Major Histocompatibility Complex Class I
As a Ligand for Natural Killer Cell Recognition

by

Ruey-Chyi Su

A thesis submitted in conformity with
the requirements for the degree of Doctor of Philosophy

Graduate Department of Medical Biophysics,
Ontario Institute for Studies in Education of the University of Toronto

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ABSTRACT

Major Histocompatibility Complex Class I
as a ligand for Natural Killer Cell Recognition

Degree of Doctor of Philosophy, 2000
Ruey-Chyi Su
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Natural killer cell (NK) -mediated cytotoxicity involves two families of receptors: activating receptors that trigger lysis of the target cells being recognized and inhibitory receptors specific primarily for Major Histocompatibility Complex Class I (MHC-I) on the target cell surface that can override the activating signal. Cells expressing reduced surface MHC-I have been shown to be targets for NK-mediated cytotoxicity. MHC-I molecules on the cell surface can be classified into molecules made stable by the binding of high affinity peptide (pH-MHC-I) or unstable molecules potentially capable of being stabilized by binding high affinity peptide (peptide receptive, PR-MHC-I). It has been previously shown that the Ly49A inhibitory receptor recognizes pH-D^d. Our data suggest the existence of other NK inhibitory receptor(s) recognizing PR-K^b, -D^b, -D^d, or -K^d. During the search for such receptor(s), we found that the inhibitory receptor Ly49C^B6 recognizes PR-K^b but does not recognize K^b once they have bound high affinity peptide. Furthermore, we have measured the stability of surface pH-K^b and PR-K^b molecules in the presence of BFA. pH-K^b has a t_{1/2} of \( \sim 45\pm 3\)h and surface PR-K^b has a t_{1/2} of approximately 30\pm 4 min at 37°C.

These observations suggest a possible explanation as to why some virus infected cells become targets for syngeneic NK cells even without a major reduction in total surface MHC-
I expression. Our recent study on cells infected with adenovirus types 5 (Ad5) or 12 (Ad12) shows that Ad5 infected cells lose surface PR-K^b expression and become sensitive to NK-lysis within 9h post-infection while Ad12 infected cells have normal PR-K^b expression and remain resistant to NK-lysis until 42h post-infection. Furthermore, the total K^b expression remains unaltered on cells infected by either Ad5 or Ad12. These observations agree with our hypothesis and further suggest that recognition of PR-MHC I may be one of the mechanisms employed by NK cells to detect viral infection.
This thesis is dedicated to my family:

Grandpa & Grandma

my dad, Wei-Jei & my mom, Chun-Yu

and my naughty sisters,

Grace, Eva, and Yi-Ting
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ABBREVIATIONS

<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>α chain</td>
<td>α chain of MHC-I</td>
</tr>
<tr>
<td>β2m</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>Ad5</td>
<td>adenovirus type 5</td>
</tr>
<tr>
<td>Ad12</td>
<td>adenovirus type 12</td>
</tr>
<tr>
<td>AIRL</td>
<td>antibody induced redirected lysis</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BPV</td>
<td>bovine papilloma virus</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CByB6 F1</td>
<td>(BALB/c x B6)</td>
</tr>
<tr>
<td>x% CM</td>
<td>complete medium (α-MEM, 50mM 2-ME, x% FCS)</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DAP (KARAP)</td>
<td>killer-activating receptor associated protein</td>
</tr>
<tr>
<td>E1A</td>
<td>Early transcribed genes encoded by adenovirus</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>E/T or E:T</td>
<td>effector to target ratio</td>
</tr>
<tr>
<td>F(ab')2</td>
<td>antigen-binding fragment of Ig</td>
</tr>
<tr>
<td>Fc</td>
<td>crystalline fragment of Ig</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLICE (Caspase 8)</td>
<td>FADD (Fas-associated death domain)-like ICE (IL-1β converting enzyme)</td>
</tr>
<tr>
<td>v-FLIPs</td>
<td>Viral FLICE-inhibitory proteins</td>
</tr>
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Flu-NP: $D^b$-restricted epitope of Influenza Nucleoprotein, ASNENMETM

Flu-NP-$K^d$: $K^d$-restricted epitope of Influenza Nucleoprotein, TYQRTRALV

GAD-Flu-NP: Flu-NP with 3 additional amino acids added to the N-terminus, GADASNENMETM

H-2: MHC class I molecules

HCMV: human cytomegalovirus

HIV: human immunodeficiency virus

HIVp: $D^d$-restricted epitope of HIV gp160, RGPGRAFVTI

HLA: human leukocyte antigen (human MHC)

HSV: herpes simplex virus

IFN-$\gamma$: interferon gamma

Ig: immunoglobulin

IL-2: Interleukin 2

ILT: Immunoglobulin-like transcript

iNOS: inducible nitric oxide synthase

ITAM: immunoreceptor tyrosine-based activating motif

ITIM: immunoreceptor tyrosine-based inhibitory motif

JAK: Janus kinase

KAR: killer cell activating receptor (human)

KIR: killer cell inhibitory receptor (human)

LAK: lymphokine activated killer cell

LAIR-1: leukocyte-associated immunoglobulin-like receptor-1

LCMV: lymphocytic choriomeningitis virus

LFA-1: leukocyte function-associated antigen-1

LGL: large granular lymphocyte

LIR-1: leukocyte Ig-like receptor

LMP: low molecular mass protein subunits of proteasomes

LN: lymph nodes

mAb: monoclonal antibodies
MCMV  murine cytomegalovirus
MFI   mean fluorescence intensity
MHC-I class I major histocompatibility complex
MHC-II class II major histocompatibility complex
MICA MHC class I chain-related gene A
MICB MHC class I chain-related gene B
NK   natural killer cell
NKC natural killer cell gene complex
NKIR natural killer inhibitory receptor
NO   nitric oxide
NWNA nylon wool non-adherent
OVAp K\textsuperscript{b}-restricted epitope of Chicken Ovalbumin, SIINFEKL
OVAp\textsubscript{K-bio} biotinylated OVAp, SIINFEK(bio)L
OVAp\textsubscript{X-bio} biotinylated OVAp, bio-XSIINFEKL
p peptide
PBL  peripheral blood lymphocyte
PBS  phosphate buffered saline
PE   phycoerythrin
pH  MHC-I specific peptide with high binding affinity
pL  MHC-I specific peptide with high binding affinity
pM  MHC-I specific peptide with high binding affinity
PR  peptide-receptive
PR-MHC-I peptide-receptive MHC-I
SA   streptavidin
SA-PE SA conjugated with phycoerythrin
SH2  Src-homology domain 2
SHP  SH2-containing tyrosine phosphatase
SCID severe combined immunodeficiency
SJL  SJL/J
TAP  Protein product encoded by transporter gene associated with antigen presentation
TCR  T cell receptor
TGF-β  transforming growth factor-beta
TNF-α  tumor necrosis factor type alpha
TM  transmembrane
Tum  L<sup>d</sup>-restricted epitope, ISTQNHRALDLVAAK
VLA-4  very later activating molecule -4
VSVp  K<sup>b</sup>-restricted epitope of Vesicular Stomatitis Virus nuclear protein, RGYVYQGL, (VSVp<sub>52-59</sub>)
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CHAPTER 1

INTRODUCTION
1.1 NK CELLS - HISTORICAL OVERVIEW

Natural killer (NK) cells were first functionally defined in the mid-1970s by their ability to mediate MHC-unrestricted cytotoxicity against some tumour cells in vitro (1, 2); (3). However, this definition has offered no means to isolate this small population of lymphocytes (~5% of splenocytes) for detailed immunological analysis. A better operational definition of NK was reached at the Fifth International Workshop on Natural Killer Cells in 1988 (4). NK cells were defined as “large granular lymphocytes that do not express on their surface the CD3 antigen or any of the known T cell receptor chains (α, β, γ, or δ) but do express CD16 and NKH-1 (Leu-19) cell surface markers in humans and NK1.1/NK2.1 in mice and mediate cytolytic reactions even in the absence of MHC-I or MHC-II expression on the target cells.” With this definition, the understanding of NK cells rapidly progressed.

Three major lines of research were undertaken to study how NK cells recognize their target cells. First, a general “survey” of NK-sensitive and -resistant target cells was made in an attempt to formulate a testable hypothesis for NK recognition. It was found that NK cells could kill tumour cell lines that have no or only low levels of MHC-I (5-8). These observations, together with the findings that NK cells reject both allogeneic cells (including lymphoma and bone marrow grafts) and semi-allogeneic cells (as in F1-hybrid anti-parent resistance) (9, 10), led Kärre et al. to hypothesize that NK cells recognize and eliminate cells that fail to express self-MHC-I (the “missing self” hypothesis) (11).

The second line of research involved the search for NK target antigen(s), based on the assumption that NK cells recognize and are activated by a specific foreign antigen on the target cell surface. To this end, foreign antigens encoded by endogenous murine retroviruses (12, 13), membrane-associated glycoproteins from NK-sensitive targets (14), transferrin receptor (15, 16), and MHC-I (specifically H-2D^d, (17)) were identified as putative target structures and shown to activate NK cells. However, none of the above could be shown to be expressed universally on all NK-sensitive target cells. Perhaps, the “target structures” which trigger NK cytotoxicity are diverse and varying from one target cell type to another.

The third approach was the search for NK receptors. The first NK activation receptor studied was the receptor for Fc of IgG type IIIA (FcγRIIIA or CD16) (18). Through CD16, NK cells recognize IgG antibody-coated target cells and mediate antibody-dependent
cytotoxicity (ADCC) (19). In the absence of antibodies, CD16 could not induce NK cytotoxicity; therefore, the MHC-unrestricted cytotoxicity against tumour cells appears to involve other NK receptors that could trigger NK lysis in the absence of antibodies. In 1992, Karlhofer et al. made the seminal finding that interaction of Ly49 (now, Ly49A) with classical MHC-I molecules, (H-2D\textsuperscript{d} or D\textsuperscript{k}) delivers an inhibitory signal to NK cells, thus acting as an inhibitory NK receptor (20). Since then, other NK receptors (activation or inhibitory receptors) have been reported in mice and humans (21-25). The recent characterization of these receptors seems to suggest that NK recognition is a balance between activation and inhibitory signals and that the inhibitory receptors seem to play a major role in the specificity of NK recognition (26).

Despite our knowledge of NK receptors, their ligands and the mechanism of how these ligands are implicated in NK recognition remains poorly understood. Most known ligands recognized by NK inhibitory receptors are MHC-I molecules encoded by different alleles (22, 27, 28), seeming to provide strong support for Kärre’s “missing-self” hypothesis. However, many studies have shown no inverse correlation between MHC-I expression and NK sensitivity (29-31). Tumour cell lines expressing normal level of MHC-I molecules have been shown to be NK targets (31); and others with a low level of MHC-I expression have been shown to be resistant to NK lysis (29, 30).

The “missing-self” hypothesis did not take into account the structural basis of MHC-I molecules. With growing knowledge about MHC-I, it is now known that MHC-I can be found in four different forms on the cell surface, each possibly possessing a different conformation (32-35). Several attempts have been made to investigate which region(s) and which form(s) of MHC-I are important in NK-recognition. By performing exon-shuffling and point-mutation experiments on human MHC-I, Storkus et al. showed that the α1-α2 region of the α chain appears to be critical in determining the specificity of MHC-I as an inhibitory ligand (36), and that the amino acids in the peptide binding groove of MHC-I are important in conferring NK resistance (37). The addition of peptide that could bind to the protective MHC-I results in the lysis of target cells.

For mouse MHC-I, Chadwick and Miller (38), and Chadwick et al. (39) found that normal non-transformed lymphoblasts could be lysed by syngeneic mouse NK cells in the presence of peptide specific for their MHC-I, suggesting that binding of MHC-I-specific
peptide removes NK inhibitory ligand(s). On the contrary, the prototypic NK inhibitory receptor, Ly49A was shown to recognize peptide-bound MHC-I, H-2D\textsuperscript{d}. Furthermore, Karlhofer and (20) and Sundbäck et al. (40) have mapped the determinant recognized by the inhibitory receptor Ly49A to the α2-region of the D\textsuperscript{d} molecule.

There are more puzzles than answers in how MHC-I is involved in regulating NK functions; for example, both Ly49A (an inhibitory receptor, (17, 41)) and Ly49D (an activating receptor, (42)) recognize H-2D\textsuperscript{d} in the same animal. Many of the ligands for inhibitory and activating receptors have not yet been identified; more work is required to better characterize the identified ligands. Until we have a better understanding of the structural basis for the interaction between inhibitory receptors and MHC-I molecules, how NK cells distinguish normal autologous cells from abnormal transformed cells remains in shadow.

1.2 MISSING-SELF HYPOTHESIS

The idea of self-recognition existed before the discovery of NK cells; several families of flowering plants employ the self-recognition of polymorphic products in the control of self-fertilization. The most inspiring biological creature in relation to missing-self recognition was the colonial tunicate Botryllus. This organism attempts to transplant itself to other members of the species as a part of its daily lifestyle. By the end of the 1970s, it was already clear that the success of this colony-fusion was controlled by a single, highly polymorphic locus. Sharing of a self-allele in this locus was sufficient to prevent rejection and promote fusion between two Botrylli. At the germ cell level, this led to the prevention of fusion, thus promoting outbreeding of this hermaphroditic species. Clearly, the idea that recognition of the self-allele of a highly polymorphic locus can be used to prevent a cellular interaction has occurred to many organisms. In the case of NK recognition, Kärre hypothesized that recognition of the self-allele of MHC-I (a highly polymorphic locus) is used to prevent NK-mediated lysis of the cell (11) (Fig 1-1).

It is well established that lysis of virally infected or transformed cells by cytotoxic T cells (CTL) requires that both effector and target share identical MHC-I alleles, a phenomenon known as "MHC restriction" (43). However, it was clear that NK cells could kill tumour cell lines differing in MHC-I, or even with no or low level of MHC-I (44).
Together with the observation that NK cells from F1 (AxB) mice are capable of rejecting an infusion of either parent (A or B) cells, a phenomenon known as hybrid resistance (45, 46), suggested that NK cells used an alternative strategy (different from T cells) to discriminate between normal and aberrant cells (Fig1-2). In an attempt to seek out situations in which NK cells did not kill or reject, Kärre compared all the disparate resistant targets and found that high levels of a complete set of autologous MHC-I was a common denominator for several resistant targets. The "missing-self hypothesis" is then established that NK cells recognize and eliminate cells that fail to express self-MHC-I (32, 47) (Fig 1-1). While T cells recognize "foreign" antigenic peptides presented by a specific MHC, NK cells recognize target cells that have reduced MHC-I expression. Consequently, these would be a backup system for the detection of cells that fail to present antigen to T cells by virtue of their reduced MHC-I expression. For example, several viral proteins have been shown to down-modulate MHC-I expression (48-51). This hypothesis not only provided a conceptual framework for the study of NK recognition, it also provided testable predictions for how MHC-I might influence NK sensitivity.

Although the "missing-self" hypothesis has today found its molecular substrate in receptors identified in mice, rats, and men, which are expressed preferentially on NK cells, and which inhibit NK effector function when bound to certain MHC-I (23, 27, 52), there are observations that cannot be explained by this hypothesis (29-31). More knowledge on how NK cells are triggered and a better characterization of NK ligands, specifically the structural basis of MHC-I in recognition, may complement the existing "missing-self" hypothesis in elucidating the mechanism of NK recognition.

1.3 CLASSICAL MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I
1.3.1 Overview

It was Peter A. Gorer who in 1937 was the first to demonstrate a histocompatibility antigen, which led to the discovery of the H-2 histocompatibility complex in mice. Later a similar complex was found in man, called HLA (first defined on human leukocytes, hence the name human leukocyte antigens), and in many other animals. Together they are called the major histocompatibility complex (MHC) because the corresponding antigens are major
Figure 1-2

Hybrid-Resistance Hypothesis

TARGET

No Lysis

Lysis

Lysis

MHC-I (A) [Parent A]

MHC-I (AxB)

MHC-I (B) [Parent B]

NK
histocompatibility antigens (i.e., they induce strong alloimmune responses and are mainly responsible for rejection of allografts).

It became quickly accepted that MHC antigens were not created solely to embarrass transplantation surgeons. Their biological function remained, however, an enigma until the early 1970s, when their important role in antigen recognition by T cells was discovered, particularly through the work of Doherty and Zinkernagel (43). In 1990s, the detailed study of T cell receptor, and the solution of the crystal structure of peptide-MHC-I binary complex collectively yielded some important insight into the role of MHC-I as informers for T cells. In 1985, the role of MHC-I in innate immunity, independent of T cells, was proposed (47). And recently, it was confirmed that MHC-I is involved in NK recognition through the identification of NK receptors recognizing MHC-I (53).

A short summary of our present knowledge of the structure and function of the peptide-presenting ("classical") MHC molecules is given here to aid the readers to better comprehend this thesis. No attempt will be made to cover completely the vast amount of literature in this area.

