β sheet. Residues in the stem region of CD94 are critical for the dimerisation of this subunit, with the stemless CD94 construct existing in a monomeric form (Boyington et al., 2000b).

Derre et al. assessed CD94 and NKG2A expression on CD8+ T cells following anti-CD3 mAb stimulation in the presence of IL-12 (Derre et al., 2002). A proportion of cultured peripheral blood lymphocytes were CD94+ but NKG2A+. To distinguish whether this was due to CD94 homodimers or CD94 pairing with other NKG2C or NKG2E a functional assay was performed. Cross-linking of CD94 by a specific mAb had no effect on redirected lysis, suggesting that these cells do not express a CD94 linked inhibitory or activating NKG2 moiety. The authors conclude that IL-12 stimulation induces the expression of non-functional CD94 homodimers on these CD3-stimulated peripheral blood lymphocytes.

Jabri et al. assessed peripheral blood CD8+ T Cell expression of CD94 and NKG2A in relation to TCR clonotypic specificity (Jabri et al., 2002). While the majority (90%) of CD94+ NKG2A− CD8+ T cell clones expressed NKG2C or NKG2E, there existed a CD94+ population that lacked transcripts for NKG2 signalling partners. This CD94+ NKG2A/C/E population, thought to express CD94 homodimers, was shown to be inert following CD94 cross linking, in contrast to NKG2 expressing CD94+ subsets. Furthermore, this study reveals that CD8 T cells demonstrate a clonal commitment to NKG2A expression. NKG2A−, NKG2A committed cells express CD94 homodimers, but following TCR stimulation in vitro, these cells express CD94:NKG2A. IL-15 stimulation has also been shown to induce increased expression of CD94 but not NKG2A (Jabri et al., 2000).
Our findings suggest that target cells loaded with a synergistic peptide induce the formation of CD94 homodimers on the surface of effector cells at the immune synapse. By engaging CD94 homodimers, such peptides would not trigger any activating or inhibitory effect due to the lack of a signalling domain. However, in the presence of a MHC class I signal peptide:HLA-E complex that is inhibits at CD94:NGK2A, the presence of a synergistic peptide amplifies this effect. The precise mechanism for this has not been fully elucidated. One possibility would be that the formation of CD94 homodimers stimulated by synergistic peptides stabilises the immune synapse between effector and target cell, thereby potentiating signalling through HLA-E:MHC-class I signal peptide complex at CD94:NKG2A. However, this effect appears specific to HLA-E binding peptides and the CD94:NGK2A receptor, as the HLA-Cw*0102:VAP-DA peptide complex interacting with KIR2DL2/2DL3 also forms an immune synapse, but does not induce synergy at CD94:NKG2A in the presence of host MHC class I leader peptides. Additionally, HLA-E binding synergistic peptides do not synergise with MHC class I leader peptides to inhibit at KIR2DL2/2DL3.

There may be various explanations as to why synergistic peptides recruit CD94 homodimers in preference to NGK2 molecules. Binding of CD94:NKG2A to HLA-E is peptide dependent, with a very fast association/ dissociation rate constant for the interaction (Vales-Gomez et al., 1999). CD94 forms 80% of the interface with the HLA-E bound peptide (Petrie et al., 2008). Kaiser et al. showed that minor changes in peptide sequences have equivalent effects on the affinities of NKG2A, NKG2C, and NKG2E (Kaiser et al., 2005). They reason that CD94 provides the majority, if not all, of the contacts to the C-terminal half of the presented peptide. This would mean that peptide substitutions most likely have the same effect on the different NKG2 molecules since the CD94 interface is equivalent across the family. Certain peptide sequences such as those of the synergistic peptides may permit binding of CD94 molecules but not NKG2A. However, interaction with CD94 is abrogated by position 5 lysine substitution, whereas other residues such as arginine, leucine and valine are tolerated.

CD94 has no inter-chain salt bridges but there are two regions of charge complementarity across the modelled CD94:NKG2 interface: one between Asp106 of CD94 and Lys 135 of NKG2A, and the other between Arg69 of CD94 and Glu122 of NKG2A. Lys 135 and Glu122 are conserved sequences.
across NKG2A, C and E, but are replaced by Ser and Lys respectively in CD94, creating an unfavourable Arg69-Lys64 interaction across the CD94/CD94 interface. This may partly explain why CD94 preferentially forms heterodimers with NKG2 molecules, compared to CD94 homodimers.

It is possible that synergistic peptide induced CD94 clustering defined in microscopy represents a low affinity interaction which may not be measurable by surface plasmon resonance assays. This is true for VAP-DA interaction with KIR.
Figure 4.18. Ribbon models showing two views of the CD94 dimer

Each monomer is coloured a different shade of green. The regions that have low sequence identity with NKG2 molecules are shown in purple. The top view is rotated 90° from the bottom view along the horizontal axis. In the bottom view, residues in the hydrophobic core of the dimer interface (V66, Y68, I75, F107 and M108) are represented by ball-and-stick models. These residues are coloured red in one monomer and yellow in the other. Figure taken from Boyington et al. 1999.
Chapter 5. A functional comparison of CD94:NKG2A and KIR

5.1. Introduction

NK cells are kept in check by inhibitory receptors for MHC class I. The majority of these receptors belong to the KIR family, but also include the C-type lectin receptor CD94:NGK2A. Successful ligation of these inhibitory receptors triggers recruitment of the tyrosine phosphatase SHP-1 to ITIM motifs within their cytoplasmic domain, leading to abrogation of NK cell activation signals. NK cells sequentially express these receptors during development (Beziat et al., 2010). A reduction in CD56 expression is a feature of increasing NK cell maturation, as evidenced by shorter telomere length in CD56\textsuperscript{dim} NK cells compared to their CD56\textsuperscript{bright} precursors (Ouyang et al., 2007; Romagnani et al., 2007). Furthermore, CD56\textsuperscript{bright} cells represent the majority of NK cells that initially appear following haemopoietic stem cell transplantation (Nguyen et al., 2005; Vago et al., 2008). CD94:NKG2A receptors are expressed by virtually all CD56\textsuperscript{bright} NK cells, while the variegated KIR are expressed in a stochastic fashion with increasing NK cell maturation to CD56\textsuperscript{dim} NK cells (Figure 5.1). Beziat et al. (2010) elegantly showed that CD56\textsuperscript{bright} NK cells give rise to CD56\textsuperscript{dim} NKG2A\textsuperscript{+} KIR\textsuperscript{−} population, which in turn mature into CD56\textsuperscript{dim} NKG2A\textsuperscript{+} KIR\textsuperscript{+} phenotype followed by terminal differentiation into CD56\textsuperscript{dim} NKG2A\textsuperscript{−} KIR\textsuperscript{+} cells. Furthermore, they demonstrated that CD56\textsuperscript{dim} NKG2A\textsuperscript{−} NK cells can reacquire NKG2A expression following IL-12 and IL-18 stimulation, leading to an increase in IFN-γ production by this CD56\textsuperscript{dim} population.
Figure 5.1. Sequential expression of CD94 then KIR during NK cell maturation

CD56\textsuperscript{bright} NK cells represent an immature subset that can differentiate into CD56\textsuperscript{dim} NK cells. CD56\textsuperscript{bright} NK cells are KIR\textsuperscript{−} but express high levels of CD94. With increasing NK cell maturation, CD94 expression is lost while KIR receptors are acquired in a sporadic manner.

The monomorphic CD94:NKG2A receptor binds HLA-E, a non-classical MHC class Ib that has limited polymorphism and a broad tissue distribution. Moreover, this receptor system has an ancient origin in evolution, present in both rodents and primates, albeit with some species-specific variation (Shum et al., 2002). In contrast to the highly conserved CD94:NKG2A/HLA-E system, the KIR receptors and their classical MHC class I ligands, HLA-A, -B and -C, show extensive diversity at both locus and allelic levels, and are only expressed in simian primates (Figure 5.2). As a result, individual KIR are expressed in a stochastic fashion, resulting in a diverse NK cell repertoire with NK cell subsets expressing different combinations of receptors. Recognition of MHC class I by CD94:NKG2A and KIR and consequent NK cell inhibition is peptide specific. The cognate peptides for HLA-E constitute a restricted set of nonamers derived from the signal peptide of other MHC class I (HLA-A, -B and -C and HLA-G) molecules. Thus NKG2A\textsuperscript{+} cells can recognise down-regulation of several MHC class I alleles, if they are no longer synthesized, or translated, but not if the down-regulation is post-translational. In contrast, KIR\textsuperscript{+} NK cells only recognize specific MHC class I alleles, therefore any effect of MHC down-modulation would be limited to the subset of NK cells expressing a particular repertoire of receptors.
Figure 5.2. Comparison between CD94:NKG2A and KIR receptor systems

The C-type lectin receptor CD94:NKG2A is encoded by the natural killer complex (NKC) on Chromosome (Ch) 12 whereas the genes for immunoglobulin-like KIR are located in the leukocyte receptor complex (LRC) on Ch 19. The corresponding HLA-ligands are encoded by the HLA complex on Ch 6. The blue text boxes demonstrate the contrasting diversity of the two receptor systems. Figure adapted from Parham et al. (2012).