1.3.2 MHC-I gene location and structure

The major histocompatibility complex (MHC) is a cluster of genes spanning ~4 million DNA base pairs (54, 55). The entire MHC complex comprises at least three known and functionally distinct classes of genes as indicated by the roman numerals I-III. Gene products involved in antigen processing (TAP-1, -2 peptide transporter, LMP proteasome subunits) (56) and many other gene products that have roles (other than processing and presentation of antigens) in the immune system are also encoded by the MHC complex (Fig 1-3).

MHC-I consists of a transmembrane, heavy chain (α, ~45kDa), encoded by genes present on chromosome 6 in human (HLA-A, HLA-B, and HLA-C) or chromosome 17 in mice (H-2K, H-2D, H-2L). The α chain associates non-covalently with a light chain (~12kDa) known as β2-microglobulin (β2m) encoded on chromosome 15 in humans and chromosome 2 in mice (55) (Fig 1-4). This α-β2m complex then associates non-covalently with a short peptide (usually 8 to 10 amino acids long) derived from cellular proteins or foreign antigen (57). α chains are highly polymorphic (58), and most of the polymorphic residues are located in the two extracellular domains, α1, and α2, which form the peptide
Figure 1-3  Schematic map of the human, mouse and rat MHC complexes. This diagram shows the relative position of the principle class I and class II genes of human, mouse and rat as well as several additional MHC-linked genes believed to be involved in antigen processing and presentation (From Fundamental Immunology, 4th Ed).
Figure 1-4  A cartoon of MHC-I heterotrimer.
binding groove, giving different allelic forms of these MHC molecules the ability to bind distinct arrays of peptides (59) (Fig 1-4). Peptide binding to MHC-I is primarily determined by "anchor" residues, i.e., residues of the peptide whose side chains are buried in pockets within the MHC peptide-binding groove. For most MHC-I, the peptides have two primary anchor residues, one at the carboxyl terminus and one at an internal residue whose position varies depending on the identity of the class I allele (60-63). In general MHC-I optimally bind peptides that are 9 amino acids (aa) in length; however, peptides of up to 33 aa have been found to be associated with some MHC-I (64, 65).

1.3.3 Model of MHC-I synthesis and transport
Most nucleated cells express MHC-I. Subunits of MHC-I (i.e., α, β2m, and peptide) are assembled in the endoplasmic reticulum (ER) (Fig 1-5). Peptides associated with α are mainly derived from intracellular proteolytic degradation in the cytosol and delivered to the ER by the transporter associated with antigen processing (TAP) (54, 55). Many molecules are involved in the assembly of MHC-I and some of them are targets of viral polypeptides which retard or prevent the expression of MHC-I (56, 66, 67). The exact mechanism and molecules involved in MHC-I assembly are yet to be definitively defined. Below is a brief summary of current knowledge of MHC-I synthesis and transport.

During synthesis, α chain of MHC-I is extruded into the ER but remains anchored to the ER membrane (55). In mouse and human, α chain associates with calnexin soon after its synthesis, via interactions both with the immature glycan and with residues in the transmembrane (TM) domain of α chain. In mouse cells, β2m binds to α chain while the latter is bound to calnexin; whereas, in human cells, it appears that β2m binding displaces calnexin (68), and the resulting α-β2m binds instead to calreticulin (69). Calnexin is an ER resident type I transmembrane protein that associates transiently with a large number of glycoproteins such as newly synthesized subunits of TCR, Ig, and MHC-I and -II. The precise role of calnexin in the expression of MHC-I is unclear; calnexin is not essential for the assembly of mouse or human α-β2m heterodimer. However, calnexin might, rather, have an indirect role. Co-expression of calnexin with free α chains was found to protect them from rapid intracellular degradation. The t½ was extended 4 or 5 fold (68). Calnexin has been proposed to retain assembled intermediates of MHC-I in the ER. In β2m-deficient cells,
Figure 1-5. A cartoon of MHC-I synthesis and transport. (From Dr. D. Williams's Lecture Note, 1995)
calnexin was found to remain in association with free $\alpha$ chains that accumulate intracellularly.

Within 10 min after $\alpha$ chain assembles with $\beta_2m$, $\alpha$-$\beta_2m$ heterodimer can be found in association with TAP (70). TAP is a heterodimeric complex of TAP1 and TAP2, encoded by MHC-linked genes. Both TAP1 and TAP2 are membrane-spanning proteins with an ATP binding cassette and their function is to translocate peptides into the ER lumen (71) (72). TAP has been found to display a dramatic preference for peptides of 7-13 amino acids in length, closely matching the 8- to 11-amino acid length typically preferred by MHC-I. Shorter peptides appear not to be transported; longer peptides appear to be transported with less efficiency. Peptides not trapped by MHC-I in the ER have very short half-lives. Rapid turnover of these peptides could result either from a putative ATP-independent pump that returns peptide to the cytosol or from ER-degradation pathways. TAP also displays some sequence preference. In human, TAP preferentially excludes peptides with a C-terminal proline and possibly also glycine. In mice, TAP is more restrictive and only transports peptides with hydrophobic C-terminal residues (73). Note that all known mouse classical MHC-I bind peptides with hydrophobic C-terminal amino acids. By contrast, in humans, the more permissive TAP accommodates several HLA alleles that preferentially bind peptides with positively charged C-termini.

In TAP-deficient mouse cells, calnexin dissociates very slowly in parallel with the slow transport of $\alpha$-$\beta_2m$ heterodimers in these cells. $\alpha$-$\beta_2m$ heterodimers are unstable on the cell surface and have a $t_{1/2}$ of much less than 30 min compared to the $t_{1/2}$ of peptide-bound $\alpha$-$\beta_2m$ (assuming the peptide has a high-binding affinity) (34, 74), which is usually greater than 4h (75, 76). Consequently, TAP-deficient cells have a lower level of MHC-I expression and the MHC-I are usually not recognized by the T cell receptor (77).

Proteasomes are important contributors to the MHC-I antigen presentation pathway. The 26S proteasome complex present in the cytoplasm consists of a 20S proteasome catalytic core and a 19S regulatory particle (78). This structure is thought to be primarily responsible for the degradation of cytosolic proteins and has been implicated in the generation of peptides for the class I antigen presentation system. The 26S proteasome is integrally involved in ubiquitin-dependent processing of polypeptides (78), and ubiquination of intact antigens enhances their efficiency of presentation (79). When peptide aldehyde inhibitors
inhibit both the 20S and 26S proteasomes, MHC-I presentation is also inhibited (80, 81), as also seen in TAP-deficient cells. The overall composition of the central (β subunit) rings within the 20S-proteasome complex is controlled by the cytokine IFN-γ. IFN-γ up-regulates the transcription of LMP-2 and LMP-7 genes, located within the MHC-II region adjacent to the TAP-1 and TAP-2 genes and LMP-10 (82-84). The increased incorporation of LMP-2, -7 and -10 β subunits into proteasomes by IFN-γ results in the reduced presence of 3 other constitutively produced β subunits in the complex. As the consequence of the replacement, heterogeneous proteasome structures with different catalytic specificity were observed (84). Therefore, IFN-γ induction of cells could have a major impact on the types of peptides produced. Cumulatively, these reports implicate the proteasome as the predominant cytoplasmic structure responsible for the generation of MHC-I-restricted peptides.

The ATP-dependent delivery of peptides to the TAP-HC-β2m complex results in the release of MHC-I from TAP. The release is peptide-specific and is dependent on the MHC-I involved. The rate of release from TAP also mirrors the release of p-HC-β2m complex into the secretory pathway (85).

Recently, a TAP-independent mechanism of MHC-I assembly in human has been discovered with the identification of an ER residential protein, Jaw1 (86). Jaw1 lacks an NH2-terminal-signal sequence, and is inserted into the membrane post-translationally by a hydrophobic TM region. It consists of a large cytosolic domain of several coiled coils, a TM region, and a 35aa lumenal tail. It can efficiently deliver a carboxy terminal-antigenic peptide to MHC-I in TAP-deficient cells or cells in which TAP is inactivated by the ICP47 protein. Expression of Jaw1 is limited to hematopoietic cells. The findings demonstrate two novel routes of antigen processing: 1) highly efficient peptide-liberation from the carboxy termini of membrane proteins in the ER, and 2) delivery of a cytosolic protein to the ER.

1.3.4 Binding of peptide and MHC-I conformation

The α chain of MHC-I forms a peptide-binding cleft in its membrane-distal domain, where the walls consist of two α-helices and the floor is a β-pleated sheet (Fig 1-6). The structural features necessary for peptide binding are fully contained in the α1/α2 region, as an MHC-I missing the entire α3 domain shows neither changes in α1/α2 conformation nor altered
Figure 1-6  Color ribbon representation of HLA-A. (A) Side view. (B) Top View. (From Fundamental Immunology, 4th Ed.).
peptide binding. The peptide-binding cleft of MHC-I has closed ends, and bind short peptides (p). In contrast, the peptide-binding cleft of MHC-II has open ends and therefore, can accommodate longer peptides of 12-15 aa. Peptides are mainly bound in the cleft of MHC-I by hydrogen bonds between main-chain atoms along the peptide and MHC-I residues in the peptide-binding cleft. High affinity binding of peptides is mediated through specific anchor residues, bound in complementary MHC-I pockets (59-61, 87, 88). Anchor residues differ with regard to either the position of the anchors within the peptide or the chemical nature of the residue at a position that acts as an anchor. Although optimal binding requires octameric or nonameric peptides (60, 61), longer peptides can be accommodated in the binding groove, either by bulging out in the middle or by extending out from the carboxy (C)-terminal end (64, 65, 89, 90). The amino-terminal is much more restrictive in its positioning requirements (91). The F-pocket of the peptide-binding cleft accommodates the C-terminal end and the C-terminal side-chain of the peptide ligand, and its contacts with the bound peptide contribute significantly to the overall binding strength (92-94). For example, the specificity of peptide binding is largely controlled by the polymorphic residue 116 of HLA-A2, which lies at the bottom of the F-pocket. Usually, three hydrogen bonds are made between conserved residues lining the F-pocket and the peptide-ligand backbone. Self-peptides, i.e. peptides derived from proteins native to the cell, bind to MHC-I, but these are overridden by viral peptides following viral infection (59, 95, 96).

Most MHC-I on the normal cell surface exist as ternary complexes, p-α-β2m (55). The stability of the complex depends primarily on the binding affinity of the peptide. The ternary complex pH-α-β2m (associated with high-affinity peptide, pH) is most stable, with a half-life of 4h or more (75). The complex pL-αβ2m, in which the peptide is either too long or lacks the proper binding motif and thus binds with low affinity (therefore, pL), is also present and is less stable (59). Three other forms of MHC-I, all unstable, α-β2m, p-α and α (perhaps in decreasing order of stability) can also be found on the cell surface (33, 34, 97). Collectively, these have a half-life of 30 min or less at 37°C (34, 97) and rapidly denature (98). Such denaturation can often be detected by mAbs specific for the α1α2 domains: the binding of such mAbs to live cells provides a measure of the capacity of cells to produce MHC-I with stable peptide ligands.

Due to the instability of pL-α-β2m, α-β2m, p-α and α, only the crystal structure of pH-
α-β2m has been studied. The three-dimensional structure of a MHC-I molecule was first obtained in 1987 after the successful crystallization of HLA-A2 (99). This structure provided a conceptual framework for understanding some of the characteristics of MHC-I and subsequent structural determination of several other MHC-I molecules (HLA-B27, HLA-Aw68, H-2Kb, H-2Db) has confirmed the overall molecular architecture of MHC-I (88, 100-104).

1.4 NON-CLASSICAL MHC-I

As the sequencing of the MHC loci progressed in a variety of mammalian species, it became clear that many other class I genes existed that showed sequence homology to the class Ia loci but differed both with regard to expression and polymorphism. These loci came to be called “non-classical” class I or class Ib in the mouse (105, 106) (Fig 1-3). Class Ib loci were found to lack the polymorphism of class Ia loci. Furthermore, expression of class Ib loci is often restricted to certain tissues, such as the thymus, liver, intestine or placenta (107, 108).

In the mouse, class Ib proteins were identified during studies undertaken to define the antigens associated with normal T cell development. The thymus leukemia antigens (TL) encoded by H-2Q and H-2T genes were discovered in 1963, while other antigens such as Qa2 and Qa1 were discovered much later (109-111). These were joined by gene products of a third and more distal class I subregion, H-2M (112) (named for the well-studied resident gene H-2M3), which has undergone recent characterization.

These non-classical MHC-I molecules were found to possess some interesting modifications in their structures and/or peptide-binding capabilities. For example, the Qa2 antigen, encoded by the Q7 gene, was found to be tethered to the cell membrane by a glycosylphosphoinositol (GPI) linkage, while a second H-2Q region gene, Q10, encoded a transmembraneless, soluble protein. Further, H-2M3 was reported to bind N-formylated peptides produced either in the mitochondria or during bacterial infection (113).

In human, three loci, termed HLA-E, HLA-F and HLA-G were first identified in the late 1980s (114, 115) (Fig 1-3). This was followed by the discovery of the MIC genes (MICA-E), which exhibited a greater divergence from class Ia than HLA-E, -F and -G. Of the five members of this family, only the MICA and MICB genes, situated between the TNF and HLA-B loci, are expressed (116). MICA requires neither β2m nor bound peptide for
cell-surface expression and has been shown to be the ligand for the activating NK receptor complex, CD94/NKG2D (117). A putative empty class I molecule in the mouse (T22) was identified and shown to communicate with T cells (118).

Similar to other classical MHC-I molecules, the HLA-E heavy chain is expressed on the cell surface as a non-covalent complex with \( \beta_2m \) and a peptide. For most MHC-I, the peptide is an octameric or nonameric fragment derived from degradation of cytoplasmic proteins, such as housekeeping enzymes, or viral proteins in infected cells processed by the proteasome. For HLA-E and Qa-1, however, there are additional constraints (119, 120). Although HLA-E expression is TAP-dependent, its peptide repertoire is largely comprised of the leader sequences from other class I molecules. In mouse, Qa-1\(^b \) has been found preferentially associated with a peptide called Qdm (AMAPRTLLL), also derived from MHC-I signal sequences. Furthermore, the leader sequences for HLA-E or Qa-1 and certain other MHC-I do not bind HLA-E or Qa-1, respectively (120). Interestingly, HLA-E and Qa-1 share an unusual feature involving the replacement of otherwise highly conserved Thr\(_{143} \) and Trp\(_{147} \) by Serine residues. This could change one of the side pockets (F) in the peptide binding cleft, perhaps accounting for the restricted repertoire of peptides. Hence, expression of HLA-E or Qa-1 requires not only the normal production of these molecules but also synthesis of at least classical MHC-I molecules.

721.221 cells are HLA-A, -B, and -C negative mutant B-lymphoblastoid cell line (121). 721.221 cells do not express intact HLA-E molecules on the cell surface because they lack an adequate peptide supply due to deficient expression of other HLA molecules (122, 123). Transfection of an HLA molecule into 721.221 cells may result in expression not only of the transfected HLA molecule but also of endogenous HLA-E (124). Moreover, there was a good correlation between capacity of a specific HLA molecule to confer resistance to NK lysis and the ability of its leader peptide to bind HLA-E.

1.5 NK RECEPTORS
1.5.1 Overview
The molecules that modulate NK function appear to segregate into two basic categories: activating receptors and inhibitory receptors (25, 125, 126). Activating receptors can trigger lysis of the target cell being recognized while inhibitory receptors can override the activating
signal upon interaction with their ligand(s) (if present) on the same target cell.

Almost all NK inhibitory receptors identified in human, mouse and rat have one or more immunoreceptor tyrosine-based inhibitory motif (ITIM, V/IxYxxL) regions in their cytoplasmic domain (23, 127, 128). When NK inhibitory receptors are cross-linked either by specific antibodies or ligands, the tyrosine (Y) residue in the ITIM regions will be phosphorylated. The phosphorylated ITIM regions enable the recruitment of phosphatases (e.g. SHP-1 and perhaps SHP-2) to counteract the activating signalling cascade and abort NK cell cytotoxicity (127).

Unlike the inhibitory receptors, NK activating receptors have a very short cytoplasmic tail with a charged residue in the transmembrane sequence, through which they associate with a small transmembrane adapter molecule (e.g., DAP12 and FcRγ III) that mediates an activating signal upon receptor cross-linking. DAP12 exists as a disulfide-linked homodimer and has one immunoreceptor-based tyrosine-activating motif (ITAM, D/ExxYxxL/Ix6-8YxxL/I) in its cytoplasmic tail (129, 130). When NK activating receptors are cross-linked by specific antibody or their ligands, the Y residue in the ITAM region will be phosphorylated to enable the recruitment of src homology domain-2 (SH2)-containing signalling kinases (e.g. lck, ZAP70 and Syk), resulting in a rapid rise in \([Ca^{2+}]_i\) and an increase in phosphoinositide turnover (131). Importantly, mAbs against NK activating receptor should trigger resting or IL-2 activated NK cells to kill target cells expressing the Fc receptor. In this process, termed “redirected lysis,” mAbs bind specifically to NK activating receptors while their Fc domains bind target-cell Fc receptors, thus providing cross-linking and bridging effects (132) (Fig 1-7). “Redirected lysis” is widely used as a functional assay in searches for NK activation receptors.