Genetic studies implicating KIR/ HLA polymorphisms in infectious diseases suggest that rapidly evolving pathogens may be driving the co-evolution of this receptor ligand system (Alter et al., 2007a; Khakoo et al., 2004; Wauquier et al., 2010). Given that KIR/ HLA genes segregate independently on different chromosomes it is possible that an individual fails to express the cognate HLA class I ligands for any given KIR receptor (Gumperz et al., 1996). The evolutionary persistence of CD94:NKG2A/HLA-E may therefore pertain to the ability of this system to recognize highly diversified MHC class I molecules, a subset of which encode remarkably similar signal peptides. Collectively, this may exert a strong selection pressure thereby guarding against auto-reactivity of NK cells. In the absence of KIR/ HLA pairings, this system may also ensure NK cells are licensed to recognise and be responsive to MHC class I down-modulation.

Our data, and that from other groups, demonstrates that certain viral and host derived peptides can also bind to HLA-E but, in isolation, these peptide:HLA-E complexes are insufficient to inhibit NKG2A⁺ NK cells (Brooks et al., 1999; Michaelsson et al., 2002). However, when these host- and virus-derived non-inhibitory peptide:HLA-E complexes are combined with cognate leader peptide:HLA-E
complexes on target cells they augment the inhibition of the NKG2A⁺ NK cell population. We have termed his observation “peptide synergy”. Synergistic peptides recruit the non-signalling CD94 moiety but not ITIM-containing NKG2A to the immune synapse, in contrast to MHC-class I leader peptides which recruit both CD94 and NKG2A.

This observation of CD94:NKG2A/ HLA-E peptide synergy induced by peptides that are otherwise non-inhibitory, contrasts that seen at KIR⁺ NK cells. Peptides that stabilize HLA-C but do not themselves inhibit through KIR, perturb rather than augment the inhibition of NK cells (Fadda et al., 2010). The exact mechanism for peptide antagonism at KIR is yet to be elucidated. Despite not inducing inhibition of KIR⁺ NK cells in degranulation assays, such antagonistic peptides still promote KIR clustering at the immune synapse. However, there is a lack of microtubule-organizing centre (MTOC) polarization to the synapse and a failure of dephosphorylation of the SHP-1 substrate Vav1. It is possible that antagonistic peptide:HLA-C interfere with the KIR clustering induced by inhibitory peptide:HLA-C. The overall significance of this observation is that KIR⁺ NK cells may be activated by changes in the peptide repertoire within a cell as can occur during viral infections.

In this chapter we explore some of the differences between CD94:NKG2A and the KIR inhibitory receptors. We assess the effect of HLA-Gsp on HCV core35-44 mediated inhibition of KIR2DL2/ 2DL3⁺ NK cells. Immunogenetic analyses have revealed that KIR and HLA-C polymorphisms influence the outcome of HCV infection. Homozygosity for KIR2DL3 in combination with its HLA-C1 group of ligands has a protective effect against chronic HCV infection (Khakoo et al., 2004; Romero et al., 2008). We screen our immunogenetic data set to determine whether individuals who possess a lower number of HLA-E/ CD94:NKG2A binding MHC class I leader peptides might be protected from HCV as their NK cells may be less inhibited. We also investigate the stoichiometry of KIR/ HLA-C and CD94:NKG2A/ HLA-E interaction.
5.2. Results

5.2.1. HLA-G<sub>sp</sub> antagonises the inhibitory effect of HCV core<sub>35-44</sub> at CD158b

In chapter 3 we demonstrated that HCV core<sub>35-44</sub> peptide and HLA-G<sub>sp</sub> bind both HLA-E and HLA-Cw*0102, with similar affinity for the latter. However, each peptide:MHC class I complex induces contrasting recognition by KIR and CD94:NKG2A. In CD107a degranulation assays, HCV core<sub>35-44</sub>:HLA-Cw*0102 has a direct inhibitory effect at KIR2DL2/2DL3 (CD158b), whereas HLA-G<sub>sp</sub>:HLA-Cw*0102 has no effect. We measured the effect of these peptides on the CD158b<sup>+</sup> NK cell subpopulation, to determine whether the non-KIR-binding HLA-G<sub>sp</sub> could relieve HCV core<sub>35-44</sub>:HLA-Cw*0102 mediated inhibition at KIR2DL2/2DL3. In our preliminary experiments we pulsed .174 cells overnight with 100 μM HLA-G<sub>sp</sub>, 100 μM HCV core<sub>35-44</sub>, 100 μM of both HLA-G<sub>sp</sub> and HCV core<sub>35-44</sub> or no peptide (Figure 5.3). Un-pulsed .174 cells stimulated 19.2% of the NKG2A<sup>-</sup>CD158b<sup>+</sup> NK cells to express CD107a. .174 cells pulsed with HLA-G<sub>sp</sub> did not induce inhibition at NKG2A<sup>-</sup>CD158b<sup>+</sup> NK cells (20.3%) in contrast to strong inhibition induced by HCV core<sub>35-44</sub> (4.5%). However, when both peptides were mixed, HLA-G<sub>sp</sub> perturbed HCV core<sub>35-44</sub> mediated inhibition at CD158b (14.4%). The effect of HLA-G<sub>sp</sub> on HCV core<sub>35-44</sub> inhibition at CD94:NKG2A was confirmed at a range of peptide concentrations using 1:1 ratio of peptides (Figure 5.4). There was a greater frequency of activated NKG2A<sup>-</sup>CD158b<sup>+</sup> NK cells in response to .174 cells loaded with HCV core<sub>35-44</sub> in the presence of HLA-G<sub>sp</sub> compared with in the absence of HLA-G<sub>sp</sub>.
Figure 5.3. HCV core\textsubscript{35-44} and HLA-G\textsubscript{sp} peptide mix CD107a assay gated on CD158b\textsuperscript{+} NK cells

CD107a assay performed using .174 target cells incubated alone (no peptide), with 100 µM HCV core\textsubscript{35-44}, 100 µM HLA-G\textsubscript{sp}, or 100 µM of both peptides. Histograms gated on CD3\textsuperscript{−} CD56\textsuperscript{−} NKG2A\textsuperscript{−} CD158b\textsuperscript{+} NK cells. Percentage indicates frequency of CD107a positive events
Figure 5.4. CD107a expression in response to increasing concentrations of HCV core<sub>35-44</sub> alone or with 1:1 ratio HLA-G<sub>sp</sub>.<sup>174</sup> target cells incubated alone (black bar), or with HCV core<sub>35-44</sub> alone (blue bars) or HCV core<sub>35-44</sub> + HLA-G<sub>sp</sub> (pink bars) at the indicated peptide concentrations. CD107a assay performed as described in Material and Methods. Percentage indicates frequency of CD107a positive events gated on CD3<sup>-</sup>CD56<sup>-</sup>NKG2A<sup>+</sup>CD158b<sup>+</sup> NK cells.
Following these preliminary experiments, peptide mix assays were repeated using a constant level of HLA-Cw*0102 occupancy. The HCV core\textsubscript{35-44}:HLA-G\textsubscript{sp} ratio was titrated using 20:80, 40:60, 60:40 and 80:20 mixes, while maintaining a final overall concentration of 100 μM peptide. Stabilisation assays confirmed that HCV core\textsubscript{35-44} and HLA-G\textsubscript{sp} share a similar affinity for HLA-Cw*0102. Peptide mix revealed a stable level of cell surface HLA-Cw*0102 as the ratio of HCV core\textsubscript{35-44}:HLA-G\textsubscript{sp} was increased (Figure 5.5 a). In CD107a degranulation assays, HCV core\textsubscript{35-44} stimulated a dose dependent inhibition of CD158b\textsuperscript{+} NK cells (p <0.0001), while HLA-G\textsubscript{sp} had no effect (p= 0.93), consistent with findings presented in Chapter 3. The peptide mix titration showed that HLA-G\textsubscript{sp} reduced the inhibitory effect of HCV core\textsubscript{35-44} (p = 0.0007) (Figure 5.5 b). HCV core\textsubscript{35-44} peptide concentration required to reach 50% inhibition was ~35 μM in the absence of HLA-G\textsubscript{sp} but increased to ~75 μM in the presence of HLA-G\textsubscript{sp} (Figure 5.5 c).

The above peptide mix experiments were carried out at a concentration of peptide where all available HLA-Cw*0102 molecules were presenting peptide, i.e. at saturating levels of peptide. To determine whether the activation of CD158b\textsuperscript{+} NK cells observed in the presence of HCV core\textsubscript{35-44} and HLA-G\textsubscript{sp} was due to displacement of HCVcore\textsubscript{35-44} by HLA-G\textsubscript{sp}, peptide mix assays were repeated at sub-saturating final concentrations of peptide (40 μM) (Figure 5.6). At this peptide concentration, free HLA-Cw*0102 molecules are available for unbound HCV core\textsubscript{35-44} and HLA-G\textsubscript{sp} to bind. The presence of HLA-G\textsubscript{sp} perturbed inhibition of CD158b\textsuperscript{+} NK cells by HCV core\textsubscript{35-44} (two way ANOVA p= 0.003). The confirmation of this effect at sub-saturating levels of peptide, while maintaining a constant level of HLA-Cw*0102 occupation indicate that the observed effect of HLA-G\textsubscript{sp} is not due to displacement of HCV core\textsubscript{35-44}. 

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Figure 5.5. HLA-G<sub>sp</sub> / HCV core<sub>35-44</sub> peptide mix experiments including stabilisation assay

(a) HLA-Cw*0102 stabilisation was detected using the HLA-Cw*0102 specific antibody VP6G3 for .174 cells loaded with HLA-G<sub>sp</sub> and HCV core<sub>35-44</sub> alone or together. In peptide mix wells (shown in red), the concentration of HCV core<sub>35-44</sub> is indicated on the x-axis, with the final concentration of peptide made to 100 μM using HLA-G<sub>sp</sub> (20:80, 40:60, 60:40, 80:20). Data shown are mean +/- SEM of three consecutive experiments. 

(b) CD107a<sup>+</sup> NK cells in response to .174 cells loaded with the indicated peptides, gated on CD3<sup>-</sup>CD56<sup>+</sup>NKG2A<sup>-</sup>CD158b<sup>+</sup> NK cells. Data shown are mean +/- SEM of triplicates performed using PBMCs from one donor. 

(c) Data from (b) was normalised to the frequency of CD107a<sup>+</sup> NK cells following co-incubation with unloaded .174 cells (no peptide). Broken black line indicates a 50% reduction in CD107a expression.
Figure 5.6. HLA-Gsp/ HCV core<sub>35-44</sub> peptide mix experiments performed at sub-saturating levels of HLA-C*0102 occupation

(a) HLA-Cw*0102 stabilisation was detected using the HLA-Cw*0102 specific antibody VP6G3 for .174 cells loaded with HLA-G<sub>sp</sub> and HCV core<sub>35-44</sub> alone or together (0-40 μM). In peptide mix wells (shown in red), the concentration of HCV core<sub>35-44</sub> is indicated on the x-axis, with the final concentration of peptide made to 40 μM using HLA-G<sub>sp</sub> (5:35, 10:30, 20:20, 30:10). Data shown represent mean +/- SEM of three consecutive experiments. (b) CD107a<sup>+</sup> NK cells in response to .174 cells loaded with the indicated peptides, gated on CD3<sup>-</sup> CD56<sup>-</sup> NKG2A<sup>-</sup> CD158b<sup>+</sup> NK cells. Data normalised to the frequency of CD107a<sup>+</sup> NK cells following co-incubation with unloaded .174 cells (no peptide). Data shown represent mean +/- SEM of triplicates performed using PBMCs from one donor.
The antagonistic effect of HLA-Gsp at CD158b+ NK cells was compared with that of VAPWNSDAL (VAP-DA) peptide. VAP-DA has a higher affinity for HLA-C*0102 compared to HCV core35-44 and HLA-Gsp, and was therefore used at a 10-fold lower concentration (2, 4, 6, 8, and 10 μM) in both stabilisation and degranulation assays (Figure 5.7a and b). In degranulation assays, .174 cells loaded with VAP-DA had no effect on CD158b+ NK cell degranulation. Peptide mix wells showed that .174 cells loaded with HCV core35-44 and HLA-Gsp induced a more degranulation of CD158b+ NK cells compared with those pulsed with HCV core35-44 alone. Compared with HLA-Gsp, VAP-DA produced greater perturbation of HCV core35-44 mediated inhibition at CD158b. It is possible that this observation is due to the high affinity VAP-DA peptide displacing HCV core35-44 from HLA-Cw*0102, even at the lower concentrations of VAP-DA used. However, the shape of the curve appears similar to HLA-Gsp mediated relief of HCVcore35-44 antagonism.

The results from these HLA-Gsp/ HCV core35-44 peptide mix experiments demonstrate that a pair of peptides that synergise to inhibit at HLA-E/CD94:NKG2A, have contrasting effects at HLA-C/ KIR, where we observe antagonism. However, the in-vivo relevance of this phenomenon is unclear due to high concentration of peptides used and will need to be tested.
Figure 5.7. HCV core35-44/HLA-Gsp peptide mix assay compared to HLA-Gsp/VAP-DA

(a) HLA-Cw*0102 stabilisation was detected using the HLA-Cw*0102 specific antibody VP6G3 for .174 cells loaded with HLA-Gsp, VAP-DA, HCV core35-44, HCV core35-44/HLA-Gsp mix or HCV core35-44/VAP-DA mix. In HCV core35-44/HLA-Gsp and HCV core35-44/VAP-DA peptide mix wells the concentration of HCV core35-44 is indicated on the x-axis. For HLA-Gsp mix, the final concentration of peptide made to 100 μM using (20:80, 40:60, 60:40, 80:20 ratios of HCV core35-44:HLA-Gsp). For VAP-DA mix, the following HCV core35-44/VAP-DA ratios were used, 20:8, 40:6, 60:4, 80:2. Data shown represent mean +/- SEM of three consecutive experiments.

(b) CD107a+ NK cells in response to .174 cells loaded with the indicated peptides, gated on CD3+ CD56+ NKG2A+ CD158b+ NK cells. Data shown represent mean +/- SEM of triplicates performed using PBMCs from one donor.
5.2.2. Differences in the stoichiometry between KIR and CD94:NKG2A receptor systems

Having observed fundamental differences between how KIR$^+$ and NKG2A$^+$ NK cells respond to changes in the peptide content of MHC class I, we determined whether there was also a difference in their response to MHC class I down-regulation. We therefore studied two potent inhibitory peptides; HLA-G$_{sp}$ for NKG2A mediated inhibition and the peptide VAPWNSFAL (VAP-FA) which in a peptide screen had the highest affinity for KIR2DL2 and KIR2DL3 (Fadda et al., 2010; Llano et al., 1998). Using the MHC deficient .174 cell line, HLA-G$_{sp}$ and VAP-FA mediated stabilization of HLA-E and HLA-Cw$^*$0102 was measured at a range of peptide concentrations (0-100 μM). Degranulation assays were also performed at the same range of peptide concentrations, before normalizing the data and plotting MHC class I stabilisation against NK cell inhibition. Degranulation assays using peptide titrations showed that inhibition of NK cells by MHC class I exhibited saturation kinetics for NKG2A$^+$ NK cells ($r^2=0.98$) but was linear for KIR$^+$ NK cells ($r^2=0.96$) (Figure 5.8). Thus more NKG2A$^+$ NK cells respond to alterations in MHC class I at low HLA-E levels than at high levels. However the level of HLA-C does not appear to influence the fraction of KIR$^+$ NK cells that respond to a given change in HLA-C expression. Hence NKG2A$^+$ NK cells are tuned to respond to changes in MHC class I at low levels of MHC expression, but this is not the case for KIR$^+$ NK cells.
Figure 5.8. NKG2A+, but not KIR+, NK cells are tuned to low cell surface levels of MHC class I

(a) .174 cells were pulsed with increasing concentrations of HLA-Gsp before measuring HLA-E stabilization and performing degranulation assays gating on NKG2A+ CD158b- (NKG2A+) NK cells. Data for MHC class I expression are normalised to “no peptide” and “saturating (100 μM) peptide” concentrations (% maximal stabilisation). Degranulation data are normalised to CD107a expression to unloaded .174 cells and loading with saturating (100 μM) peptide (% maximal inhibition). Correlations were then made between the levels of cell surface MHC class I and the fraction of degranulating NK cells. (b) Similar experiments were performed with VAPWNSFAL (VAP-FA) loaded .174 cells, except staining with HLA-Cw*0102 for stabilisation assays, and gating on the NKG2A- CD158b+ (CD158b+) NK cells fraction in degranulation assays. The results of three independent experiments are shown.
5.2.3. Immunogenetic analyses: Predicted peptide binding repertoires of HLA-E and HLA-C and the outcome of HCV infection

To test the potential clinical importance of our findings, we performed a series of immunogenetic analyses.