1.5.2 *ly49 and nkpr1* gene families
Most NK receptors identified in mice are type II integral membrane proteins (external C-terminus) that contain a carbohydrate recognition domain (CRD), a common feature of the members of the C-type lectin superfamily (Fig 1-8). The first murine NK inhibitory receptor for MHC-I was described by Yokoyama and his colleagues (1989) (133). They demonstrated that Ly49 (now, Ly49A) is expressed on a subset of NK cells as a disulfide-linked homodimer, and it inhibits cytotoxicity against targets that express the MHC-I antigens,
Figure 1-7  Schematic comparison of ADCC and redirected lysis. Note that the antibody is in opposite orientation with respect to the NK cells.
Figure 1-8  MHC-I specific receptors on NK cells. (A) C-type lectin, Type II transmembrane proteins that form disulfide-linked dimers. (B) Ig superfamily, type I transmembrane protein with two or three Ig domains. Both mouse and human NK inhibitory receptors contains one or several ITIM regions at their intracellular domain. NK activating receptors (e.g., Ly49D, Ly49H, and p50) lack ITIM region and are usually associated with a signalling molecule that contains an ITAM region, through a charged residue in the transmembrane region. Examples of signalling molecule are DAP12 and CD3ζ.
H-2D\(^d\) and H-2D\(^k\), but not H-2D\(^p\), H-2K\(^d\) or H-2L\(^d\) (20). To date, at least 16 cross-hybridizing and linked genes encoding Ly49s (ly49a-p) have been identified on mouse chromosome 6. Four Ly49 homologs have also been cloned in rat, but so far only one inhibitory receptor, specific for MHC-I (RT1.A), has been characterized (134). See Appendix A for a summary of NK receptors that have been identified and characterized.

Ly49 receptors are expressed as disulfide-linked homodimers (133, 135-138). Diversity within the ly49 gene family is provided by alternative mRNA splicing and allelic polymorphism (133, 135, 139-143). The ly49 genes differ both in their extracellular and cytoplasmic domains, implying diversity in ligand binding and signal transduction functions, respectively. Furthermore, the expression of several different ly49 genes in overlapping NK subsets also provides a diverse repertoire of Ly49 receptors.

Table 1-1 summarizes monoclonal antibodies reactive with five Ly49 family members. (136, 144-147). With these antibodies the distribution of the corresponding receptors on different NK cells has been examined and reveals a complex expression pattern, which raised two great concerns that are, unfortunately, often ignored when studying Ly49 molecules (148). First, NK cells commonly express Ly49 receptors that are apparently irrelevant for the animal in the sense that they fail to react detectably with the host’s MHC-I and second, NK cells co-express two or more Ly49 receptors, which often recognize more than one MHC-I allele, each. Nonetheless, the capacity of these Ly49 receptors to bind certain MHC-I can explain most of the alloreactivity patterns of murine NK cells.

Among the first activating NK receptors to be identified were the NKR-P1s (see Appendix A). These are 60-80 kDa, disulfide-linked homodimers. There are three mouse NKR-P1 genes (MusNkrp1-A, -B and -C) and probably five in rat. The first mAb NK1.1 to define a cell surface molecule predominantly expressed on mouse NK cell was made by Koo and Peppard (1984) (149). Later the mAb 3.2.3, which identifies all rat NK cells, was developed (132). Both of these antibodies recognize products of the NKR-P1 gene family and stimulate NK cytotoxicity and degranulation (132). The mouse NK1.1 antigen is encoded by MusNkrp1-C (150). Expression of NKR-P1 varies between different strains of mice (151-153) (Table 1-2). The NK1.1\(^{-}\) strains do contain low levels of transcripts for the other isoforms of NKR-P1. Cloning of the promoter regions from these NK1.1\(^{-}\) strains showed a 95-98% identities, suggesting that the differences in expression are due to strain-specific
### Table 1-1  Monoclonal Antibodies Specific for Ly49s

<table>
<thead>
<tr>
<th>Ly49 antigen</th>
<th>mAb</th>
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<tbody>
<tr>
<td>Ly49A</td>
<td>A1, JF9.318, YE1/48, 12A8</td>
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<tr>
<td>Ly49C</td>
<td>5E6, 4LO3311, 1F8, 14B11</td>
</tr>
<tr>
<td>Ly49D</td>
<td>12A8, 4E5, 12A1</td>
</tr>
<tr>
<td>Ly49F</td>
<td>14B11</td>
</tr>
<tr>
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<td>4D11</td>
</tr>
<tr>
<td>Ly49H</td>
<td>1F8</td>
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<tr>
<td>Ly49I</td>
<td>5E6, 14B11</td>
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</table>

### Table 1-2  Strain Reactivity of mAb PK136 (NK1.1)

<table>
<thead>
<tr>
<th>NK1.1⁺ strains</th>
<th>NK1.1⁻ strains</th>
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<tbody>
<tr>
<td>C57BL/6 and congenic strains</td>
<td>A/J</td>
</tr>
<tr>
<td>C57BL/10 and congenic strains</td>
<td>AKR/J</td>
</tr>
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<td>C57BR/cdJ</td>
<td>BALB/c and congenic strains</td>
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<td>C3HeB/FeJ</td>
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<tr>
<td>CE/J</td>
<td>DBA/2J</td>
</tr>
<tr>
<td>NZB/B1NJ</td>
<td>NOD/LJ</td>
</tr>
<tr>
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<td>129/J</td>
</tr>
<tr>
<td>ST/J</td>
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</tr>
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transactivation factors. In terms of diversity, it is known that NK cells can express more than one member of the family.

Although the biological function of NKR-P1 is presently unknown, clues come from the studies of Ryan et al, who showed that loss of NKR-P1 expression by the rat RNK-16 NK leukemia cell line correlates with the inability to kill certain mouse tumour cell targets (154). Transfection of the RNK-16 mutant with rat NKR-P1A cDNA restores cytotoxicity against these targets. In addition, Kung and Miller (1995) showed that mAb NK1.1 can inhibit the killing of parental lymphoblasts by NK cells from (C57BL/6 x BALB/c) F1 mice, suggesting a role for NKR-P1C in target cell recognition (155). However, killing of most target cells does not seem to require NKR-P1C since NK1.1+ mouse strains possess normal NK cells. Perhaps, the NKR-P1 homologs in these mice are expressed but not stained by mAb NK1.1.

Until the functional characterization of Ly49D (42) and NKR-P1B (157), it was thought that mouse nkrpl genes encode NK activating receptors and mouse ly49 genes encode NK inhibitory receptors recognizing classical MHC-I. However, Ly49D were found to have all the characteristics of an NK activating receptor. It lacks the ITIM motif found in all NK inhibitory receptors and associates with DAP12 (42). Furthermore, Ly49D<sup>B6</sup> can trigger B6 NK cells to kill target cells expressing H-2D<sup>d</sup>. Recently, Ly49H was also identified as an activating receptor (130). Conversely, NKR-P1B possesses an ITIM in its cytoplasmic domain and was shown to inhibit NK cytotoxicity triggered by an NK activating receptor (1C10 antigen) (157). Thus, there are activating receptors and inhibitory receptors in both the NKR-P1 and Ly49 families.

Interestingly, both the ly49 and nkrpl gene clusters were mapped to a stretch of DNA on mouse chromosome 6 (156). The genomic region was then defined as the "NK gene complex (NKC)" (Fig 1-9). Having been identified on syntenic regions of rat and human chromosomes, NKC was found to be conserved among species (156, 158). In addition to the Ly49s and NKR1s, the NKC contains some disease resistance genes that mediate their immune protective effect via NK cells, as well as genes that encode structurally unrelated proteins that may modulate NK function.

Efforts were made to search for human homologues of Ly49 and NKR1, but with limited luck. Human NKR1-A was identified but its function was quite distinct from mouse
Figure 1-9  Composite maps of the mouse (chromosome 6) and human (chromosome 12p) NK gene complex (NKC). The chromosomal orientation of the mouse interval is inverted with respect to the human.
or rat NKRPI-A (159). The functional consequences of treating human NK cells with anti-
NKR-P1A mAb are more complex, resulting in no effect, activation, or inhibition, depending
on the NK cell population studied (159) (124). It is also interesting to note that all rodent
NKRPIs express the CxCP motif that interacts with phosphorylated p56<sup>ck</sup>, but human
NKRPI-A lacks this motif (24, 25). The structural difference and the diverse response
elicited by anti-NKR-P1 mAb suggest that additional, functionally distinct isoforms of
NKRPIs might exist in human. As yet, human homologs of the Ly49 genes have not been
identified. Instead, human NK cells express receptors belonging to the killer inhibitory
receptor (KIR) family.

1.5.3 Human NK receptors-KIRs and KARs
Members of the KIR family are structurally distinct from Ly49 receptors (160-162) (Fig 1-8).
KIRs are type-I integral membrane proteins belonging to the immunoglobulin (Ig)
superfamily. Members of the KIR family can be grouped into 3 subtypes based on HLA
specificity. HLA-C responsive KIRs contain two Ig domains; HLA-B responsive KIRs have
three Ig domains, and an HLA-A responsive KIR also has three Ig domains (160, 161).
Although KIRs and Ly49 inhibitory receptors both recognize MHC-I, KIRs differ from
Ly49s in many aspects. For example, KIRs display little polymorphism although multiple
family members do exist. Moreover, KIR genes map to chromosome 19 instead of the
human NKC located on chromosome 12 (163-166). Therefore, KIRs and Ly49 inhibitory
receptors do not subserve the identical function. KIR homologues have yet to be found in
mouse.

The molecular cloning of human KIRs revealed that some members of this gene
family also activate NK cells. These activating receptors, being close relatives of KIRs, have
been termed killer activating-receptors (KARs) (167). KIRs and KARs are expressed in
pairs. KAR (e.g., p50) and its KIR partner (e.g., p58), have identical extracellular portions
but different transmembrane and intracellular domains. KARs have a shorter cytoplasmic
domain, and lack cytoplasmic ITIMs, but possess charged amino acids in their
transmembrane segments that permit association with DAP12 (129, 168). In addition, KAR
also contains a CxCP motif in its cytoplasmic domain for interaction with p56<sup>ck</sup>. p50 KAR
displays HLA-C specificity and is co-expressed with at least one inhibitory receptor specific
for another class I allele. A measurable NK triggering via p50 KARs occurs only in the absence of inhibitory interactions. In vivo this situation may occur either following down-regulation or peptide-induced alteration of the HLA-I interacting with the inhibitory receptors. Although p50 KARs appear to display the same allele specificity of the corresponding p58 KIRs, it is possible that inhibitory and activating receptors recognize different forms of HLA class I molecules or different HLA class I-bound peptides.

1.5.4 CD94/NKG2 family
Receptors homologous to C-type lectins (Fig 1B) have been identified on human NK cells but appear not to represent Ly49 or NKR-P1 homologues (169). CD94 and a family of molecules called NKG2 (A-F) display distant homology with the murine NKR-P1 and Ly49 families and are encoded by genes clustered in the human NKC (170). CD94 forms disulfide-linked heterodimers with distinct members of the NKG2 family, these requiring CD94 for surface expression. NKG2A and B appear to be alternatively spliced from the same gene, but genomic studies have shown that other NKG2 proteins (NKG2C-F) are products of distinct genes. CD94 has a very short cytoplasmic domain (7 amino acids), and therefore, signal transduction is mainly through the NKG2 partner. NKG2A/B contain ITIMs and are inhibitory whereas NKG2C lacks an ITIM, associates with DAP12 and is stimulatory (117, 129, 171). Thus far, there is little information on the expression/function of other identified NKG2 genes: NKG2D appears only distantly related to the family, NKG2E may be an activating receptor, and NKG2F is missing the carbohydrate recognition domain, which is a characteristic of a C-type lectin protein. CD94 and NKG2 orthologues have been identified in rodents. Like KIRs, CD94/NKG2 heterodimers are selectively expressed by NK cells and a subset of CTLs. CD94/NKG2A was initially believed to be involved in NK recognition of a broad spectrum of HLA-I; nevertheless, it has now been firmly established that the specific ligand for both CD94/NKG2A and CD94/NKG2C is the non-classical MHC-I, HLA-E (123). CD94/NKG2D is a receptor for the stress-inducible MHC-I like molecule MICA (117). Hence, KIRs, NKR-P1s, Ly49s (which recognize classical MHC-I) and CD94/NKG2 receptors appear to play complementary roles for surveying the biosynthesis/expression of most if not all MHC-I.
1.5.5 Other molecules implicated in NK activation or inhibition

Many more molecules that could activate or inhibit NK functions but do not belong to Ly49, NKR-P1, KIR, or CD94/NKG2 families have been identified over the years but, for the most part, are less well characterized. I will not attempt to describe all the NK receptors identified. Following are some of them. For activating receptors, NKp46, NKp30, NKp44, 2B4 and CD16 are members of the Ig-superfamily and their masking by blocking mAb inhibits the NK lysis of a variety of target cells. CD16, NKp46 and NKp30 are associated with CD3ζ and/or FcRγ, whereas NKp44 is associated with DAP12. Much current work involves searching for their ligands and their signalling pathways (24, 25, 168).

In addition, several adhesion molecules (e.g. CD2, CD11a/CD18 (LFA-1) and CD49/CD29 (VLA-4) have been implicated in NK activation (172-177). It was hypothesized that changes in the quantity and activation stage of certain adhesion molecules on NK cells, and the density and arrangement of their ligands on target cells, may suffice to activate NK cells. In support of this hypothesis, some adhesion molecules are de novo expressed or up-regulated when NK cells are activated by target cells or inflammatory cytokines such as TNFα (175, 176). The cytoskeletal-membrane linker protein, ezrin, was shown to directly control target susceptibility to NK cells via intracellular control of the cell surface expression pattern of the adhesion molecule ICAM-2 (ligand for CD11a/CD18) (178).

For inhibitory receptors, Ig-like transcripts (ILTs), leukocyte associated Ig-like receptors (LAIR) and Gp49 are the ones that are currently being studied (179). ILTs includes at least eight members with different numbers of Ig domains, cytoplasmic tails with or without ITIMs, and distinct expression patterns. ILTs are expressed primarily on monocytic cells and dendritic cells but are also found on some NK and B cells (164, 180, 181). They are encoded in a gene complex centromeric to the KIR gene complex. Three of the ILT family members (ILT6, ILT7 and ILT8) contain short cytoplasmic domains and are associated with FcRγ which transduces stimulatory signals. LAIR carries a single Ig domain and is expressed on all leukocytes. Antibody cross-linking of LAIR on NK cells can inhibit NK cytotoxic function (via SHP-1 and SHP-2 signalling). Lastly, gp49 found on mouse cells, is a distant structural relative of KIR (182-184). Two gp49 genes, encoding gp49B and a non-inhibitory gp49A isoform, are linked on chromosome 10 in a region that is not syntenic.
with the human KIR gene complex (164). Gp49B has been shown to inhibit NK cytotoxicity and mast cell de-granulation (184).

1.6 MHC-I AND NK RECOGNITION

The “missing-self” hypothesis has suggested that the specificity of NK recognition is provided by inhibitory signals transduced by receptors for self-MHC-I and has been strongly supported by the recent discoveries of NK inhibitory receptors and ligands (Fig 1-1). However, not all lysis mediated by NK cells could be explained by this hypothesis. Since the proposal of the “missing-self” hypothesis, several groups have tried to better understand the structural basis of MHC-I in NK recognition to complement the hypothesis.

Several studies have focused on defining the regions of MHC-I involved in the recognition by NK inhibitory receptors. In 1989, by performing exon-shuffling and point-mutation experiments on human MHC-I, Storkus et al. showed that the α1-α2 region appears to be critical in determining the specificity of MHC-I (HLA-A and HLA-B) as an inhibitory ligand (36), and that the amino acids in the peptide binding groove of MHC-I are important in conferring NK resistance (37). In 1998, Karlhofer et al. (20), and Sundbäck et al. (40) have mapped the mouse MHC-I determinant recognized by the inhibitory receptor Ly49A to the α2-region of the Dd molecule.

There are four different forms of MHC-I (p-α-β2m, p-α, α-β2m, α) expressed on the surface of a normal cell (see “classical MHC-I” section); therefore, any of these four forms of MHC-I could be the ligand for NK receptors. MHC-I molecules capable of binding exogenously added peptides are present in a detectable level (by mAb-staining) on both normal and TAP-deficient (e.g., RAM-S) cells, and are conventionally referred to as “empty” MHC-I molecules (Chapter 2). It is essentially unknowable, however, to what extent these molecules are truly empty, i.e., contain solvent in their binding groove (e.g., α-β2m or α) versus a weakly bound peptide (e.g., pL-α-β2m) (34, 74, 185). Nonetheless, the exogenously added peptide is most likely to bind to α-β2m and/or displace the pL in the pL-α-β2m complex (after the pL dissociates). Binding to α chain alone is probably unlikely as it was shown to be highly unstable on the cell surface at 37°C (34). We, thereby, refer to all forms of MHC-I that can bind exogenous peptide of high affinity as “peptide-receptive” MHC-I (Chapters 3-
5) (74). This is an operational definition. Christinck et al. and Luscher et al. showed that approximately 10% of D\textsuperscript{b} molecules expressed on the surface of EL-4 tumour cells are peptide receptive (76, 186).

Peptide binding is known to influence the conformation of the surface of class I molecules as detected with mAbs and TCR (187-190). Using a system employing fluorescence resonance energy transfer, Catipovic et al. found that H-2K\textsuperscript{b} α-β\textsubscript{2}m heterodimers are in a relatively extended conformation, and that this conformation becomes more compact when peptide is bound (190). This is consistent with peptide-receptive MHC-I molecules (p\textsubscript{L}-α-β\textsubscript{2}m and/or α-β\textsubscript{2}m) having conformation(s) different from that of a p\textsubscript{H}-α-β\textsubscript{2}m ternary complex. It is possible that NK receptor(s) can distinguish different conformations of MHC-I.

Since 1992, several studies have attempt to address the question of whether the presence of peptide in the peptide-binding groove within the α\textsubscript{1},α\textsubscript{2} region is critical in recognition by an NK inhibitory receptor. Storkus et al., using C1R cells (an HLA-A, HLA-B null human tumor cell line) that are normally lysed by human NK cells, found that these cells could be protected from lysis by transfection of certain HLA-A or HLA-B and that protection was reversed by the addition of peptide that could bind to the protective MHC-I (191, 192). Chadwick and Miller (38), and Chadwick et al. (39) found that normal non-transformed lymphoblasts could be lysed by syngeneic mouse NK cells in the presence of peptide specific for their MHC-I. They tested 9 different peptides specific for K\textsuperscript{b}, D\textsuperscript{b}, K\textsuperscript{d}, L\textsuperscript{d}, or D\textsuperscript{d}. All of these peptides could sensitize a normal lymphoblast to be lysed if they could bind to it. There are two possibilities to explain these results: (1) binding of exogenous peptide replaces a protective self-peptide essential for NK inhibitory receptor recognition, and (2) converting the peptide-receptive form of MHC-I into p\textsubscript{H}-α-β\textsubscript{2}m complexes by binding of exogenous peptide masks (or alters) the region (or conformation) required for recognition by the NK inhibitory receptor(s).

In 1994, these two hypotheses were tested. Results of Correa and Raulet (193) and Orihuela et al (41) show that Ly49A, the prototypic NK inhibitory receptor recognizes D\textsuperscript{d} in the presence of either foreign or self-peptides specific for D\textsuperscript{d}. These groups transfected TAP-deficient RMA-S (41) or LKD8 (193) cells with D\textsuperscript{d} cDNA construct. In the absence of TAP, these cells express the α-β\textsubscript{2}m, p\textsubscript{L}-α-β\textsubscript{2}m and possibly the D\textsuperscript{d} α on their cell surface, all
known to be extremely unstable, thus resulting in low level of surface expression at 37°C. To stabilize D\textsuperscript{d} expression on the cell surface, foreign or self peptide(s) of high binding affinity for D\textsuperscript{d} were used to convert the unstable forms of MHC-I into the most stable form of MHC-I, p\textsubscript{H}-α-β\textsubscript{2}m (t\textsubscript{1/2} > 4 h). They found that D\textsuperscript{d}-transfected cells were protected from lysis mediated by Ly49A\textsuperscript{+} B6 NK cells if D\textsuperscript{d} were stabilized on the cell surface. The extent of protection correlated with the extent of stabilization of D\textsuperscript{d} expression by added peptide. Both groups suggested that the role of peptide was to promote the assembly and cell surface expression of MHC-I and that there was no peptide specificity in Ly49A recognition of D\textsuperscript{d}.

In agreement with these finding, a recent study on the crystal structure of the extracellular domains of Ly49A bound to D\textsuperscript{d} showed that the Ly49A dimer interacts extensively with D\textsuperscript{d} at two distinct sites (194). At site 1, which is less extensive, a single Ly49A binds at one side of the D\textsuperscript{d}-peptide-binding platform (α\textsubscript{1}, α\textsubscript{2} domains) but away from the peptide antigen, suggesting that Ly49A recognizes the D\textsuperscript{d} conformation stabilized by D\textsuperscript{d}-specific peptide and not the peptide antigen. At site 2, which has a more extensive interface, the Ly49A dimer is wedged into a cavity bounded by the D\textsuperscript{d} α\textsubscript{2} (60%) and α\textsubscript{3} (15%) domains and β\textsubscript{2}m (25%), suggesting that the overall D\textsuperscript{d} conformation is critical for Ly49A recognition. Moreover, while binding at site 1 is consistent with previous studies on the interaction between Ly49A on the NK cell and D\textsuperscript{d} on the target cell (trans-interaction), binding at site 2 seems to represent a possible cis-interaction between Ly49A and D\textsuperscript{d} on the same NK cells. The possible significance of this possible interaction is not known at the present time.

In studying MHC-I recognized by human NK receptors, Malnati et al used RMA-S cells transfected with HLA-B27 as targets, human NK clones expressing KIR receptors specific for HLA-B27 as effectors, and exogenous synthetic peptide ligands of HLA-B27 to stabilize surface expression of HLA on RMA-S cells (195). Surprisingly, only one of the four peptides specific for HLA-B27 tested provided protection from lysis by the specific NK clones (195). The protection was independent of the peptide binding affinity to HLA-B27. By doing further analysis of HLA-B27 specific peptides using amino acid substitutions, Peruzzi et al. found that the side chains of the 7th and 8th amino acid of "protective" peptides were conserved and may be involved in NK recognition (196). This involvement may be either indirect, by affecting the conformation of the KIR binding site, or direct through interference with KIR binding to the class I heavy chain.
These observations were apparently contradictory to what Storkus et al. and Chadwick et al. had shown. However, it remains possible that some inhibitory receptors recognize the pH-α-β2m form of MHC-I and other inhibitory receptors recognize the peptide-receptive form of MHC-I or something else, for there are many mouse NK inhibitory receptors identified and their ligands are yet to be carefully characterized. Mandelboim et al. showed that cell-surface expression of human MHC class I molecules, in the absence of peptide, was both necessary and sufficient to inhibit HLA-specific human NK lines and clones (197). Using a similar system, they transfected RMA-S cells with human HLA-C of two different haplotypes along with human β2m. Culture of the cells at 26°C without exogenous peptide allowed for high expression of the transfected HLA-I and this persisted for at least 2 hr after the cells were transferred to 37°C. Presence of a particular peptide-receptive HLA-C haplotype was sufficient to inhibit lysis by an NK clone specifically inhibited by that haplotype. Therefore, it is possible that human NK inhibitory receptors can also recognize two different forms of MHC-I.

Instead of looking at the MHC-I structure, some groups have tried to seek out the regions on inhibitory receptor that are important for the receptor binding to its ligand, mainly MHC-I. Up to now, only a few Ly49 members have been studied in detail. The lectin domain of Ly49A has been shown to be functionally important in the inhibition of natural killing, but it is not known if the lectin domain itself participates in the binding of MHC-I. Murine class I antigen express two conserved N-linked carbohydrates, but it remains to be shown whether these are required for the binding of MHC-I by Ly49 receptors. One possible, but hypothetical, mechanism for interaction with MHC-I, is that the lectin domain of Ly49 binds directly to a class I carbohydrate, thus facilitating interaction of the receptor with other parts of MHC-I. Alternatively, Ly49 receptors may interact through the lectin domain with a variety of cell surface molecules, but acquire specificity through the recognition of MHC-I. Relevant in this context are studies by Takei et al, indicating that the specificity of recognition is dependent on both the lectin domain and the adjacent extracellular stalk region of Ly49 (138, 147).

In addition to the recognition of classical MHC-I, NK cells also express receptors for non-classical MHC-I. Recent studies on human NK cells have demonstrated that NK cell CD94/NKG2 receptors bind to the non-classical MHC-I, HLA-E (198). It has been evident
that CD94/NKG2 receptors gauge the overall level of HLA class I expression on cells, one indicator of their health status. The mouse nonclassical MHC-I, Qa-1, shares several features with HLA-E, and Qa-1\(^b\) tetramers have been shown to bind to a large subset of fresh or IL-2-activated NK1.1\(^+\)/CD3\(^-\) splenocytes independently of the expression of Ly49 inhibitory receptors. Binding occurs whether NK cells have evolved in an MHC-I-expressing or in an MHC-I-deficient environment. Recently, the mouse homologue of CD94/NKG2 has been cloned and shown to recognize Qa-1\(^b\) (199). Functional studies showed that recognition of Qa-1 results in the inhibition of target cell lysis by CD94/NKG2\(^+\) NK cells.

1.7 VIRAL STRATEGIES AGAINST IMMUNE RESPONSE

1.7.1 Overview

Viruses of different strains have very distinctive ways of entering and perpetuating themselves in the host. A generalization of the mechanisms of viral entry, replication cycle, and viral interaction with host does not exist. However, many viruses take similar approaches to escape immune surveillance. These approaches are 1) evasion of MHC-I presentation, 2) restriction of MHC-II-mediated antigen presentation, 3) negative regulation of cytokine-production, and 4) inhibition of apoptosis (48).

1.7.2 Evasion of MHC-I presentation:

Cytotoxic T lymphocytes (CTL) play a crucial role in virus clearance. CTLs recognize infected cells through their T cell receptor (TCR), which bind to the MHC-I expressed on the surface of most nucleated cells. It follows that viruses that attenuate MHC-I expression have a selective advantage through making the infected cells invisible to CTLs. Every step in the assembly and trafficking of the MHC-I complex (p-α-β\(_2\)m) presents a suitable target for this strategy (Fig 1-10). The first step in the generation of an antigenic peptide for MHC-I presentation is proteolysis. Both human cytomegalovirus (HCMV) and Epstein-Barr virus
Figure 1-10  Inhibition of MHC-I antigen presentation by viral proteins.
(EBV) encode proteins (pp65 and EBNA-1 respectively) that interfere with proteasomal proteolysis to inhibit the generation of virus-specific T cell epitopes (200, 201). Furthermore, instead of inhibiting peptide-generation, variants of human immunodeficiency virus (HIV) generate an antagonist peptide, which can actively silence HIV-specific CTLs when being presented by MHC-I (202, 203).

The second step in generating MHC-I is TAP-dependent transport of cytosolic peptide into the ER lumen. Herpes simplexvirus (HSV) and HCMV both encode proteins (ICP47 and US6 respectively) that interfere with peptide-transport (204-208). ICP47 competes with peptides for the single peptide-binding site on the TAP complex while US6 attacks the TAP complex from the ER-lumenal side (207, 208). Completely assembled MHC-I must be transported to the cell surface to be recognized by T and/or NK cells. Viruses like adenovirus, HCMV and HIV synthesize proteins (E3-19K, US3, and Vpu respectively) to force the retention of MHC-I in the ER-lumen (209-215). Murine CMV (MCMV) is subtly different: viral protein gp40 causes MHC-I retention in the cis-Golgi compartment (216). In addition to retention, HCMV also encode proteins (US2 and US11) that bind to MHC-I and redirect the α chain to the cytosol for degradation (217-219).

MHC-I may be retained in or purged from the ER, but even when they reach the cell surface, they are not safe. Internalization of MHC-I is a viable strategy to avoid detection by T cells. In lymphoid cells, HIV Nef actively link the adaptor complex AP-2 to MHC-I, which leads to accelerated endocytosis and degradation of MHC-I (220).

However, elimination of MHC-I expression is not without risk to the virus: NK cells can recognize cells deficient in self-MHC products. To avoid NK attack, mouse, rat and human CMV encode their own MHC-I homologs, m144 (221), r144 (222) and UL18 (223-225), respectively, which serve as decoys for NK cells, to inhibit lysis of the infected cells (223, 226-229). Expression of UL18 (in a vaccinica vector) in Chinese hamster ovarian cells has shown that UL18 binds both β2m and peptide (230, 231). In vitro, UL18 is found to bind to NK inhibitory receptors LIR-1 and to inhibit NK lysis through CD94 (223, 232). However, the expression of UL18 in HCMV-infected cells has not yet been convincingly demonstrated, and HCMV-infected cells are highly susceptible to unfractionated NK cells in vitro (233); hence, the physiological relevance of UL18 in HCMV infection remains unclear. The in vivo relevance of MCMV m144 has been better defined: an MCMV mutant with an
m144 gene deletion is cleared more efficiently by NK cells, as confirmed by restored pathogenicity of the mutant virus in NK-depleted animals (229). NK inhibitory receptors for m144 have not yet been found; interestingly, unlike UL18, m144 does not bind peptide (223), suggesting that the receptors that bind the viral MHC-I homologs differ in their requirement for bound peptide or that the functions of m144 and UL18 are not equivalent. Another MHC-I viral homolog (MC090R) has recently been identified in the slow growing, persistent human tumourigenic poxvirus, molluscum contagiosum virus (234). Like m144, MC080R also lacks conserved amino acid residues required for peptide binding (235). Further characterization of these MHC-I homologs and the NK receptors involved might improve our understanding of NK recognition.

1.7.3 Restriction of MHC-II-mediated antigen presentation
Unlike MHC-I, MHC-II are more restricted in their expression, being found primarily on antigen presenting cells such as dendritic cells, macrophages, B cells, and (in the human) activated T cells (236). MHC-II typically display exogenously acquired peptides that are recognized by the TCR-CD4 complex, thereby activating T helper cells. Very little evidence is available that suggests specific regulation of MHC-II expression following virus infection. EBV encodes an IL-10 homolog which, like physiological IL-10, can prevent the surface display of MHC-II and so delay the call for T cell help (237-239). The bovine papilloma virus (BPV) E6 protein and HIV Nef can both affect the intracellular distribution of MHC-II or the antigens destined for presentation by MHC-II via interfering with the endocytic machinery (240-242). The E5 product of BPV can elevate endosomal pH and therefore spare certain antigens from proteolysis and prevent their presentation (243).

1.7.4 Negative regulation of cytokine-production
Cytokines can be either positive or negative regulators of immune and inflammatory responses, and for this reason, many viruses encode their own cytokines (244). For example, EBV IL-10 homolog Bcrf1 is a negative regulator of IL-12, which promotes IFN-γ production and has a profound impact on the development of Th1- and Th2- like cytokine-producing cells (237, 238). Viruses can also neutralize cytokine activities by either interfering with intracellular signalling or synthesizing soluble receptors specific for
cytokines. Adenoviruses encode at least four genes that antagonize different stages of tumour necrosis factor (TNF) induced biological activities (245-248), while members of the poxvirus family collectively encode an impressive array of soluble receptors that bind and block the activity of IFN-α, -β, and -γ, TNF, and IL-1 (48, 50). Recently, more viral proteins have been identified to be the antagonists of cytokines and of chemokines, important regulators of the immune response.

1.7.5 Inhibition of Apoptosis

Apoptosis, or programmed cell death, is a process by which a cell dies in response to certain internal or external stimuli. CTL and NK recognition of virus-infected cells results in a rapid, apoptotic death of the target cells, providing an obviously important mechanism for the elimination of virus-infected cells. Virus infection can also induce apoptosis more directly and may restrict virus infection by killing off the host cell before the release of progeny virions. Because viruses require cellular machinery to complete their replication cycle and to reproduce, they have evolved to interfere with apoptotic cell death. One example of apoptotic interference involves CrmA (249), a poxvirus protein, which resembles a serine protease inhibitor (serpin) but functionally inhibits Asp-specific cysteine proteases (caspases) and probably also Granzyme-B which is normally released by CTLs or NK cells in inducing apoptosis (250-253). Several members of the herpesvirus family encode a protein termed v-FLIPs that inhibit the recruitment and activation of FLICE, a caspase that is part of the death-inducing signal complex (254). In this manner, v-FLIPs protect against cell death induced by activation of members of the TNF-receptor family (255). Another viral protein involved in the inhibition of TNF-induced cytolysis is the E1B-19K protein encoded by adenovirus (247). There has no direct in vivo evidence yet but these factors may protect virus-infected cells from lysis mediated by CTL or NK cells.