5.2.3.1 Effect of the number of CD94:NKG2A cognate MHC class I leader peptides

The cognate peptides for HLA-E are derived from polymorphic MHC class I (HLA-A, -B, -C and HLA-G) signal peptides. Not all MHC class I derived signal peptides bind to HLA-E, and of those that do, not all of these peptide:HLA-E molecules are recognised by CD94:NKG2A. We hypothesised that individuals who possess a greater number of MHC class I signal peptides that do not permit both HLA-E and CD94:NKG2A binding might be protected from chronic HCV infection as their NK cells may be less inhibited compared to those individuals whose leader peptides are predicted to bind.

The data from 361 individuals with chronic HCV infection (cHCV) and 112 spontaneous resolvers (SR) typed for MHC class I were analysed (Figure 5.9) (Khakoo et al., 2004). Leader peptides sequences for HLA-A, -B and -C alleles were derived from the EMBL Bank database and assigned as to whether or not they would bind HLA-E and productively engage CD94:NKG2A (Goujon et al., 2010). No significant difference was noted between the average number of cognate leader peptides predicted to bind HLA-E/CD94:NGK2A between resolvers and chronically infected individuals (cHCV 3.981, SR 3.982; p=0.82; Mann Whitney test).

5.2.3.2 Influence of the number of HLA-Gsp-binding HLA-C alleles and the outcome of HCV infection.

Earlier in this chapter we have shown that HLA-Cw*0102 binds HLA-Gsp and that the resulting complexes activate KIR2DL2/2DL3+ NK cells by interfering with inhibitory peptide:HLA-Cw*0102 complexes. HLA-G has been shown to be upregulated in chronic HCV and where it may offer a protective effect (de Oliveira Crispim et al., 2012). Not all HLA-C alleles bind HLA-Gsp (Appendix Figure 9.1.). We hypothesised that individuals who possess HLA-C alleles that bind HLA-Gsp might
be protected from chronic HCV infection as their CD158b^NK cells may be activated by HLA-G upregulation. Using software to predict peptide binding to MHC class I molecules, we assessed binding of HLA-Gsp (VMAPRTLFL) to individual HLA-C alleles comparing chronic HCV to spontaneous resolvers (Hoof et al., 2009). The data from of 382 individuals with chronic HCV infection (cHCV) and 122 spontaneous resolvers (SR) typed for MHC class I were analysed (Figure 5.10). There was no significant difference in the number of HLA-C alleles predicted to bind HLA-Gsp (CHC 0.24, SR 0.26; p= 0.6; Mann Whitney test).
Figure 5.9. Absence of genetic association between the number of NKG2A cognate MHC class I leader peptides and the outcome of HCV infection

(a) Leader peptides from classical HLA-A, -B, -C were assigned either a positive or negative binding potential for HLA-E or NKG2A according to the work of Llano et al (Llano et al., 1998). 

(b) The data from 361 individuals with chronic HCV infection (CHC) and 112 spontaneous resolvers (SR) typed for MHC class I were analyzed. The number of MHC class I leader peptides in each individual was then assessed in individuals that spontaneously resolved HCV infection or remained chronically infected. (c) Mean number of NKG2A cognate peptides and results of the comparison by the Mann Whitney test are shown.
(a) Data from 382 individuals with chronic HCV infection (CHC) and 122 spontaneous resolvers (SR) typed for HLA-C were analysed. HLA-C alleles were assigned either a positive or negative binding potential for HLA-Gsp according to the NetMHCpan prediction tool (Hoof et al., 2009). The number of HLA-C alleles predicted to bind HLA-Gsp (VMAPRTLFL) in each individual (between 0-2) was then assessed comparing spontaneous resolvers and those who remained chronically infected. (b) Mean number +/- SEM of HLA-Gsp binding HLA-C alleles and results of the comparison by the Mann Whitney test are shown.

Figure 5.10. Number of HLA-C alleles predicted to bind HLA-Gsp and the outcome of HCV infection

(a) Data from 382 individuals with chronic HCV infection (CHC) and 122 spontaneous resolvers (SR) typed for HLA-C were analysed. HLA-C alleles were assigned either a positive or negative binding potential for HLA-Gsp, according to the NetMHCpan prediction tool (Hoof et al., 2009). The number of HLA-C alleles predicted to bind HLA-Gsp (VMAPRTLFL) in each individual (between 0-2) was then assessed comparing spontaneous resolvers and those who remained chronically infected. (b) Mean number +/- SEM of HLA-Gsp binding HLA-C alleles and results of the comparison by the Mann Whitney test are shown.
5.2.3.3 Influence of the number of HLA-A, –B and -C signal peptides binding HLA-C alleles and the outcome of HCV infection.

In chapter 3, we showed that leader peptides derived from HLA-A2 (HLA-A2<sub>sp</sub>; VMAPRTLVL) and HLA-B7 (HLA-B7<sub>sp</sub>; VMAPRTVLL) bind to HLA-Cw<sup>*0102</sup>, promoting inhibition of KIR2DL2/2DL3 (CD158b<sup>+</sup>) NK cells. NetMHCpan software analysis shows that HLA-Cw<sup>*0102</sup> is predicted to bind to all classical MHC class I signal peptides shown in Figure 5.9 a, but this is not true for other HLA-C alleles (Hoof et al., 2009). We therefore hypothesised individuals who possess a greater number of HLA-A, –B and -C leader peptides predicted to bind to their HLA-C alleles would be more susceptible to chronic HCV as their NK cells would be more inhibited. Data from of 363 individuals with chronic HCV infection (cHCV) and 112 spontaneous resolvers (SR) typed for MHC class I were analyzed (Figure 5.11 a& b). Leader peptides sequences for HLA-A, -B and -C alleles were derived from the EMBL Bank database (Goujon et al., 2010), followed by NetMHCpan software analysis to assess binding of these leader peptides to individual HLA-C alleles (Appendix Figure 9.2.). Contrary to our hypothesis, individuals possessing a greater number of HLA-A, –B and –C leaders binding their HLA-C alleles were significantly over-represented in the spontaneous resolver group (CHC 2.00, SR 2.50; p=0.034; Mann Whitney test).

The HLA-C1 group of alleles are predicted to bind inhibitory KIR2DL2, KIR2DL3 and activating KIR2DS2, whereas HLA-C2 group of alleles bind inhibitory KIR2DL1 and activating KIR2DS1. We therefore categorized the data into those individuals possessing a corresponding inhibitory KIR receptor (Figure 5.11 c) or activating KIR receptor (Figure 5.11 d) to their particular HLA-C alleles. For individuals with an inhibitory KIR receptor for their HLA-C group of ligands, those with a greater number HLA-A, –B and –C leader peptides binding their HLA-C alleles were over-represented in the spontaneous resolver group (mean number of binders CHC 2.04, SR 2.50; p=0.04; Mann Whitney test). However, for individuals possessing an appropriate activating KIR receptor for their HLA-C group of ligands, this significance was lost (mean number of binders CHC 2.02, SR 2.34; p=0.35; Mann Whitney test).

HLA-C1 homozygosity is observed at higher frequency in individuals who resolve HCV infection compared with those with chronic infection (Khakoo et al., 2004; Romero et al., 2008), although the effect is limited to individuals also homozygous for the gene encoding the KIR2DL3 receptor. We
speculated that the peptide content of HLA-C1 could influence the outcome of infection, therefore we analysed our data comparing individuals homozygous for the C1 group of alleles (C1C1), heterozygous (C1C2), or homozygous for the HLA-C2 group (C2C2) (**Figure 5.1**e). Individuals with a higher number of HLA-A, -B and -C leader peptides binding their HLA-C alleles were over-represented in the spontaneous resolver group in C1C1 individuals (mean number of binders CHC 2.74, SR 3.78; p=0.011; Mann Whitney test), but not C1C2 individuals (mean number of binders CHC 1.99, SR 1.74; p=0.43; Mann Whitney test) or C2C2 individuals (mean number of binders CHC 0.8, SR 0.3; p=0.58; Mann Whitney test). Furthermore, this protective effect of leader peptide binding at C1C1 was present in individuals possessing either a KIR2DL3 allele (mean number of binders CHC 2.65, SR 3.74; p=0.008; Mann Whitney test) or a KIR2DL2 allele (mean number of binders CHC 2.66, SR 3.96; p=0.03; Mann Whitney test) (**Figure 5.12**). No significant difference in outcome of the C1C1 group was seen between individuals homozygous for KIR2DL3 or those homozygous for KIR2DL2.