1.8 NK RESPONSE DURING VIRAL INFECTION

1.8.1 Overview

The innate immune system consists of pre-existing or rapidly inducible effector components as a first line of defence against pathogens to which the host has not previously been exposed. Such a system is necessary as the B and T cell specific responses take several days
to develop. An unimpeded replication of a virus producing $10^5$ progeny per cell could potentially infect all of the cells in the host after only 3 or 4 replication cycles (256). Without the innate immune system holding infection in check, pathogens could overwhelm the host before the T and B cell response became effective.

1.8.2 Cytotoxicity
The role of NK-mediated cytotoxicity in the anti-viral immune response remains controversial. It has been observed that, although NK cells are activated to mediate elevated cytotoxicity during LCMV infections, the deletion of NK cells from infected mice has no significant effect on viral resistance (257). In the case of NK cytotoxicity during MCMV infections, studies have shown that mice lacking the cytotoxic pathway (i.e., perforin$^{-}$) when infected have substantially higher virus titres in the spleens, compared to perforin wild-type controls (258, 259). These experiments suggest that NK cytotoxicity might be essential in controlling viral replication of certain but not all strains of virus.

NK cytotoxicity can be activated when the IFN-α/β cytokines are induced (260) and also when there is direct contact between the virus-infected cells and NK cells (261), as well as in the presence of positive (but absence of negative) signalling from the target to the effector cells for release of cytolytic molecules. As an apparent strategy to avoid recognition by T cells, certain viruses reduce expression of MHC-I capable of stimulating negative receptors on the NK cell surface (52, 224, 226, 227). Nevertheless, not all virus-infected cells that are also NK targets express low level of MHC-I and not all virus-infected cells expressing low level of MHC-I can activate NK cells. Several studies found that adenovirus type 12 (Ad12) can efficiently shut down host MHC-I expression to weaken T cell-mediated anti-viral response (262, 263). Early viral proteins encoded by the E1A gene segment were implicated for this reduced MHC-I expression (264-266). Surprisingly, E1A gene products also confer resistance to NK-mediated cytotoxicity. On the contrary, Ad5 –infected cells express normal level of host MHC-I (267) and are shown to be NK targets in cytotoxic assays (268-270). Till now, no MHC-I homolog has been found encoded by Ad12; perhaps, the understanding of the structural basis of MHC-I in NK recognition might aid in solving this puzzle.
1.8.3 Cytokine secretion

NK cells can make cytokines with antiviral functions, including IFN-γ and TNFα (244, 271, 272). Cytokine response is elicited at detectable protein levels during some but not all virus-infections. NK IFN-γ production is observed during MCMV and influenza virus, but not LCMV, infections (273). Cross-linking NK activating receptors or the presence of IL-12 (produced by virus-infected macrophages) can also induce NK cells to produce IFN-γ (261). During MCMV infection, the NK IFN-γ response is systemic, with serum levels reaching 10,000 pg/ml at peak times. Depletion of IFN-γ or IL-12 with mAbs increased the incidence of MCMV-induced hepatitis and virus replication in liver (273-275).

One of the ways in which IFN-γ can exert its antiviral effects is by inducing the expression of the gene for inducible nitric oxide synthase (iNOS) in cells such as macrophages, Kupffer cells, and hepatocytes (276). iNOS then catalyzes the production of a free radical gas, nitric oxide (NO), from the guanidine nitrogen of L-arginine. NO production has been shown both in vivo and in vitro to inhibit the replication of vaccinia virus, ectromelia virus, and HSV-1 (277, 278). Recently, it was also shown that mice treated with an inhibitor of NOS, Nω-methyl-L-arginine, had greatly enhanced MCMV synthesis in the liver by 3 days post-infection. However, even under conditions of high systemic IFN-γ production during MCMV infection, peak antiviral functions in liver required the proximity of NK cells (259).

TNFα also can be produced by NK cells (279). However, in contrast to IFN-γ, a wide range of cell types can make this factor at early times after infection (280). Nevertheless, it is possible that NK cell produced TNF-α feeds back onto its own regulatory pathway and synergizes with IL-12 to stimulate the production of more IFN-γ (272). In the liver, macrophage/Kupffer cell produce IL-12 and TNF-α might act on NK cells to stimulate the production of IFN-γ, which in turn stimulates the macrophages, Kupffer cells, and hepatocytes to produce NO to control MCMV replication in that organ. Human recombinant TNF-α has been shown to selectively induce the lysis of cells infected with vesicular stomatitis virus (VSV), HCMV, Theiler’s murine encephalomyelitis virus, or HSV-1 (256, 279).
1.9 OUTLINE OF THE THESIS

One of the major interests in the Miller laboratory has been to investigate how NK cells distinguish self-cells from infected or transformed cells. Specifically, how do initially NK-resistant, normal self-cells become NK-sensitive target cells? Ljunggren and Karre suggested that NK cells recognize and eliminate cells that fail to express self MHC class I molecules (the “missing self” hypothesis), thus providing a conceptual framework and testable predictions for how MHC class I molecules influence NK sensitivity (11, 47). In 1992, Chadwick, a formal Ph.D. student in the Miller lab made the initial observation that if a peptide specific for the MHC-I on normal non-transformed lymphoblasts was present in the culture, the lymphoblasts were lysed by added syngeneic mouse NK cells. This observation suggests that binding of MHC I-specific peptide either triggered NK cells by creating an activating ligand(s) or removing a “self marker” (inhibitory ligand(s)) from lymphocyte surface and hence, made the lymphoblasts susceptible to NK lysis. Since then, there has been a tremendous progress in our understanding of MHC-I biology (structure, antigen presentation, binding of MHC-I specific peptides). Relevant to our work are the findings that there are more than one form of MHC-I expressed on the cell surface (32, 34, 97) and that 10% of total surface H-2Db are peptide-receptive (76, 186). Only peptides that have the correct binding motifs and the right length (~8-9 a.a.) can bind to MHC-I with high affinity and within a short period of time (61, 186). When I joined the Miller laboratory in 1995, we decided to revisit Chadwick’s system. We reasoned that a better understanding of how exogeneous MHC-I specific peptides modify surface MHC-I molecules and thereby convert a normal syngeneic lymphoblast into a NK target would provide insights into the NK recognition mechanism of self-cells, and/or the immune surveillance of virally infected cells in innate immunity. In my project, I have adopted and optimized the NK assay first described by Chadwick and Miller (39). The modified assay conditions and the use of high affinity peptides specific for several different MHC-I molecules allowed us to better define the binding and modification of target cells, and therefore, to characterize this phenomenon in greater details. In this thesis, we provide evidence to support the notion that surface peptide-receptive MHC-I (PR-MHC-I) might be the ligand for NK inhibitory receptor, and that binding of specific MHC-I peptides to the PR-MHC-I molecules destroys the inhibitory
signal delivered to NK cells. In search of the corresponding NK inhibitory receptor(s), we have identified Ly49C_{B6} as the NK inhibitory receptor that recognizes "peptide-receptive" H-2K^{b} molecules. We have extended the work by studying the stability of surface "peptide-receptive" H-2K^{b} on splenocytes and the physiological relevance of such a recognition mechanism in viral infection.

Chapter I: Introduction
This chapter summarizes recent advances in NK inhibitory receptors and MHC-I expression involved in NK recognition.

Chapter II: NK cells can recognize different forms of class I MHC
In collaboration with Dr. Brian H. Barber and Dr. Jean Gariepy who provided us with MHC-I specific peptides and experimental suggestions, we have confirmed Chadwick et al.'s observation that lymphocytes pre-pulsed with high affinity peptide become sensitive to NK mediated lysis. This study also suggested that surface peptide-receptive MHC-I (PR-MHC-I), which is converted to peptide-bound MHC-I upon the addition of exogenous high affinity peptide, might be a ligand for NK inhibitory receptors. Using the same system, we also showed that the prototypical NK inhibitory receptor, Ly49A does not recognize PR-D^{d}. Lymphocytes pulsed with D^{d}-specific peptide became sensitive to lysis by syngeneic Ly49A^{-} NK cells but not by Ly49A^{+} NK cells. Dr. Sam K.P. Kung provided technical help and advice for some of the experiments. This chapter has been published: Su, R-C., Kung, S.K.P., Barber, B.H., Gariépy, J. and Miller, R.G. (1998) NK cells can recognize different forms of class I MHC. J. Immunol. 161(2): 755-66.

Chapter III: Ly49C_{B6} NK Inhibitory Receptor Recognizes "Peptide-Receptive" H-2K^{b}
In searching for receptor(s) that would recognize PR-MHC-I, we found that lymphocytes pre-pulsed with K^{b}-specific peptide became sensitive to lysis mediated by syngeneic NK cells expressing 5E6 antigens (Ly49C and Ly49I) but not by 5E6^{-} NK cells. In collaboration with Dr. Suzanne Lemieux who provided us with mAb 4LO3311 (recognizing Ly49C but not Ly49I), we found that Ly49C is probably the inhibitory receptor for PR-K^{b}. Furthermore, in collaboration
with Ms. Elizabeth T. Silver in Dr. Kevin P. Kane's lab, who provided us with Ly49C- and Ly49I- cDNA constructs, we showed that COS-7 cells that express Ly49C bound to Kb-expressing lymphocytes in the absence of Kb-specific peptide, but not in the presence of Kb-specific peptide. These observations strongly suggest that the Ly49C<sup>6</sup> inhibitory receptor recognizes PR-K<sup>b</sup>. Dr. Sam K.P. Kung provided valuable critiques and advice in this project. This chapter has been published: Su, R.-C., Kung, S.K.P., Silver, E.T., Lemieux, S., Kane, K.P., and Miller, R.G. (1999) Ly49C<sup>6</sup> NK inhibitory receptor recognizes “peptide-receptive” H-2K<sup>b</sup>. J. Immunol. 163(10): 5319-5330.

Chapter IV: Stability of Surface H-2K<sup>b</sup>, H-2D<sup>b</sup>, and “Peptide-Receptive” H-2K<sup>b</sup> on Splenocyte Surface MHC-I expression plays important roles in CTL activation and NK recognition. In this chapter, we have studied the stability of surface K<sup>b</sup>, D<sup>b</sup>, and PR-K<sup>b</sup> and found that there are at least two sub-populations of both K<sup>b</sup>, and D<sup>b</sup>. An unstable sub-population was lost within 4h after the continuous export of MHC-I was stopped whereas the quasi-stable sub-population had a t<sub>1/2</sub> of ~20h. Furthermore, ~10% of total surface K<sup>b</sup> are PR-K<sup>b</sup> with a t<sub>1/2</sub> less than 1h. PR-K<sup>b</sup> generation and the kinetics of peptide binding to PR-K<sup>b</sup> were also examined. This chapter will be published: Su, R.-C., and Miller, R.G. (2000) Stability of surface H-2K<sup>b</sup>, H-2D<sup>b</sup>, and "peptide-receptive" H-2K<sup>b</sup> on splenocytes.

Chapter V: A new mechanism in NK-mediated recognition of virally infected cells In searching for a physiological situation, which might result in down-regulation of PR-MHC-I and as a result, activation of NK cells, we came across adenoviruses type 5 (Ad5) and type 12 (Ad12). In collaboration with Dr. Frank L. Graham who provided us with Ad5 and Ad12 viruses, we found that Ad5-infected cells expressed reduced level of PR-K<sup>b</sup> (albeit normal levels of surface K<sup>b</sup> and D<sup>b</sup>) and became sensitive to NK mediated lysis. In agreement with our previous studies, Ad12-infected cells expressed normal levels of surface K<sup>b</sup>, D<sup>b</sup>, and PR-K<sup>b</sup> and remained resistant to NK lysis until 42h post-infection. We further showed that NK1.1 antigen (activating receptor) and 5E6 antigens (Ly49C, and Ly49I inhibitory receptors) were important in the recognition of Ad5-infected cells by NK. This chapter will be published: Su, R.-C., Graham, F.L., Shannon, J., and Miller, R.G. (2000) Infection by Adenovirus type 5 leads to reduction in “peptide-receptive” H-2K<sup>b</sup> expression
and activation of NK cells.

*Chapter VI: Discussion*

This chapter discusses how the results of our studying could complement the "missing-self" hypothesis if PR-K^b were considered as one of "self-markers" for NK recognition.
CHAPTER 2

NK CELLS CAN RECOGNIZE DIFFERENT FORMS OF CLASS I MHC

2.1 ABSTRACT

NK recognition and lysis of targets is mediated by activation receptor(s) whose effects may be over-ridden by inhibitory receptors recognizing MHC-I on the target. Incubation of normal ConA blasts with a peptide that can bind to their MHC-I renders them sensitive to lysis by syngeneic NK cells. By binding to MHC-I, the peptide alters or masks the target structure recognized by an inhibitory NK receptor(s). This target structure is most likely an "empty" dimer of class I heavy chain and β2-microglobulin as opposed to a "full" class I trimer formed by binding of specific peptide that is recognized by CTL.
2.2 INTRODUCTION

It is now widely accepted that NK cells recognize and lyse target cells through the interplay of two families of receptors (27, 156, 281). Activating receptors, when occupied, trigger lysis of the target cell being recognized. The activating signal, however, can be overridden by a dominant negative signal from an inhibitory receptor when the latter interacts with its ligand (if present) on the target cell. The ligand(s) for activating receptors remain unclear, but there is a general agreement that the ligand for some (perhaps all) inhibitory receptors is associated with class I major histocompatibility complex (MHC-I) alleles with a particular receptor being specific for a limited number of class I alleles. C1R is an HLA-A, HLA-B null human tumor cell line sensitive to lysis by polyclonal human NK cells. Storkus et al. found that transfection of some (but not all) HLA-A or HLA-B molecules into C1R protected it from NK lysis (191). By doing exon shuffling and point mutation experiments, Storkus et al. showed that the α1-α2 region of MHC-I appears to be critical in determining the specificity of MHC-I as an inhibitory ligand (36), and that the amino acids in the peptide binding site of MHC-I molecules appear to be important in the protection (37). In addressing whether occupation of the peptide-binding site was important, Storkus et al., using C1R cells transfected with protective human HLA-A or HLA-B MHC-I molecules, found that addition of peptide that could bind to a protective MHC-I reversed protection, i.e. sensitivity to lysis was restored upon peptide binding (192). Similar observations have been obtained in a more physiological setting in which normal, untransformed ConA blasts and syngeneic (polyclonal) mouse NK cells were used, respectively, as target and effector cells (38, 39). They found that the ConA blasts, which are resistant to lysis by syngeneic mouse NK cells, could be rendered sensitive to lysis if peptides that could bind to the MHC-I of the normal cells were included in the assay. Eight peptides, capable of binding K\(^b\), D\(^b\), K\(^d\), or L\(^d\) class I molecules, were tested. All 8 peptides tested (7 of which included CTL epitopes and 1 of which did not) could sensitize normal targets for lysis if they could bind to the MHC-I of the target, but otherwise had no effect (39). One possible explanation of these results, consistent with those of Storkus et al. (192), is that binding of peptide to MHC-I is altering or masking an inhibitory ligand recognized by an inhibitory receptor and thus sensitizing the cells to lysis.
Identification of human KIRs (p58.1, p58.2 or p70) and murine Ly49A molecules as NK inhibitory receptors specific for particular MHC-I alleles facilitates a detailed study of specific receptor-ligand interactions. Ly49A is known to recognize D\textsuperscript{d} (17, 20) and recent evidence indicates that recognition requires that D\textsuperscript{d} is loaded with peptide (41, 282). Both groups used mutant cell lines lacking functional peptide transporter molecules so that only empty (and unstable) MHC-I molecules appear on the cell surface. These can bind and be stabilized by high affinity class I-binding exogenous peptide (32, 97). Both groups used Ly-49A\textsuperscript{+} NK cells as effector cells and the mouse mutant cell lines RMA-S (282) or LKD8 (41), transfected with D\textsuperscript{d} as target cells. Addition of peptide that could bind to D\textsuperscript{d} was shown to protect the cell lines from lysis by Ly49A\textsuperscript{+} mouse NK cells. The extent of protection correlated with the extent to which the added peptide stabilized D\textsuperscript{d} expression (282). Both groups suggested that the role of peptide was to promote the assembly and cell-surface expression of MHC-I and that there was no peptide-specificity in Ly49A recognition of the D\textsuperscript{d} molecule. In a similar study, Malnati et al. used RMA-S cells transfected with HLA-B27 as targets, human NK clones expressing KIR receptors specific for HLA-B27 as effectors, and exogenous synthetic peptide ligands of HLA-B27 to stabilize surface expression of the HLA molecules on RMA-S cells (195). One of the four peptide ligands specific for HLA-B27 tested provided protection from lysis by the specific NK clones (195). The protection was independent of the peptide binding affinity to HLA-B27. By doing further analysis of HLA-B27 specific peptides using amino acid substitutions, Peruzzi et al. found that the side chains of the 7th and 8th amino acid of "protective" peptides were conserved and may be involved in NK recognition (196). This involvement may be either indirect, by affecting the conformation of the KIR binding site, or direct through interference with KIR binding to the class I heavy chain (23).