Further analysis showed that in our cohort, HLA-C1 group of alleles are more likely to bind HLA-A, -B and -C leader peptides than HLA-C2 group of alleles (mean number of binders C1C1 3.07, C2C2 0.72; p<0.0001; Mann Whitney test), see also **Appendix Figure 9.3.**
Figure 5.11. Number HLA-A, -B and -C leader peptides predicted to bind HLA-C alleles and the outcome of HCV infection

Data from 363 individuals with chronic HCV infection (CHC) and 112 spontaneous resolvers (SR) typed for MHC class I were analysed. HLA-A, -B and -C leader peptides were assigned either a positive or negative binding potential for binding to each individual HLA-C allele according to the NetMHCpan prediction tool (Hoof et al., 2009). (a) The number of predicted binders (2 HLA-C alleles per individual, therefore the potential number of binders varied between 0-12) was then assessed comparing spontaneous resolvers and those who remained chronically infected. Mean number +/-SEM of HLA-A, -B and -C alleles binding HLA-C alleles and results of the comparison by the Mann Whitney test are shown for (b) all data (CHC 363 patients, SR 112 patients); (c) individuals matched for an appropriate inhibitory KIR receptor (CHC 337 patients, SR 111 patients); (d) individuals matched for an appropriate activating KIR receptor (CHC 199 patients, SR 64 patients); (e) subset analysis for individuals possessing C1C1 alleles (CHC 104 patients, SR 49 patients), C1C2 alleles (CHC 178 patients, SR 53 patients) and C2C2 alleles (CHC 55 patients, SR 10 patients). p values calculated using Mann Whitney test.
Individuals homozygous for C1 alleles were subcategorised based on their 2DL2 and 2DL3 alleles. (a) individuals possessing a KIR2DL3 allele (CHC 91 patients, SR 46 patients) or a KIR2DL2 allele (CHC 59 patients, SR 23 patients); (b) homozygous for KIR2DL3 (CHC 45 patients, SR 26 patients) or KIR2DL2 (CHC 36 patients, SR 7 patients). \( p \) values calculated using Mann Whitney test.

Figure 5.12. Number HLA-A, –B and –C leader peptides predicted to bind HLA-C alleles and the outcome of HCV infection: C1C1 subset analysis
5.3. Discussion

Our original peptide mix experiments sought to determine whether HCV core\textsubscript{35-44} could perturb the inhibitory effect of HLA-G\textsubscript{sp} at CD94:NKG2A, analogous to peptide antagonism observed at KIR where HLA-C\textsuperscript{*}0102 bound peptides that induce weak KIR binding depress the inhibitory effect of a strong KIR binding peptide (Fadda et al., 2010). Rather than interfering with CD94:NKG2A binding inhibitory peptides, HCV core\textsubscript{35-44} augments the inhibitory effect of host MHC-class I derived HLA-G\textsubscript{sp}, HLA-A2\textsubscript{sp} and HLA-B7\textsubscript{sp}, a phenomenon we have termed peptide synergy. However, HLA-G\textsubscript{sp} and HCV core\textsubscript{35-44} have an opposite effect at HLA-Cw\textsuperscript{*}0102, consistent with the peptide antagonism model described at KIR. HLA-G\textsubscript{sp} and HCV core\textsubscript{35-44} both bind to HLA-Cw\textsuperscript{*}0102. Whereas HCV core\textsubscript{35-44}:HLA-Cw\textsuperscript{*}0102 inhibits NK cell degranulation via CD158b, HLA-G\textsubscript{sp}:HLA-Cw\textsuperscript{*}0102 has no effect. This observation was confirmed using blocking antibodies, as shown in Chapter 3. In peptide mix experiments, HLA-G\textsubscript{sp} relieved HCV core\textsubscript{35-44} mediated inhibition at KIR2DL2/2DL3. Thus there are fundamental differences in the way in which the KIR and the NKG2A systems respond to changes in the peptide content of MHC class I. Alterations in the peptide repertoire of HLA-E can favour inhibition of NKG2A\textsuperscript{*} NK cells, whereas peptide repertoire changes at HLA-C favour activation of KIR\textsuperscript{*} NK cells.

The original data demonstrating peptide antagonism at KIR used model peptides based on a variant of a TIMP-1 peptide (Fadda et al., 2010). Our findings presented here may represent a biologically plausible mechanism for KIR\textsuperscript{*} NK cell activation. HLA-G\textsubscript{sp} is derived from processing of the MHC class Ib molecule HLA-G, which in health has a tissue distribution limited to the placenta. HLA-G has long been regarded as a tolerogenic molecule, contributing to materno-fetal tolerance through engagement of LILRB-1 and KIR receptors on NK cells, DCs and T cells. However, recently HLA-G has been shown to be upregulated in the serum and liver of patients with chronic HBV and HCV, but not healthy controls (de Oliveira Crispim et al., 2012; Souto et al., 2010; Weng et al., 2011). De Oliveira et al. (2012) observed HLA-G expression in 51% of liver specimens from patients with chronic HCV, but no control liver samples. In view of the known immunosuppressive effect of HLA-G they suggest that expression of this molecule could contribute to viral persistence. Paradoxical to this model, HLA-G expression was more frequent in milder necroinflammatory stages of chronic hepatitis compared to moderate/severe, implying a protective effect of HLA-G.
Furthermore, Cordero et al. have shown that polymorphisms affecting HLA-G influence the susceptibility of HCV infection in patients with sickle cell disease (Cordero et al., 2009). They looked at two polymorphisms in a highly polymorphic promoter region thought to influence microRNA binding, thus regulating RNA turnover and miRNA-mediated repression of translation of HLA-G. The expression of polymorphisms associated with higher levels of HLA-G had a dose dependent protective effect. A protective effect of HLA-G was also seen against influenza virus. At first glance, this idea seems counter-intuitive since HLA-G mediated immune tolerance may be expected to predispose to viral persistence. However, we speculate that it is the expression of HLA-G<sub>sp</sub> not HLA-G that leads to the protective effect. Alterations in the peptide repertoire of infected cells leading to an increase in HLA-G<sub>sp</sub> could theoretically lead to NK cell activation by increasing MHC class I occupancy by antagonist peptide. HLA-G<sub>sp</sub> mediated NK cell activation would be limited to the subset of NK cells that express KIR2DL2/2DL3, possibly in the absence of other receptors that bind HLA-G e.g. LILRB-1. However, given that each individual NK cell expresses a random subset of inhibitory KIRs with distinct MHC-I specificity, antagonism of any one of those KIRs may be sufficient to mount an antiviral response. Therefore increased expression of HLA-G<sub>sp</sub> may represent an attempted protective host response in viral infection.

Immunogenetic analyses have revealed that polymorphism in KIR genes and their HLA-C ligands may offer protection against chronic HCV infection (Khakoo et al., 2004; Romero et al., 2008). Individuals homozygous for KIR2DL3 and its HLA-C1 group of ligands are less likely to develop chronic infection following exposure to HCV. The protective effect of this gene combination may be due to KIR2DL3 binding HLA-C1 ligands with a lower avidity than other inhibitory KIR, thereby reducing the threshold for activation for NK cells that express this specific inhibitory receptor (Fadda et al., 2010; Moesta et al., 2008). Screening of our immunogenetic dataset of individuals exposed to HCV demonstrates no effect of the classical MHC class I leader sequences predicted to bind HLA-E/CD94:NKG2A and the primary outcome of HCV infection. This contrasts to KIR in which KIR2DL3 in combination with its HLA-C ligand is protective (Knapp et al., 2010). Thus at least for HCV, consistent with our <em>in vitro</em> data there is <em>in vivo</em> evidence for HCV being better adapted to CD94:NKG2A than to KIR.
We further analysed our data set HLA-C alleles for their predicted binding to HLA-Gsp. We hypothesised that individuals with a greater number of HLA-Gsp binding alleles would be protected from chronic HCV infection as HLA-G upregulation in these individuals may result in HLA-Gsp functioning as an antagonistic peptide at HLA-C. Although HLA-Cw*0102 binds strongly to HLA-Gsp, this is not true for the majority of HLA-C alleles which are predicted to be weak binders. This may explain why no significant difference was observed between chronic HCV and spontaneous resolvers in the number of HLA-C alleles predicted to bind HLA-Gsp. HLA-Cw*0102 is predicted to bind all classical MHC class I leader peptides. However, other HLA-C alleles vary in their ability to bind these leader peptides. We speculated that individuals who possess a greater number of MHC class I leader sequences that bind to their HLA-C alleles may be more susceptible to chronic HCV infection as their NK cells would be more inhibited compared to those with fewer binders. Paradoxical to our model, individuals with greater numbers of HLA-A, -B and -C leader peptides predicted to bind to their HLA-C molecules are over-represented in the spontaneous resolver group compared to those with chronic HCV infection. Furthermore, this protective effect is restricted to individuals encoding an appropriate inhibitory KIR receptor for their HLA-C group of ligands, and also to those individuals homozygous for HLA-C1 alleles. There are various possible explanations for this observation. The HLA-C1 group of alleles are more likely to bind HLA-A, -B and -C leader peptides than the HLA-C2 group (Appendix Figure 9.3.), therefore there may be a greater expression of HLA-C1 than HLA-C2 during NK cell development. NK cells necessitate interaction of inhibitory receptors with HLA-class I molecules during development to achieve functional status, otherwise known as “licensing” (Kim et al., 2005). It is possible that the quantity of cell surface HLA-C influences NK cell licensing, and therefore influences HCV outcome through factors pertaining to NK cell education. An alternative explanation is that HLA-A, -B and -C alleles function as altered peptide ligands at CD158b. In our experiments presented in Chapter 3, although HLA-A2sp and B7sp bind HLA-Cw*0102, the resulting complexes have lower affinity for CD158b+ NK cells than VAP-FA peptide and could therefore behave as weak inhibitors.