In summary, binding of peptide to MHC-I has been shown to sensitize targets to NK lysis (38, 39, 192), as well as to protect targets from NK lysis (41, 195, 196, 282). We here try to reconcile these apparently contradictory findings by assessing the possibility that NK cells can recognize different forms of MHC-I molecules. There are four possible forms of MHC-I molecules expressed on the normal cell surface. The majority exist as trimolecular complexes, each composed of a properly folded heavy chain (\(\alpha\)) containing the peptide-binding groove, a non-covalently-associated \(\beta_2\)-microglobulin molecule (\(\beta_2\text{m}\)) and a peptide (\(p\)) that can bind to MHC-I with high affinity (therefore, \(p_H\)) in the peptide-binding groove (thus \(p_H-\alpha-\beta_2\text{m}\)) (34).
Three other unstable forms of MHC-I, \( \alpha-\beta_2m \), \( \alpha-p_H \), and \( \alpha \) (perhaps, in decreasing order of stability) can be found (33, 34, 187, 283). In addition, \( p_L-\alpha-\beta_2m \) molecules in which the peptide is either too long or lacks the proper binding motif and thus binds with low affinity (therefore, \( p_L \)) are probably also present. For the cell line RAM-S (H-2\(^b\)), \( \alpha-\beta_2m \) and \( \alpha \) have been shown directly to have half lives of less than 30 min and at least one particular \( p_L-\alpha-\beta_2m \) has been inferred indirectly to have a comparably short lifetime and is likely to give rise to \( \alpha-\beta_2m \) whereas \( p_H-\alpha-\beta_2m \) appears to have a lifetime much greater than 4h (34). Only two of these four forms of MHC-I molecule (the trimolecular complex of \( \alpha, \beta_2m \) and \( p_H \), and the bimolecular complex in which the \( p_H \) is not present) are likely to be expressed in appreciable numbers on the surface of a normal cell (33, 34, 97, 283). Approximately 10\% of \( D^b \) molecules expressed on the cell surface are likely to be bimolecular MHC-I (\( \alpha-\beta_2m \)) molecules because: 1) About 10\% of \( D^b \) molecules on EL4 cells can be bound very rapidly by exogenous peptide (half-time of 9.3 +/- 1.1 min at 37\(^\circ\)C (186)), and 2) the binding of exogenous peptide to purified \( D^b \) \( \alpha \) chain was measured to have a half time of 13h, presumably because most of it was denatured, while binding of peptide to purified \( \alpha-\beta_2m \) bimolecules had a half time of less than 10 min at 22 \(^\circ\)C (283). Although both \( \alpha \) chain and \( \alpha-\beta_2m \) bimolecule can potentially bind exogenous peptide, added peptide is most likely to bind to \( \alpha-\beta_2m \) bimolecular MHC-I because \( \alpha \) chain is very unstable at 37\(^\circ\)C. We thereby refer to \( \alpha-\beta_2m \) bimolecular MHC-I as "empty MHC-I". All four forms of MHC-I molecule, but particularly \( p_H-\alpha-\beta_2m \) and empty \( \alpha-\beta_2m \) molecules (because of their appreciable abundance on the cell surface), might be recognized by NK inhibitory receptors involved in self-recognition.

In this report, we first reinvestigate and further characterize the experimental system developed by Chadwick et al. in which incubation of normal ConA blasts with class I-binding peptide sensitizes them to lysis by syngeneic NK cells (38, 39). We conclude that the peptide is most likely altering or masking the ligand recognized by an inhibitory receptor. This ligand appears to be empty MHC-I, as defined above. Second, to reconcile this conclusion with the fact that the inhibitory receptor Ly49A recognizes the trimolecular complex of class I \( D^d \) plus peptide, we have investigated the lysis of normal ConA blasts by syngeneic Ly49A\(^+\) and Ly49A\(^-\) NK cells in the presence and absence of class I binding peptide. The results are consistent with the conclusion that Ly49A recognizes \( D^d \) plus peptide but, at the same time, suggest that there
are additional inhibitory receptors that recognize empty MHC-I molecules. We propose a model in which a small change in the total inhibitory signal delivered by several inhibitory receptors can switch a cell from resistance to sensitivity to lysis by NK cells.
2.3 MATERIALS AND METHODS

2.3.1 NK generation:
The method used for producing activated NK cells (LAK cells) was identical to that used previously (38, 39, 155). Briefly, 2x10⁶ nylon wool non-adherent spleen cells from B6 athymic nude mice (Jackson Laboratory, Bar Harbor, ME) were cultured at 37°C for 3-4 days in 5 ml α-MEM supplemented with 10% FCS, 50 μM 2-ME, and 10 mM HEPES buffer (hereafter referred to as 10% CM), containing 500 U/ml mouse rIL-2. In some experiments, as specified, B6 CD8 knock out mice (284), BALB/c athymic nude mice (Jackson) or normal BALB/c mice (Jackson) depleted of T-cells using anti-CD4/CD8 antibodies and Dynabeads (Dynal A.S., Oslo, Norway), all depleted of nylon wool adherent cells, were used. Mouse rIL-2 was obtained as a supernatant from a cell line transfected with the IL-2 gene (285). These cells were cultured in 25 cm² flasks at 37°C in a 10% CO₂ in air incubator. Yields typically exceeded 5000 U/ml of rIL-2.

2.3.2 Target cell generation:
Target cells were Concanavalin A (ICN Pharmaceuticals Canada Ltd., Montreal, Que.) activated B6 lymphoblasts (B6 ConA blasts) produced by incubating 10⁷ B6 splenocytes for 3 days in 10 ml 10% CM supplemented with ConA (2 μg/ml). On day 3, ConA blast cells were harvested on Lympholyte M (Cedarlane Lab., Hornby, ON) and ⁵¹Cr-labelled by incubating about 6x10⁶ cells for 90 min at 37°C with 360 μCi Na⁵¹CrO₄ (NEN Life Science Products, Boston) in 150 μl of PBS containing 67% FCS. They were then washed 3 times with 1% CM (containing 1% FCS instead of 10% FCS), to remove non-incorporated Na⁵¹CrO₄.

2.3.3 MHC-I binding Peptides:
The effect of MHC-I binding peptides on normal ConA blasts sensitivity to NK lysis was assessed by pulsing ConA blasts with the experimental peptide (at a concentration of 1 ng/ml in 10% CM, unless stated otherwise) for 45 min at 4°C before the assay. Peptides utilized were a Dᵇ-restricted epitope of influenza nucleoprotein, ASNENMETM, (Flu-NP366-374) (286), a Kᵇ-restricted epitope of Chicken Ovalbumin, SIINFEKL, (OVAP258-265) (287), a Dᵈ-restricted epitope of HIV gp160, RGPGRAFVTI, (HIVp318-327) (288), a Kᵈ-restricted epitope of influenza nucleoprotein, TYQRTRALV, (Flu-NP147-155) (286, 289), and an Lᵈ-restricted epitope referred
to as Tum', ISTQNHALDLVAAK, (Tum-12-26) (290). Both Flu-NP peptides (>90% purity) were synthesized and purified by the Alberta Peptide Institute (Edmonton, Alberta, Canada). Chicken Ovalbumin, SIINFEKL (OVAp258-265), and its derivatives, biotinylated Ovalbumin peptide, (bio)-XSIINFEKL where X is aminocaproic acid (a linker between biotin and the peptide), and SIINFEK(bio)L were prepared by the Ontario Cancer Institute Biotechnology Laboratory, using an Applied Biosystems Peptide Synthesizer (Applied Biosystems, Foster City, CA). HIVp (>90% purity) was a gift from Dr. D. Williams (University of Toronto). Flu-NP (D\textsuperscript{b}), OVAp, HIVp, and Flu-NP (K\textsuperscript{d}) peptides are natural ligands for D\textsuperscript{b}, K\textsuperscript{b}, D\textsuperscript{d} and K\textsuperscript{d} respectively and bind to D\textsuperscript{b}, K\textsuperscript{b}, D\textsuperscript{d} and K\textsuperscript{d} with high affinities (286-289). Tum' peptide binds specifically to L\textsuperscript{d} molecules after being processed to its optimal length by proteases in serum. During the pulsing condition used in the current study, unprocessed Tum' peptide cannot bind to L\textsuperscript{d} (39).

2.3.4 Peptide Pulsing and Cytotoxicity Assay:
Methods for measuring lytic activity were identical to those used previously (39, 155). After 3 washes, \textsuperscript{51}Cr-labeled ConA blasts were incubated with peptide in 3 ml of 1% CM for 45 min at 4°C and washed again before being used in a 4.5h \textsuperscript{51}Cr release assay performed in 96-well V-bottom microtiter plates using 2000 targets/well, dispensed in 100 µl aliquots and effectors at an E:T ratio as indicated or at 30:1, also added in 100 µl aliquots. For experiments where preincubation of NK cells with F(ab')\textsubscript{2}, anti-Ly49A mAb (JR9-318) was required, the preincubation was done at 37°C for 30-45 min while preparing target cells for the assay. Specific lysis was calculated as % specific lysis = (E-S)/(T-S) x 100 where each value represents the mean ± S.E.M. of five replicates. E is the experimental mean of \textsuperscript{51}Cr released; S, the amount of \textsuperscript{51}Cr released when the target cells were cultured in medium alone; and T, the total amount of \textsuperscript{51}Cr released in the presence of 2% acetic acid. Dialyzed FCS (12 KDa cut off) was regularly used in place of regular FCS during the \textsuperscript{51}Cr labeling, pulsing and assay stages (186, 291).

2.3.5 CTL generation and maintenance:
Generation of peptide-specific CTL was done as described previously (292). Briefly, lymphocytes from normal C57BL/6 (B6) mice were depleted of B cells by passage through
nylon wool and cultured at 5-6 x10^6 cells/ml in 10 ml 10% CM in the presence of 1 ng/ml of peptide (Flu-NP or OVAp) and 5 U/ml of mouse rIL-2 (285). On day 7, CTL were harvested on Lympholyte M (Cedarlane Lab., Hornby, ON) and used in the cytotoxicity assay. To maintain a CTL line, 10^6 cells were harvested after 7-10 days of culture, and cultured with 2x10^6 irradiated (15 grey) B6 spleen cells in the presence of 1 ng/ml of peptide and 5 U/ml of mouse rIL-2 as above.

2.3.6 Cold-Target competition assay:
Radiolabelled B6 ConA blasts, either pre-pulsed or non-pre-pulsed with peptide (1 ng/ml), were tested as targets using either B6 NK cells or peptide specific B6 CTL lines as effectors, as described in the cytotoxicity assay except that unlabelled B6 ConA blasts, either pulsed (1 ng/ml) or unpulsed, were included in the wells at 0, 1, 3, or 5-fold multiplicities of the labeled targets as indicated. Cold and hot targets were premixed prior to the addition of effector cells (i.e. NK cells or CTL lines). A 4.5h ^{51}Cr release assay was performed in 96-well V-bottom microtiter plates and specific ^{51}Cr release was measured. Specific lysis was calculated as described in the cytotoxicity assay section.

2.3.7 Conjugate Formation Assay:
FITC (green dye, Sigma) labeled LAK cells were prepared as described by Kung et al. (293). Briefly, Day3 or Day4 B6 LAK cells (10-12 x10^6) were incubated with a FITC solution (10 μg/ml PBS final) at 37°C for 18 min. Excess FITC was removed by centrifuging the cells through 5 ml 6% BSA/PBS. The cells were then washed twice with 1% BSA/PBS. PKH26 (red dye, Sigma) labeled target cells were prepared according to the manufacturer’s protocol. Briefly, YAC-1 and B6 ConA blasts were washed twice with serum free medium, and then incubated with PKH26 dye (4x10^6 M) in labeling buffer (Diluent C, 10^7 cells/ml) at 25°C for 3-5 min. The staining reaction was stopped by adding an equal volume of 1% BSA/PBS. The cells were washed 3 times with 10% CM to remove excess PKH26 dye. The conjugation formation assay used was described by Cavarec et al. (294). FITC labeled LAK cells were pelleted and incubated with the PKH26 labeled target cells (B6 ConA blasts, B6 ConA blasts pulsed with OVAp peptide, or YAC-1) at an E:T=3:1 for 10 min at 37°C. At the end of incubation, the effector-target mixture was resuspended in 1 ml of 1% BSA/PBS and kept at 4°C
before being analyzed for its fluorescence. For negative controls, LAK cells and target cells, at a ratio E:T=3:1, were mixed and vortexed without any co-centrifugation prior to the analysis with FACSscan.

2.3.8 Time Delay Experiment with or without Brefeldin A (BFA):
B6 ConA blasts were pulsed with peptide and washed free of unbound peptide as described in the cytotoxicity assay section. The cells were then incubated in 10% CM at 37°C with or without BFA (5 µg/ml, Sigma-Aldrich Canada Ltd. Oakville, ON) for varying lengths of time before being tested as targets in a 4.5h ⁵¹Cr release assay using either syngeneic B6 NK cells or peptide specific B6 CTL lines.

2.3.9 Flow cytometry/FACs Analysis:
To measure newly emerged empty MHC-I molecules, day 3 ConA blasts were pre-pulsed with non-labeled OVAp peptide (10 ng/ml) for 45 min to fill empty Kᵇ molecules, washed free of unbound peptide and then incubated at 37°C for 0 min or 90 min in the presence or absence of BFA before being pulsed with biotinylated OVAp peptide (100 ng/ml). To measure the effect of BFA on the existing empty MHC-I molecules, day 3 ConA blasts were incubated at 37°C for 0 or 4h in the presence or absence of BFA before being pulsed with biotinylated OVAp peptide (100 ng/ml). FITC-conjugated mAb 5F1, purified from the hybridoma 5F1-2-14 (295), was used to detect the expression of peptide-Kᵇ complexes on the cell surface immediately after the pulsing with biotinylated OVAp peptide as it has been shown (295) that this mAb does not recognize empty Kᵇ molecules. OVApₓ-bio (does not bind to MHC-I), and OVAp𝐾-bio were used in the staining assay. The binding of biotinylated OVAp was visualized with R-phycoerythrin conjugated streptavidin (Sigma) which binds to biotin, and analyzed using LYSIS II program (Becton Dickinson).

2.3.10 Cell Sorting for Ly49A⁺ and Ly49A⁻ NK subsets:
Day 3 BALB/c LAK cultures were harvested and resuspended in 1% BSA/PBS (10⁷ cells/ml). The cells were then incubated with 4µg of JR9-318 mAb (146), obtained from Dr. D. Raulet with permission of Dr. J. Roland (Pasteur Institute, Paris, France), per 10⁶ cells at 4°C on a rotator for 45 min. JR9-318 mAb recognizes the NK inhibitory receptor, Ly49A (146). Stained
cells were washed with cold 1% BSA/PBS and then incubated with sheep anti-mouse IgG conjugated to Dynabeads (Dynal A.S, Oslo, Norway) (1 bead per cell) for 45 min at 4°C on a rotator. Ly49A⁺ cells, bound to the magnetic beads, were separated from Ly49A⁻ cells and both were cultured in 5 ml 10% CM containing 500 U/ml mouse rIL-2 (285) for an additional 3-4 days as above. Ly49A⁺ cells that were bound to the beads dissociated from the beads during the overnight incubation and the beads were then removed.

2.3.11 F(ab')₂ fragment generation:

For F(ab')₂ fragment generation, 2 mg of affinity purified anti-Ly49A mAb (JR9-318) was resuspended in 1 ml of 0.1M sodium citrate buffer (pH 3.5) and then digested with 10 μg pepsin (Boehringer Mannheim, Mannheim, Germany) at 37°C for 4-5 h. The reaction was stopped by adding 1/10 volume of 1M Tris to the mixture. After a centrifugation at 10,000 rpm for 30 min, the supernatant was collected and mixed with Protein A-Sepherose beads (Sigma Chemical Corporation, St. Louis, MO) to remove undigested Ab and Fc fragments. The purity and the binding activity of F(ab')₂ fragments were checked by 10% SDS-PAGE and flow cytometry, respectively.
2.4 RESULTS

2.4.1 Normal ConA blasts become sensitive to lysis mediated by syngeneic NK cells after being pulsed with MHC-I binding peptide.