Differences in the stoichiometry of CD94:NKG2A and KIR mediated inhibition of NK cells was also assessed. Our assessment of CD94:NKG2A inhibition relative to MHC class I expression implies that NKG2A+ NK cells are more easily modulated at low levels of HLA-E expression. Accordingly subtle
alterations in HLA-E expression result in a greater change in the fraction of responding NK cells when cell surface levels of HLA-E are low, than when they are high. This is different to inhibition by KIR in which the stoichiometry is linear across the tested range, which in our experiments reflect endogenous MHC levels. We therefore propose that NK cells expressing the NKG2A receptor respond efficiently to changes in the quantity of cell surface MHC class I expression, but not peptide, and those expressing KIR respond more efficiently to alterations in peptide repertoire.

Thus there are fundamental differences in the way in which the KIR and the NKG2A systems respond to changes in the peptide content of MHC class I. Modification of the peptide repertoire of HLA-E can favour inhibition of NKG2A+ NK cells through a mechanism of synergy, but peptide repertoire changes at HLA-C favour activation of KIR+ NK cells. This difference is related to the ability of CD94 to bind to HLA-E:peptide complexes in the absence of the inhibitory signalling moiety NKG2A. Viruses can therefore exploit the tolerance of the HLA-E:CD94:NKG2A system, by generating HLA-E binding peptides to enhance inhibition of NKG2A+ NK cells. The evolution of additional receptor families, either KIR or Ly49, provides the host with a second inhibitory receptor family for MHC class I. Peptide antagonism is a mechanism by which KIR+ NK cell can respond efficiently to changes in peptide repertoire and consequently provide the host with selective advantage over the NKG2A system (Fadda et al., 2010). Indeed NKG2A+, but not KIR2DL2/3+ (CD158b+) NK cells are inhibited by the naturally occurring HCV variant YLLPRRGPKL, and so for this specific peptide there is evidence for host benefit of the KIR over the CD94:NKG2A system.

Most species tested to date have two distinct inhibitory receptor systems for MHC class I, one of which is CD94:NKG2A. In primates there has been an expansion of the KIR locus which is under strong positive selection. The fundamental differences in response to changes in MHC class I level or bound peptide between these two receptor:ligand systems provides a rationale for their maintenance in extant species.
Chapter 6. Final Discussion

NK cells are a pivotal component of the innate immune response, monitoring and defending cells from viruses and tumorigenesis. Their protective role in viral infection is highlighted by reported cases of NK cell deficiency, where viruses that normally cause mild disease instead result in severe and life-threatening illness (Biron et al., 1999). NK cell functions include cytotoxicity and the production of cytokines, which can regulate downstream innate and acquired immune responses in addition to having a direct effect on target cells.

NK cell activity is regulated by the integration of signals derived from a repertoire of activating and inhibitory cell receptors. As activating ligands are frequently expressed on healthy cells, to prevent autoreactivity NK cells receive a dominant inhibitory signal from receptors for MHC class I. These MHC class I specific receptors include the KIR family and CD94:NKG2A. Inhibitory KIRs bind polymorphic HLA-A, -B and -C molecules, with both receptor and ligands showing huge diversity. The consequences of KIR/HLA diversity are still being unravelled, but we now understand that certain polymorphisms may influence the outcome of HIV and HCV infection, autoimmune disease and neoplasia (Carrington et al., 2005; Khakoo et al., 2004; Martin et al., 2002; Martin et al., 2007; Nelson et al., 2004).

KIR receptors are a rapidly evolving, with a diversified family of receptors present in simian primates. Human KIR genes can be divided into two haplotypes which are present in all human populations, suggesting they are maintained by balancing selection (Parham and Moffett, 2013). Haploype A contains almost exclusively inhibitory KIR, compared to haplotype B which has several activating KIR receptors. Activating KIR may be essential in the successful outcome of pregnancy, but KIR2DL3, belonging to haplotype A, has been shown to be protective against chronic viral infection (Khakoo et al., 2004).

In contrast to KIR/HLA-A, B and –C, CD94:NKG2A and its ligand HLA-E have been highly conserved throughout evolution. The fact that CD94:NKG2A has persisted following the generation of a new family of MHC class I specific NK cell receptors suggests that this system offers complementary
functions to KIR. As KIR and their MHC class I ligands are highly polymorphic and segregate on different chromosomes it is possible that an individual may lack an inhibitory receptor-ligand interaction, which could impact on NK cell education subsequent function. HLA-E is found constitutively on the surface of healthy cells but this expression requires the presence of a peptide derived from proteosomal processing of the highly conserved leader sequence of certain MHC-A, -B and -C molecules, and HLA-G (Braud et al., 1998). NK cells can therefore utilise CD94:NKG2A/HLA-E interaction to observe the presence of polymorphic MHC class I molecules and, as a surrogate marker, the cell capacity to process and present these peptides.

The crucial role of NK cells in protecting against viruses is further illustrated by the numerous mechanisms employed by viruses to evade NK cells (Orange et al., 2002). Human CMV (HCMV) encodes for the UL18 protein, a homolog of MHC class I which is capable of binding to β2m and inhibiting NK cells through the LiLRB1 receptor (Chapman et al., 1999; Cosman et al., 1997; Prod'homme et al., 2007). Viruses can escape CTL detection by encoding for proteins that inhibit target cell expression of HLA-A and HLA-B, but by selectively preserving expression of HLA-C and HLA-E they can also suppress the activity of the majority of NK cells through engagement of CD94:NKG2A and KIR, respectively (Bonaparte and Barker, 2004; Cohen et al., 1999; Williams et al., 2002). Several viruses have been shown to encode for peptides that increase expression of HLA-E (Nattermann et al., 2005a; Nattermann et al., 2005b; Tomasec et al., 2000; Ulbrecht et al., 2000; Ulbrecht et al., 1998). This led us to investigate the effect of HLA-E binding viral and host peptides on NK cell activity, and whether these peptides also interacted with HLA-C/ KIR.

Consistent with published observations, we confirmed that peptide sequences encoded by HCV (HCV core\textsubscript{35-44} and its variant HCV core\textsubscript{R9K}), HIV (HIV p24\textsubscript{14-22}), EBV (EBV bzlf\textsubscript{38-47}), and the host encoded stress protein hsp60 derived peptide (hsp60\textsubscript{sp}) all bound to HLA-E, enhancing its cell surface expression on an MHC class I deficient cell line. However, all of these peptides, along with the MHC class I derived signal peptides HLA-G\textsubscript{sp}, HLA-A2\textsubscript{sp} and HLA-B7\textsubscript{sp} also bound to HLA-Cw*0102.

In single peptide degranulation (CD107a) assays we observed that HCV core\textsubscript{35-44}, a HLA-A2 restricted T cell epitope, protected target cells from NK cell degranulation. Blocking antibodies showed that this
effect was mediated by interaction between KIR2DL2/2DL3 (CD158b) receptors and HLA-C/ HCV core\textsuperscript{35-44}. None of our other HLA-Cw*0102 binding viral peptides inhibited the CD158b\textsuperscript{+} population, including, HCV core\textsubscript{R9K}, the only known variant of HCV core\textsuperscript{35-44}, containing a lysine for arginine substitution at P9 (Nattermann et al., 2005a). KIR mediated recognition of HLA-C is influenced by residues 7 and 8 of nonameric peptides (Fadda et al., 2010; Rajagopalan and Long, 1997). Being a decamer, HCV core\textsuperscript{35-44} may have a slightly different conformation in the peptide binding groove such that lysine substitution at position 9 is not tolerated by CD158b\textsuperscript{+} NK cells.

Binding of CD94:NKG2A to HLA-E is also sensitive to the nature of the bound peptide (Michaelsson et al., 2002). CD94:NKG2A usually recognises HLA-E containing MHC class I derived signal peptides (MHC-I\textsubscript{sp}) leading to NK cell inhibition, which we confirmed in our degranulation assays using HLA-A\textsubscript{2}\textsubscript{sp}, HLA-B7\textsubscript{sp} and HLA-G\textsubscript{sp}. Despite binding to HLA-E, none of our viral peptides or hsp60\textsubscript{sp} induced inhibition of the NKG2A\textsuperscript{+} NK cell population in single peptide experiments. However, when target cells were loaded with both MHC-I\textsubscript{sp} and either viral peptide or hsp60\textsubscript{sp}, we observed an increase in NKG2A\textsuperscript{+} cell inhibition compared to inhibition produced with MHC-I\textsubscript{sp} alone. We have termed this observation peptide synergy. Confocal microscopy suggests that HLA-E bound synergistic peptides (HCV core\textsuperscript{35-44}, HIV p24\textsubscript{14-22}, EBV bzlf\textsubscript{38-47} and hsp60\textsubscript{sp}) stimulate the recruitment of CD94 to the immune synapse, in the absence of NKG2A. This is in contrast to HLA-E bound to MHC-I\textsubscript{sp} that induce aggregation of both subunits. In the next part of this chapter we speculate on how CD94 recruitment, likely to be in the form of homodimers, may enhance inhibition at NKG2A.