The effect of pulsing normal ConA blasts with MHC-I-binding peptide was studied. Effector cells were splenocytes from B6 (H-2 K\(^b\), D\(^b\)) athymic nude (T-cell deficient) or CD8 knock out mice (284) depleted of B cells by passage through nylon wool and cultured for 3-4 days in a high concentration of mouse rIL-2 (38, 155). This procedure produces a population of highly enriched and activated NK cells (often referred to as LAK or lymphokine activated killer cells). Target cells were B6 ConA blasts pulsed for 45 min at 4\(^\circ\)C with either the D\(^b\)-binding peptide Flu-NP\(_{366-374}\) (Flu-NP) (286), the K\(^b\)-binding peptide OVA\(_{258-265}\) (OVAp) (287), or the D\(^b\)-binding peptide GAD-Flu-NP (Flu-NP with 3 additional amino acids added to the N-terminus of the Flu-NP\(_{366-374}\) peptide). Both Flu-NP and OVAp are natural ligands of an optimum length that can bind with high affinity to D\(^b\) or K\(^b\), respectively, in less than 30 min (75). In contrast, GAD-Flu-NP peptide might bind to D\(^b\) with a relatively low affinity (75, 186). On varying E:T ratio, significant lysis of Flu-NP or OVAp pulsed target cells was always observed for E:T ratios of 3:1 to 10:1, and usually reached maximum value by 10:1 to 30:1 (Fig. 2-1A). Normal ConA blasts pulsed with medium alone were, as expected, resistant to NK-mediated lysis. When normal ConA blasts pulsed with varying concentrations of Flu-NP or OVAp peptide were used in the assay, significant lysis over background of Flu-NP or OVAp pulsed target cells was seen for peptide concentrations as low as 1 pg/ml with the lysis values plateauing in the 10-100 pg/ml range (Fig. 2-1B). When normal ConA blasts were pulsed with a too long peptide, GAD-Flu-NP, no increase in lysis was observed over the whole dose-response range (Fig. 2-1B). Pulsing normal ConA blasts with peptides that could not bind to either D\(^b\) or K\(^b\) did not sensitize these target cells to lysis mediated by syngeneic NK cells (data not shown). Furthermore, no significant lysis of normal ConA blasts was observed when NK cells were pulsed with Flu-NP for 45 min at 4 \(^\circ\)C and then used as effector cells (data not shown). Thus, sensitization to lysis required that the target cells be exposed to the added peptide and that the added peptide have both the correct length and motif to bind to a MHC-I molecule expressed.

The added peptide might sensitize normal ConA blasts to NK lysis by altering the level of overall MHC-I expression rather than through direct binding to MHC-I. It is well established
Figure 2-1. Normal cells become more sensitive to NK lysis after being incubated with peptide that can bind to their MHC-I molecules. (A) Percent lysis vs. E:T ratio for target cells pulsed with Flu-NP (closed square), OVAp (closed diamond) or no peptide (open diamond). B6 ConA blasts were pulsed with the peptide indicated (10 ng/ml) and then tested as targets at varying E:T ratios as indicated on the abscissa in a 4.5h $^{51}$Cr release cytotoxicity assay using syngeneic B6 NK cells. This experiment is representative of more than 40 such experiments. (B) Peptide dose-response curve for Flu-NP (closed square), OVAp (closed diamond) or Flu-NP with GAD added on the N-terminus (open circle). B6 ConA blasts were pulsed with varying concentrations of peptide as indicated on the abscissa, washed free of unbound peptide, and then tested as targets in a 4.5h $^{51}$Cr release cytotoxicity assay using syngeneic B6 NK cells. Middle parts of both dose-response curves have been reproduced at least 3 times for both peptides using B6 NK cells derived from B6 normal mice, B6 CD8 knock out mice and B6 athymic nude mice. This experiment is representative of 2 independent experiments.
that there is an inverse relationship between sensitivity to NK lysis and MHC-I expression (11, 191). Thus, as little as a two-fold decrease of MHC-I can double the amount of lysis observed for a particular target cell (191). It is possible that binding of the added peptide to MHC-I on the target cell surface induces NK sensitivity by inducing a relatively modest down regulation of MHC-I expression. However, we found, if anything, a slight increase (<10%) in the level of expression of K\textsuperscript{b} or D\textsuperscript{b} after B6 ConA blasts were pulsed with OVAp peptide or Flu-NP peptide, respectively (data not shown). Hence, we conclude that the added peptide most likely exerts its effect through direct binding to the MHC-I expressed on the target cell surface.

2.4.2 Peptide binding to MHC-I appears to alter or mask an inhibitory signal.

NK recognition is thought to be mediated by an activating receptor whose effects may then be overridden by an inhibitory receptor (27, 156, 281). Our results are most easily explained by assuming that binding of the added peptide to the MHC-I on the target cell surface altered or masked an inhibitory signal recognized by NK cells. However, in principle, the peptide binding to MHC-I might either create a target structure that is recognized by an NK activating receptor (i.e., similar to T cell-recognition), or alter or mask an inhibitory structure that is recognized by an NK inhibitory receptor. In an attempt to distinguish between these possibilities, we did cold-target competition experiments. Radiolabelled, peptide-pulsed normal B6 ConA blasts were incubated with B6 NK cells and varying numbers of cold B6 targets that had or had not been pulsed with peptide. The results (Fig 2-2) show that the cold targets were equally effective competitors whether or not they were pulsed with OVAp-peptide (Fig. 2-2A). This implies that both peptide pulsed and unpulsed cold targets were equally effective in forming conjugates with NK cells. We tested this directly by measuring the ability of B6 NK cells to form conjugates with B6 ConA blasts pulsed or not pulsed with OVAp and, as a control, with YAC-1 (Fig. 2-3). The NK cells were stained with a green fluorescent dye (FITC) and the ConA blasts with a red fluorescent dye (PKH26), mixed, and centrifuged together (experiment, conjugates should form) or kept suspended (control, conjugates much less likely to form). Events detected in the flow cytometer that showed both green and red fluorescence were scored as conjugates. In agreement with the competition results (Fig 2-2), comparable numbers of conjugates formed using both pulsed and unpulsed ConA blasts (Fig. 2-3). As only peptide-pulsed ConA blasts are lysed, the observations are consistent with the conclusion that binding of the added peptide to
Figure 2-2. Cold targets compete for NK-mediated lysis of hot targets whether or not they have been pulsed with peptide whereas only peptide pulsed cold targets can compete for CTL-mediated lysis. (A) B6 radiolabelled ConA blasts, either pulsed (filled symbols) or unpulsed (open symbols) with OVAp (1 ng/ml) were tested as targets using B6 NK cells as in Fig 2-1B except that unlabelled B6 ConA blasts, either pulsed (1 ng/ml, diamonds) or unpulsed (circles) were included at 0, 1, 3, or 5-fold multiplicities of the labeled targets as indicated on the abscissa. Cold and hot targets were premixed prior to the addition of effector cells (i.e. NK cells). The figure is representative of 6 independent experiments. (B) As in A except that an OVAp-specific CTL line was used at an effector to hot target ratio of 50 to 1. An additional group was included in which Flu-NP-pulsed (1 ng/ml) targets were tested as cold competitors (filled square). The figure is representative of 4 independent experiments.
Figure 2-3. B6 ConA blasts, whether pulsed or not pulsed with peptide, are equally effective in forming conjugates with B6 LAK cells. Targets: A) B6 ConA blasts, B) B6 ConA blasts pulsed with OVAp, or C) YAC-1 cells (labeled with PKH26: detected by FL2; fluorescence intensity is shown on Y-axis) were pelleted and incubated with effectors: B6 LAK cells (labeled with FITC: detected by FL1; fluorescence intensity is shown on X-abscissa), at an E/T=3. Effector-target conjugates were detected as PKH26"FITC" events, shown in the boxed area. As negative controls, FITC-labeled B6 LAK cells and PKH26-labelled targets: D) B6 ConA blasts, E) B6 ConA blasts pulsed with OVAp, or F) Yac-1 cells, at a ratio E/T=3, were mixed and vortexed without any co-centrifugation prior to the analysis with FACScan analyzer. Fewer effector-target conjugates (PKH26"FITC" events) were formed when the effectors and targets were not brought together by centrifugation. Note that 10,000 events per sample were analyzed, but only the first 2,000 events were presented in the plot for the clarity and neatness of the presentation. G) and F) showed LAK cells and B6 ConA blasts alone, respectively. 10,000 events per sample were shown in panels G) and F). The figure is representative of 3 independent experiments.
Figure 2-3

A  31.30%

B  34.06%

C  48.77%

D  2.33%

E  3.16%

F  8.76%

G

H

B6 LAK cells

Log Fluorescence Intensity- FITC

Log Fluorescence Intensity- PKH26

B6 Con A

B6 Con A + OVAp

YAC-1
the MHC-I on ConA lymphoblast target cells is altering or masking an inhibitory structure recognized by NK cells, leading to the lysis of target cells (see Discussion).

As a control for the cold target competition experiment, the same cold-target competition experiment was done using an OVAp-specific CTL line as the effector cells (292). Here, as expected, only OVAp-pulsed cold targets (and not Flu-NP pulsed or unpulsed targets) were effective competitors (Fig. 2-2B), as it is known that a specific peptide-MHC complex (α-β2m-pH) forms the target structure that is recognized and activates the CTL to lyse the target cell (296). Similar cold target competition results for NK and CTL were obtained using the K\textsuperscript{b}-binding Flu-NP peptide (data not shown).

2.4.3 Peptide-pulsed target cells remain sensitive to lysis mediated by CTL for a much longer period of time than by NK cells.

To gain insight into the nature of the effect produced by peptide-pulsing, the lifetime of the lysis-sensitive state was measured. Flu-NP pulsed B6 ConA blasts were washed free of unbound peptide and incubated for increasing lengths of time at 37°C before NK cells were added. The results show that sensitivity of these Flu-NP pulsed target cells to NK-mediated lysis returned to that of unpulsed targets following an incubation of 60-90 min (Fig. 2-4A). As a control, a CTL line specific for the same peptide was generated (292) and tested against the same target cells pulsed with the same peptide in the same assay. The peptide-pulsed targets retained full sensitivity to CTL-mediated lysis following up to 4h of incubation (Fig. 2-4B).

To verify that the short lifetime of the peptide-induced sensitive state was not unique to Flu-NP peptide, we also tested the OVAp peptide in the same manner (Table 2-1). Again, sensitivity to lysis of OVAp-pulsed ConA blasts had returned to that of normal ConA blasts after 90 min of incubation in the absence of exogenous peptide. To test whether such targets could be re-sensitized to lysis, the same peptide (OVAp, 1ng/well) was added into assay wells containing OVAp pulsed ConA blasts incubated for 2h in the absence of peptide (Table 2-1, line 5): Sensitivity to NK-lysis was restored to that of targets tested immediately after the initial peptide pulsing (line 1). As a control, we also generated an OVAp-specific CTL line and found that, as for Flu-NP, OVAp-pulsed target cells retained full sensitivity to lysis after 4h of pre-incubation (data not shown).
Figure 2-4. The NK target structure formed by pulsing with peptide is short-lived compared to the CTL target structure formed by pulsing the same targets with the same peptide. (A) Time delay experiment -- NK cells: B6 ConA blasts were pulsed with 1 ng/ml Flu-NP (filled square), a non-binding peptide, Tum- (filled circle), or no peptide (open diamond) and washed free of unbound peptide as in Fig. 2-1. The cells were then incubated in 10% CM at 37°C for varying lengths of time, as indicated on the abscissa, before being tested as targets in a 4.5h 51Cr release assay using syngeneic B6 NK cells as in Fig. 2-1. The figure is representative of 8 independent experiments. (B) Time delay experiment -- CTL: B6 ConA lymphoblast targets were pulsed with Flu-NP (filled square) or no peptide (open diamond), washed free of unbound peptide and incubated for varying lengths of time in 10% CM exactly as in A. They were then tested for their ability to be lysed by a CTL line specific for the peptide using conditions identical to those used for the NK cytotoxicity assay except that the E:T ratio was 10:1. The figure is representative of 3 independent experiments.
Table 2-1: Time delay experiment for NK cells using OVAp peptide.

B6 Con A lymphoblasts were pulsed with 1 ng/ml OVAp and washed free of unbound peptide, as in Fig. 2-1. They were then incubated in medium at 37°C for various lengths of time as indicated under Time-Delay before being used as targets in a 4.5h ⁵¹Cr-release assay, as in Fig. 2-1. The bracketed entry after each % Specific Lysis entry is the background lysis observed using targets that were not pulsed with peptide before the time-delay but were otherwise treated identically. After a 2h time-delay step, one set of target cells was co-cultured with NK cells in the presence of added peptide (1ng/well) during the ⁵¹Cr-release assay (line 5). Data from one of two identical experiments are shown.

<table>
<thead>
<tr>
<th>Time-Delay (h)</th>
<th>% Specific Lysis</th>
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<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
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*Same as line 4 except 1 ng of OVAp peptide was added to each well at the start of the ⁵¹Cr release assay.
2.4.4 **Brefeldin A prevents the loss of sensitivity to NK-mediated lysis.**

It is possible that the expression of newly synthesized MHC-I molecules is involved in the loss of sensitivity to NK-lysis of peptide-pulsed target cells after the 90 min of pre-incubation. Brefeldin A (BFA) is a fungal metabolite which reversibly disrupts the Golgi apparatus resulting in the blocking of transport to the cell surface of newly synthesized protein (297). In particular, BFA has been shown to block the transport of MHC-I molecules to the cell surface (298). We tested the effect of including BFA in the pre-incubation step of the experiments of Fig. 2-4: Flu-NP pulsed B6 ConA blasts were incubated in the absence of free exogenous peptide with or without BFA for varying lengths of time before NK cells or CTL were added. In the presence of BFA, the sensitivity of the Flu-NP pulsed target cells to NK-mediated lysis remained high for at least 2h instead of rapidly falling (Fig. 2-5A). CTL mediated lysis of Flu-NP pulsed target cells was not affected in the presence of BFA (Fig. 2-5B). Furthermore, background lysis of normal ConA blasts was not affected by BFA; BFA did not sensitize normal cells to NK-lysis in the absence of peptide. The presence of BFA also prevented the loss of sensitivity to NK lysis for OVAp-pulsed ConA blasts (Fig. 2-5C). Thus, we conclude that preventing the appearance of newly synthesized proteins, most likely MHC-I, on the cell surface prevents the loss of sensitivity to NK lysis of peptide-pulsed target cells. The possibility that BFA is having some other effect on MHC-I expression is explicitly addressed in the following section.

2.4.5 **Brefeldin A prevents the appearance of newly synthesized empty MHC-I but has little effect on overall MHC-I expression.**

One explanation for the peptide-induced sensitization to NK lysis is that NK inhibitory receptors recognize empty MHC-I molecules on the target cell surface and that the addition of high affinity peptide fills the empty MHC-I molecules. Therefore, cells become sensitive to NK lysis because the added peptide blocks the NK recognition of inhibitory ligand. In the absence of exogenous peptide, newly synthesized empty MHC-I molecules emerge onto the target cell surface and regenerate the inhibitory signal, thus preventing lysis.

To test directly for a correlation between the absence of empty MHC-I and sensitivity to NK-lysis, and the reappearance of empty MHC-I and the loss of sensitivity to lysis, we measured the relative number of full and empty K<sup>b</sup> molecules on ConA blasts pulsed with OVAp (K<sup>b</sup>-specific). OVAp-pulsed ConA blasts were washed free of unbound OVAp and
Figure 2-5. The NK target structure formed by incubation with peptide is stable in the presence of Brefeldin A (BFA). (A) B6 ConA blasts were pulsed at 4°C with 1 ng/ml Flu-NP (squares) or no peptide (triangles), washed free of peptide, and incubated in 10% CM at 37°C for varying lengths of time (abscissa) as in Fig 2-3A with (filled symbols) or without (open symbols) added BFA (5μg/ml) before being used as targets for B6 NK cells as in Fig. 2-1. BFA (0.4μg/ml) was also included in the cytotoxicity assay for those groups (filled symbols) for which it had been used previously. The figure is representative of 6 independent experiments. (B) Same as A except that the effector cells were Flu-NP specific CTL generated and analyzed as in Fig. 2-3B. The figure is representative of 4 independent experiments. (C) Same as A except that OVAp (1 ng/ml, squares) was used to pulse the B6 ConA blasts. The figure is representative of 2 independent experiments.
incubated at 37°C for 90 min in the presence or absence of BFA. The expression of peptide-K\textsuperscript{b} complex was measured before and after the 90 min incubation using the mAb, 5F1, which recognizes specifically the trimolecular K\textsuperscript{b} complex (α-β\textsubscript{2}m-p) (189, 295). We found that the level of peptide-K\textsuperscript{b} complex expression was not affected by the 90 min incubation in the absence of BFA (Fig. 2-6a,b), but fell slightly (~10-20%) in the presence of BFA (Fig. 2-6c), perhaps because BFA prevents the transport of newly synthesized trimolecular MHC-I to the cell surface while having no effect on the endocytosis of cell surface proteins. Empty K\textsuperscript{b} molecules appeared only in the absence of BFA (Fig. 2-6e; Table 2-2). To detect empty K\textsuperscript{b} molecules, we used an OVAp peptide in which the lysine (K) at position 7 was biotinylated (OVAp\textsubscript{K-bio}). This lysine side chain is known to be one of the CTL epitopes in OVAp and is therefore expected to protrude from the peptide-binding groove (103, 299). We found that this peptide binds specifically to K\textsuperscript{b} and can be readily detected by the addition of streptavidin-PE (Fig. 2-6 and data not shown). As a control peptide, we used OVAp to which a biotinylated aminocaproic acid was added to the N-terminus (OVAp\textsubscript{x-bio}). This peptide did not bind (Fig. 2-6 and data not shown).