Ligation of CD94:NKG2A by HLA-E containing MHC-I\textsubscript{sp} triggers the phosphorylation of tyrosine residues within each of the two ITIM motifs attached to the cytoplasmic tail of NKG2A. This permits the recruitment of src homology 2 domain bearing tyrosine phosphatases (SHP)-1 and/or SHP-2 (Borrego et al., 2006; Kabat et al., 2002). Following activation, these phosphatases dephosphorylate the Vav guanine nucleotide exchange factor and ERM (erzin/ radixin/ moesin) family proteins leading to actin cytoskeletal rearrangements which in turn disrupts activation signalling (Borrego et al., 2005; Masilamani et al., 2006). In contrast to NKG2D and many other receptors which are down-regulated upon ligand binding, CD94:NKG2A is continuously renewed at the cell surface (Groh et al., 2002; Maxfield and McGraw, 2004). Given that HLA-E is expressed constitutively on healthy cells this
constant recycling of CD94:NKG2A may serve to guarding against autoreactivity by maintaining a
dominant inhibitory signal. To achieve continuous expression at the cell surface despite binding to
HLA-E, it has been demonstrated that CD94:NKG2A utilises an uncommon means of endocytosis,
termed pinocytosis, which is associated with rapid recycling to the cell surface (Masilamani et al.,
2008). CD94:NKG2A receptor trafficking has been shown to be independent of ligand ligation
(Borrego et al., 2002).

In the absence of HLA-E, CD94:NKG2A receptors are evenly distributed and free to diffuse along the
plasma membrane. On interaction with a target cell bearing HLA-E bound to a MHC class I derived
signal peptide, CD94 and NKG2A accumulate at the contact site between NK cell and target cell,
termed the immune synapse, as supported by our microscopy findings. This localisation of receptors
to the immune synapse is similar to that observed for inhibitory KIR+ NK cells incubated with HLA-C
bearing target cells (Davis et al., 1999; Fadda et al., 2010). Using a fluorescent imaging method,
Sanni et al. demonstrate that the mobility of GFP tagged CD94:NKG2A away from the immune
synapse is markedly reduced following receptor binding to HLA-E (Sanni et al., 2004).

The reduced mobility of inhibitory NK cell receptors at the immune synapse may be a more complex
process than passive binding of MHC class I, as suggested by studies looking at KIR2DL2/ HLA-Cw3
interaction. It has been shown that KIR/ HLA clustering is in the formation of a doughnut surrounding
a central cluster of adhesion molecules such as LFA-1 and ICAM-1 (Davis et al., 1999). The crystal
structure of KIR/ HLA interaction demonstrates a 1:1 stoichiometry (Boyington et al., 2000a).
Boyington and Sun proposed models for how this doughnut is formed at the immune synapse,
centred on KIR/ HLA receptor oligomerisation (Boyington and Sun, 2002). The first model proposes
that D1 domain of one receptor interacts with D2 domain of the neighbouring receptor, as observed in
the crystal structure analysis of KIR2DL2/ HLA-Cw3 complex, leading to receptor oligomerisation
and aggregation (Snyder et al., 1999). The second oligomerisation form was also observed in the crystal
structure of the KIR2DL2/HLA-Cw3 complex (Boyington et al., 2000a). Apart from the functional
interface with HLA-C, each KIR molecule within the crystal lattice makes a Van der Waals interaction,
with a symmetry related HLA-C molecule in a peptide independent manner. The resulting oligomer
maintains a 1:1 receptor ligand stoichiometry while maintaining the same orientation for all the KIR/HLA complexes.

Thus, in addition to accumulating at the immune synapse, inhibitory NK cell receptors may need to be tightly clustered to achieve an inhibitory signal. This model could be used to explain augmented inhibition at NKG2A induced by certain synergistic peptides (Figure 6.1). At low concentrations (1 μM) of MHC-class Isp there may be insufficient HLA-E expression to achieve sufficient CD94:NKG2A immobilisation at the immune synapse, leading to a relatively weak inhibitory signal. Synergistic peptides induce upregulation of HLA-E; however, they may permit recruitment and immobilisation of CD94 (in the form of homodimers) but not NKG2A. As CD94 does not possess a signalling motif, these homodimers alone would be of no functional significance. Though when synergistic peptides are mixed with low concentration MHC-class Isp the recruitment of additional CD94 complexes may lead to multimerisation with CD94:NKG2A aggregates. The resulting immobilisation of CD94:NKG2A/HLA-E/ MHC class Isp enhances the inhibitory signal generated through NKG2A subunits. Furthermore, this effect would be dose related as increased concentrations HLA-E-bound synergistic peptide would promote the recruitment of further CD94 homodimers leading to stabilisation of the NKG2A-containing oligomeric complex. At high concentrations of MHC class Isp there is already sufficient cross linking of CD94:NKG2A due to an abundance of HLA-E, therefore additional CD94 complexes in the form of synergistic peptide induced CD94 homodimers may not further augment the inhibitory signal.

In our model, two potential aggregate interfaces for CD94 are possible, either between adjacent HLA-E molecules, or between adjacent CD94/NKG2A moieties. An aggregation interface independent of HLA-E could theoretically limit lateral movement of receptor subunits within the plasma membrane of resting NK cells, thereby impairing polarisation of CD94:NKG2A during formation of the immune synapse. Therefore the alternative theory that the ligand HLA-E plays a part in oligomerisation may be more attractive, as has been postulated for KIR.
The three cartoons depict the interface between NK and target cell. (a) In the presence of a target cell bearing HLA-E containing a permissive MHC-I signal peptide (MHC-I_sp), there is accumulation of CD94:NKG2A heterodimers at the immune synapse. This functional interface triggers ITIMs in the tail of NKG2A mediating NK cell inhibition. An oligomeric aggregate of CD94:NKG2A is formed with CD94 interacting with either neighbouring HLA-E in a peptide independent manner (aggregate interface 1), or the adjacent receptor (aggregation interface 2). (b) HLA-E bound synergistic peptides permit recruitment of CD94 but not NKG2A, leading to aggregation of non-inhibitory CD94 homodimers. (c) In the presence of both MHC-I_sp and synergistic peptide there is accumulation of functional CD94:NKG2A. The additional CD94 homodimer/HLA-E interaction may facilitate multimerisation of receptor complexes at the synapse through either aggregation interface 1 or 2. This would ultimately lead to immobilisation of CD94:NKG2A and augmented inhibition.
The oligomerisation model may also explain why synergy mediated by HLA-E binding peptides is specific to NKG2A+ cells, and not observed at KIR. HLA-A2\textsubscript{sp} and HLA-B7\textsubscript{sp} also bind to HLA-Cw\textsuperscript{*}0102, inducing inhibition at KIR2DL2/2DL3. Synergistic peptides (other than HCV core\textsubscript{35-44} and hsp60\textsubscript{sp} which bind HLA-Cw\textsuperscript{*}0102 inducing inhibition at KIR2DL2/2DL3) do not augment inhibition at KIR2DL2/2DL3. In this instance, the additional CD94 complexes recruited by the synergistic peptide may be unable to interact with KIR therefore immobilisation is not enhanced and no synergistic inhibition is observed at this receptor group. Alternatively, it is possible the failure of synergy at KIR may have been due to the low concentration of MHC class I signal peptide used. MHC-I\textsubscript{sp} have a low affinity for HLA-Cw\textsuperscript{*}0102, with 1 µM peptide insufficient to inhibit at KIR2DL2/2DL3. To exclude an effect at KIR, further assays looking at CD158b+ NK cell-inhibiting concentrations of MHC-I\textsubscript{sp} in the presence or absence of synergistic peptides could be performed.

This is not the first description of non-signalling NK cell receptors augmenting inhibition at other co-expressed NK receptors. Kirwan and Burshtyn demonstrated that ITIM-deficient KIR can inhibit at ILT-2 (LILRB-1) receptors in a mechanism that is dependent on KIR/ HLA-C interaction (Kirwan and Burshtyn, 2005). Using blocking antibodies, they show that the inhibitory effect mediated by ITIM deficient KIR requires the interaction between the relatively conserved α3 domain of MHC-class I and LILRB-1. Synergistic inhibition observed at CD94:NKG2A is independent of LILRB-1, as shown in our experiments using blocking antibodies. The discrepancy may be related to the fact that LILRB-1 does not bind HLA-E, whereas is shares ligands with KIR, binding a broad range of MHC class I ligands, including HLA-A, -B, C and HLA-G (Chapman et al., 1999; Cosman et al., 1997; Fanger et al., 1998; Shiroishi et al., 2003). Therefore ITIM-deficient KIR may induce aggregation of MHC-class I enabling engagement with LILRB-1 contributing to receptor oligomerisation.

Antagonism at KIR offers a discrepancy with our model, suggesting there may be a difference between how these two receptor systems operate. It has been shown that two variants of the TIMP-1 peptide, VAP-FA (VAPWNSFAL) and VAP-DA (VAPWNSDAL) both upregulate HLA-Cw\textsuperscript{*}0102 expression on MHC deficient target cells. However, whereas HLA-Cw\textsuperscript{*}0102\textsubscript{VAP-FA} inhibits at CD158b,
HLA-Cw*0102_{VAP-DA} does not. Although HLA-Cw*0102_{VAP-DA} does not induce inhibition of KIR^+ NK cells, imaging studies show that it still promotes KIR clustering at the immune synapse comparable to HLA-Cw*0102_{VAP-FA}. However, when VAP-DA is mixed with VAP-FA, it interferes with VAP-FA mediated inhibition at CD158b, termed peptide antagonism. Therefore, in the case of these particular peptides, the additional KIR/HLA-C complexes induced by VAP-DA do not augment the inhibitory effect at KIR. This would suggest that the receptor oligomerisation model for inhibition does not apply to all situations. This could pertain to downstream differences beyond extracellular receptor oligomerisation. It has been demonstrated that HLA-Cw*0102_{VAP-DA} induces KIR recruitment of SHP-1 but fails to induce an inhibitory signal (Borhis et al., 2013; Fadda et al., 2010). However, in peptide mix conditions, HLA-Cw*0102_{VAP-DA} may uncouple inhibitory signalling induced HLA-Cw*0102_{VAP-FA} by competing for SHP-1 recruitment, leading to an antagonistic effect. As CD94 homodimers do not possess and intracellular domain, such downstream effects may not be relevant.

In our study, HLA-G_{sp} increased cell surface HLA-Cw*0102 without inducing inhibition at CD158b^+ NK cells. In peptide mix experiments, HLA-G_{sp} was able to relieve CD158b^+ NK cell inhibition mediated by HCV core_{35-44}. Thus, this pair of peptides which synergise to inhibit NK cells at CD94:NKG2A have contrasting interactions at KIR, where antagonism may serve to activate NK cells. HLA-G has recently been found to be upregulated in chronic HCV, where it is associated with lower necroinflammatory scores (de Oliveira Crispim et al., 2012). Additionally, in a high risk population, polymorphisms associated with potentially higher levels of HLA-G are found more frequently in exposed uninfected individuals compared to those with chronic HCV (Cordero et al., 2009). This may be surprising given that HLA-G is regarded to promote NK cell tolerance, through interaction with LILRB-1. We propose that HLA-G_{sp}, a by-product of HLA-G expression, could contribute to this protective effect and represent a protective host response to viral infection. Increased HLA-G_{sp} expression could activate CD158b^+ NK cells by functioning as an altered peptide ligand leading to antagonism of other HLA-Cw*0102 bound peptides.

While this may represent a plausible mechanism, as with all of our peptide work, one must consider peptide abundance and affinity. Antagonism of HCV core_{35-44} mediated inhibition required relatively high (>25 μM) concentrations of HLA-G_{sp}. In our assays, we used external peptide loading of an
MHC deficient cell line. It is possible that the intracellular loading pathway is more efficient at presenting peptides and therefore requires smaller quantities. This could be assessed by measuring HLA-Cw*0102 expression in cell line transfected with HLA-G and HLA-Cw*0102. Furthermore, HLA-Gsp appears to have lower affinity for HLA-Cw*0102 than TIMP-1 derived peptides (TIMP-1 being the most abundant indentified peptide eluted from HLA-Cw*0102 expressing 721.221 cells) (Barber et al., 1996; Fadda et al., 2010). The same reasoning may be applied to HLA-E bound peptides. It is possible that abundance of MHC-Isp may limit the range of peptides presented by HLA-E. This may be true under physiological conditions; however, in cases of viral infection downregulation of MHC-class I, particularly –A and –B, have been described. In our MHC class I stabilisation assays, HLA-B7sp and HLA-A2sp have a higher affinity for HLA-E compared to our virus derived peptides and hsp60sp. Down-regulation of these MHC class I molecules may enable HLA-E to bind and present peptides derived from other sources. Indeed, cell surface HLA-E expression has been demonstrated to be increased on hepatocytes that are HCV core positive compared to those that are HCV core negative, and is also upregulated on lymphocytes that are HIV p24 positive compared to those that are HIV p24 negative (Nattermann et al., 2005a; Nattermann et al., 2005b). To establish the physiological relevance of our findings, future experiments looking at cells transfected with various viruses could be performed.

Our finding that viral peptides can synergise with endogenous peptides to increase inhibition is novel and has implications for viral evasion. Based on our experiments, one could speculate that viruses may be better adapted to escape CD94:NKG2A mediated surveillance. Given that changes in peptide repertoire at HLA/ KIR can favour activation of NK cells, this system may offer a selective advantage in fighting infection. This theory if further supported by our finding that MHC class I polymorphisms that could affect peptide repertoires of HLA-C influence the outcome of HCV infection, whereas those that affect HLA-E/ CD94:NKG2A do not. This would lend support to current opinion that the evolution of KIR/HLA is driven by pathogen mediated selection.
Chapter 7. Conclusion

In this thesis, we have examined the peptide selectivity of inhibitory NK cell receptors that interact with MHC class I to regulate NK cell activity. Using a TAP-deficient cell line we have loaded exogenous peptides to enhance cell surface MHC class I expression. Certain non-inhibitory viral and host derived peptides that bind HLA-E can synergise to inhibit NKG2A⁺ NK cells when combined with an inhibitory self-peptide. Confocal microscopy showed these synergistic peptides promote the selective recruitment and clustering of CD94, but not NKG2A at the immune synapse in both an NKL cell line and primary NK cells. Using a CD94 transfected/ NKG2A negative cell line we confirmed that HLA-E bound synergistic peptides induce clustering of CD94. Although synergy is seen for several peptides, there remain features of peptide selectivity. Position 5 lysine substitution abrogated the synergistic effect of these peptides in degranulation assays, accompanied by a loss of CD94 clustering.

These findings provide new insight into the participation of HLA-E-binding peptides to CD94-mediated inhibition of NK cells. Although cell surface CD94 homodimers have previously been described, no biological role independent of NKG2 molecules has been identified. This study reveals a novel mechanism for NK cell regulation by demonstrating that in the absence of direct signalling capacity, CD94 molecules can contribute to the inhibition of NKG2A⁺ cells. CD94:NKG2A is upregulated in several viral infections, such as HIV and HCV. Therefore, viruses could be better adapted evade NK cells through this receptor ligand system.

Important directions for future studies would aim to determine the biological relevance of our findings. Cell lines could be transfected with HLA-E, along with HCV core and other MHC class I, to determine whether peptide synergy occurs in an endogenous peptide loading model. All of the PBMC/ primary NK cell experiments presented in this thesis were derived from uninfected (healthy control) donors. It would be interesting to investigate how NK cells from individuals with chronic HCV compare to healthy control NK cells in their response to peptide combinations. The higher level of CD94:NKG2A found on HCV-NK cells may mean that they are calibrated differently. These experiments could lead to greater understanding of how viruses can modulate NK cell activity.
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Figure 9.1. Predicated binding of HLA-G<sub>sp</sub> peptide to HLA-C alleles

HLA-G<sub>sp</sub> (VMAPRTLFL) was assigned either a positive (lighter green boxes) or negative (darker green boxes) binding potential for binding to each individual HLA-C allele according to the NetMHCpan prediction tool (Hoof et al., 2009). nM IC 50 binding affinities shown for binders.
<table>
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<th>HLA-C allele</th>
<th>VMAPRTLLL</th>
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<th>VMAPRTLVLL</th>
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**Figure 9.2. MHC class I leader peptide binding to HLA-C alleles**

HLA-A, -B and -C leader peptides were assigned either a positive (lighter green boxes) or negative (darker green boxes) binding potential for binding to each individual HLA-C allele according to the NetMHCpan prediction tool (Hoof et al., 2009). nM IC 50 binding affinities shown for binders.
### Figure 9.3. Comparison between the number of HLA-C1 and HLA-C2 group alleles predicted to bind MHC class I signal peptides

Data derived from Figure 9.2.

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