Newly expressed empty K\textsuperscript{b} molecules were clearly detectable on pulsing OVAp-pulsed ConA blasts with OVAp\textsubscript{K-bio} after the 90 min incubation in the absence of exogenous peptide (Fig. 2-6e; Table 2-2) but were not detectable when BFA was present during the 90 min incubation (Fig. 2-6f; Table 2-2). The total number of OVAp\textsubscript{K-bio} bound K\textsuperscript{b} complexes was also measured and found to decline after the 90 min incubation (about 55% in the absence of BFA, and 53% in the presence of BFA, staining data not shown); nevertheless, the important point was that the decline was not affected by the presence of BFA. The large decline might be a result of the OVAp\textsubscript{K-bio} peptide having a greatly reduced binding affinity to MHC-I as a result of the modification. This appears to be the case: Approximately a ten fold higher concentration of OVAp\textsubscript{K-bio} was required to stabilize K\textsuperscript{b} molecules on the cell surface of RMA-S cells, compared to that of OVAp (data not shown).

Clearly, the loss of empty K\textsuperscript{b} molecules after peptide pulsing correlated with the sensitivity of these target cells to NK lysis, and the re-appearance of empty K\textsuperscript{b} molecules after the 90 min incubation at 37°C coincides with the loss of sensitivity to NK lysis. Thus, these data are fully consistent with our hypothesis that NK inhibitory receptors recognize empty MHC-I molecules.
Figure 2-6. Total peptide-K\(^b\) expression was little affected by BFA but newly synthesized empty K\(^b\) molecules emerged only in the absence of BFA during a 90 min incubation. (A) FACs profiles for peptide-K\(^b\) expression (left panels) and for empty K\(^b\) expression (right panels). The left panels (a,b,c) show peptide-K\(^b\) complex expression (measured with mAb 5F1 staining) immediately after peptide pulsing (panel a), and 90 min later in the absence (panel b) or presence (panel c) of BFA. The right panels (d,e,f) show binding of biotinylated OVAp immediately after peptide pulsing (panel d), and 90 min later in the absence (panel e) or presence (panel f) of BFA. OVAp\(_X\)\(\text{bio}\) where X is an aminocaproic acid serving as a linker between biotin and the peptide (does not bind to MHC-I) and OVAp\(_{K\text{-bio}}\) which binds to K\(^b\) were used in the staining assay. The binding of biotinylated OVAp was visualized with R-Phycoerythrin conjugated streptavidin (Sigma) which binds to biotin, and analyzed using LYSIS II program (Becton Dickinson). The figure is representative of 6 independent experiments.
Table 2-2  Summary of the mean channel value ± SD of three replicates of the nine profiles shown in Fig 2-6 right panels (d-f).

<table>
<thead>
<tr>
<th></th>
<th>Panel</th>
<th>BFA</th>
<th>No peptide</th>
<th>OVA&lt;sub&gt;PK&lt;/sub&gt;-bio</th>
<th>OVA&lt;sub&gt;PX&lt;/sub&gt;-bio</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;d&lt;/sub&gt; = 0</td>
<td>d</td>
<td>-</td>
<td>3.18 ± 0.03</td>
<td>3.34 ± 0.05</td>
<td>3.72 ± 0.06</td>
</tr>
<tr>
<td>T&lt;sub&gt;d&lt;/sub&gt; = 90 min</td>
<td>e</td>
<td>-</td>
<td>3.05 ± 0.07</td>
<td>6.31 ± 0.02</td>
<td>2.80 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>+</td>
<td>2.82 ± 0.07</td>
<td>2.92 ± 0.04</td>
<td>2.78 ± 0.06</td>
</tr>
</tbody>
</table>
A potential problem with this model is the observation (Fig 2-5) that ConA blasts not pulsed with peptide and incubated with BFA remained resistant to lysis. Empty MHC-I molecules are known to be unstable and if BFA blocks expression of new empty MHC-I, one might expect all empty MHC-I to disappear over the time of the assay, thus rendering the ConA blasts sensitive to lysis. To address this possibility, we measured the relative number of empty K\textsuperscript{b} molecules on ConA blasts not pulsed with peptide and incubated with or without BFA for \( t=0 \), and \( t=4\text{h} \) using OVA\textsubscript{K,bio} as in Fig 2-6. The relative number of empty K\textsuperscript{b} fell by 31±7% in the presence of BFA and by 11±12% in the absence of BFA over the 4h incubation. We hypothesize that the remaining empty MHC-I molecules are sufficient to provide protection from NK lysis. (See Discussion)

2.4.6 The Ly49A molecule does not recognize empty D\textsuperscript{d} molecules.

In contrast to our observations, other groups have shown that under appropriate conditions, addition of H-2D\textsuperscript{d} specific peptide creates an inhibitory signal which protects NK-susceptible target cells expressing H-2D\textsuperscript{d} from being lysed by Ly49A\textsuperscript{+} B6 NK cells (41, 282). To attempt to reconcile this difference, we studied the recognition of D\textsuperscript{d} molecules by Ly49A\textsuperscript{+} and Ly49A\textsuperscript{−} NK cells in our syngeneic experimental system. The ConA blasts and NK cells used were derived respectively from normal and athymic nude BALB/c (H-2\textsuperscript{d}) mice. Day 3, rIL-2 activated NK cells were sorted into Ly49A\textsuperscript{+} and Ly49A\textsuperscript{−} subsets using the mAb JR9-318 which recognizes Ly49A molecules on both B6 and BALB/c NK cells (146, 300). BALB/c ConA blasts, both pulsed and not pulsed with a D\textsuperscript{d}-specific peptide, HIVgp160\textsubscript{318-327} (HIV\textsubscript{p}) (288), were examined for sensitivity to lysis mediated by either Ly49A\textsuperscript{+} NK cells or Ly49A\textsuperscript{−} NK cells. The results show that BALB/c blasts whether or not pulsed with HIV\textsubscript{p} were resistant to lysis mediated by the Ly49A\textsuperscript{+} NK cells but when F(ab\textsuperscript{′})\textsubscript{2} anti-Ly49A mAb (JR9-318) was included in the assay, both normal, and HIV\textsubscript{p}-pulsed blasts were lysed by Ly49A\textsuperscript{+} NK cells (Fig 2-7A). (See Discussion) In contrast, when Ly49A\textsuperscript{−} NK cells were used, they lysed HIV\textsubscript{p}-pulsed blasts and spared unpulsed blasts whether or not F(ab\textsuperscript{′})\textsubscript{2} anti-Ly49A mAb was present (Fig 2-7B). When a K\textsuperscript{d}-specific peptide (Flu-NP-K\textsuperscript{d}) was used for pulsing BALB/c blasts, both Ly49A\textsuperscript{+} and Ly49A\textsuperscript{−} NK populations could produce lysis. Interestingly, a mixture of both Flu-NP-K\textsuperscript{d} and HIV\textsubscript{p} peptide in the absence of F(ab\textsuperscript{′})\textsubscript{2} anti-Ly49A mAb (JR9-318) enabled lysis by Ly49A\textsuperscript{+} as well as Ly49A\textsuperscript{−} NK cells (Fig 2-7C,D). (See Discussion)
Figure 2-7. Ly49A⁺ NK cells lyse Flu-NP (Kᵈ-specific) pulsed ConA blasts, but not HIVp (Dᵈ-specific) pulsed ConA blasts. (A) BALB/c ConA blasts whether pulsed with HIVp (filled square) or with 10% CM alone (open square) were resistant to lysis mediated by Ly49A⁺ NK cells. The addition of F(ab')₂ anti-Ly49A mAb (JR9-318) in the assay made both HIVp-pulsed ConA blasts (closed triangles) and 10% CM-pulsed ConA blasts (open triangles) susceptible to lysis by Ly49A⁺ NK cells. The figure is representative of 3 independent experiments. (B) Normal ConA blasts pulsed with 10% CM alone (open symbols) were resistant to lysis by Ly49A⁻ NK cells either in the presence (triangles) or the absence (squares) of F(ab')₂ anti-Ly49A mAb (JR9-318), while HIVp-pulsed ConA blasts (closed symbols) were lysed by Ly49A⁻ NK cells whether or not the F(ab')₂ anti-Ly49A mAb (JR9-318) was added. (C) Both 10% CM-pulsed (open circle) and HIVp-pulsed (filled squares) ConA blasts were resistant to lysis by Ly49A⁺ NK cells, while ConA blasts pulsed either with Flu-NP-Kᵈ peptide (Kᵈ-specific, closed circles) alone or with a mixture of both Flu-NP peptide and HIVp (closed diamonds) were lysed. (D) ConA blasts pulsed either with Flu-NP-Kᵈ (closed circles), HIVp (closed squares), or both (closed diamonds) were lysed by Ly49A⁻ NK cells, while normal ConA blasts (open circles) remained resistant to lysis. The figure is representative of 3 independent experiments.
Figure 2-7

Ly49A⁺ LAK

Ly49A⁻ LAK

% specific lysis

Effector : Target ratio
The observation that HIVp-pulsed targets were not lysed by Ly49A⁺ NK cells is fully consistent with the hypothesis that Ly49A recognizes peptide-loaded D⁰ molecules and prevents lysis that would otherwise have occurred. That these targets were lysed when Ly49A molecules on the NK cells were covered up by F(ab')₂ anti-Ly49A mAb and were also lysed when Ly49A⁻ NK cells were used as NK effectors is consistent with the existence of inhibitory receptors recognizing empty MHC-I as hypothesized in the preceding sections. An explicit model that is consistent with all the lysis results of Fig 2-7, in which resistance to lysis depends upon the total number of possible inhibitory signals is given in the Discussion (Table 2-3).
2.5 DISCUSSION

Self-recognition involves empty MHC-I molecules. We here confirm previous results (38, 39) that normal ConA blasts become sensitive to lysis by syngeneic NK cells when incubated with peptide that can bind to their MHC-I molecules (Fig. 2-1). The process does not seem to be peptide-sequence specific in that all peptides tested sensitized targets for NK lysis provided that they could bind to MHC-I in the peptide pulsing procedure. Concentrations of 1-10 pg/ml of the peptide tested (all of which bind with high affinity) were sufficient to produce significant sensitization (Fig 2-1). For comparison, concentrations 100-fold lower are sufficient to sensitize ConA blasts to lysis by CTL lines specific for the same or similar high affinity peptides (75).

It is now widely accepted that if an activating receptor on an NK cell recognizes a cell, that cell will be killed unless an inhibitory receptor on the NK cell also recognizes the cell. According to this model, the added peptide in our system must be altering the target cell either by creating a new ligand recognized by an activating receptor or by altering or masking a ligand recognized by an inhibitory receptor (or possibly both). To distinguish between these two possibilities, the most powerful approach is to use specific mAb F(ab')2 fragments against the ligand as has been done to block the inhibitory-MHC-I interaction (20, 301). However, this approach cannot yet be used in this study because the putative receptor involved in our system has yet to be identified (except that Ly49A is not involved). We have relied on cold target competition as an alternative for providing insight into the nature of the ligand affected by peptide-pulsing and found that unlabelled ConA lymphoblast targets, whether or not peptide pulsed, were equally effective competitors for NK-mediated lysis of labeled peptide-pulsed targets (Fig. 2-2). We also found, using flow cytometry, that ConA blasts pulsed or not pulsed with peptide were equally effective in forming conjugates with FITC-labeled NK cells (Fig. 2-3). Similarly, Ljunggren et al. found that the cell line RMA (moderately resistant to NK lysis) and RMA-S, a mutant cell line derived from it and highly sensitive to NK lysis, were equivalent in their ability to bind NK cells (125). Taken together with the fact that only peptide-pulsed targets are lysed, we conclude that the added peptide is most likely altering or masking the ligand recognized by an NK inhibitory receptor. The BFA experiments support this conclusion.
Preventing surface arrival of proteins should not affect an activating ligand, which is already there.

The observation that the ligand recognized by the NK inhibitory receptor operative in this system and the CTL receptor are affected differently by BFA leads to the hypothesis that they are recognizing different ligands and, in particular, that the inhibitory NK receptor recognizes the empty form of MHC-I molecules on syngeneic ConA blasts. The data of Figures 2-1 to 2-5 can be explained by and support this hypothesis. Thus, normal ConA blasts can be recognized by syngeneic NK cells but their lysis is normally prevented when negative inhibitory signals generated by recognition of empty MHC-I molecules are above some threshold level and override the activation signal in the NK cell. Pulsing normal ConA blasts with peptide of high binding affinity fills most (if not all) empty MHC-I molecules and thus reduces the negative inhibitory signal below the threshold level in some NK cells and renders the ConA blasts susceptible to lysis (Fig. 2-1). When peptide-pulsed ConA blasts are incubated in the absence of exogenous peptide (preincubation experiment, Fig. 2-3A), newly synthesized empty MHC-I molecules are transported to and expressed on the cell surface where they regenerate the inhibitory structure that increases the inhibitory signal above the threshold level and thus prevents NK lysis. Furthermore, if MHC-I-specific peptide is added to the target cells again, lysis can be restored (Table 2-1). If the "regeneration" of the inhibitory structure is prevented by blocking the expression of newly synthesized MHC-I in the preincubation step (BFA experiment, Fig. 2-4), the targets remain sensitive to lysis.

An understanding of the number of "empty" MHC-I molecules on the cell surface under varying conditions is central to our model. New empty MHC-I molecules might arise on the cell surface through loss of peptide from the trimolecular complex on the cell surface as both peptide and β2m can freely and independently disassociate from the trimolecular complex (33, 34, 76, 302). Alternatively, newly synthesized empty MHC-I molecules can also arrive at the cell surface (32, 97). Whether MHC-I molecules are truly empty or contain peptide binding with low affinity (pL) and readily lost is not clear. To distinguish between these possibilities, we directly examined the relative frequency of full and empty K<sup>b</sup> on B6 ConA blasts pulsed with a high affinity binding peptide (OVAp) and then incubated for 90 min in the presence or absence of BFA (Fig. 2-6). In the presence of BFA, which should prevent the emergence of new empties from the cell interior, no new empties were detected after a 90 min incubation, implying
that the trimolecular complex has a half-life much greater than 90 min and that little peptide was lost during this 90 min incubation. There is no direct measurement of the half-life of this trimolecular complex. However, a possibly similar Flu-NP-D\textsuperscript{b} trimolecular complex (the same as studied here, see Fig. 2-1) is known to have a half-life of 10h (76, 186). In the absence of BFA, a significant number of new empties appeared on the cell surface (Fig. 2-6). We assume that these are newly synthesized molecules exported from the cell interior. Their appearance correlates with the disappearance of sensitivity to NK lysis (Fig. 2-4, 2-5). In examining the effect of BFA on normal B6 ConA blasts, we found that empty MHC-I were still detected even after the cells were incubated for 4h in the presence of BFA at 37\textdegree C, although a decrease in the level of empty MHC-I expression was observed. Given that the \(\alpha\)-\(\beta\textsubscript{2m}\) forms of \(\text{D}^b\) and \(\text{K}^b\) molecules have been reported to have half-lives much less than 2h (34, 97, 283), one might have expected unpulsed ConA blasts to have lost all their empty MHC-I molecules during the incubation with BFA. That they did not has two possible explanations: (i) New empty MHC-I molecules are continuously formed through loss of low affinity peptide from trimolecular MHC-I complexes (\(p_L\)-\(\alpha\)-\(\beta\textsubscript{2m}\)) already on the cell surface. (ii) Measurements of \(\alpha\)-\(\beta\textsubscript{2m}\) half-lives have been made by extracting \(\alpha\)-\(\beta\textsubscript{2m}\) complexes from the cell surface with mAb. Molecules embedded in the membrane of a normal, viable cell may be more stable. We conclude that the ligand for the inhibitory receptor operative in our system is most likely to be empty MHC-I.

The model that added peptides might be displacing protective "self" peptides is rendered unlikely by the current data. A 45-min pulse with a high affinity peptide produced a state of sensitization (Fig 2-1). If the high affinity peptide is displacing particular protective self-peptides, then they must be bound with low affinity to be displaced in such a short time pulse (34). This then implies that control target cells not pulsed with high affinity peptide and then incubated with BFA should have become sensitive to lysis as the protective self-peptide was lost. This was not seen (Fig 2-5).

An as-yet-unidentified inhibitory receptor, differing from Ly49A, recognizes the bimolecular form of the \(\text{D}^d\) molecule. As described in the Introduction, three groups have shown that, under appropriate conditions, addition of MHC-I binding peptides to a target can prevent NK lysis (41, 195, 282). To reconcile the difference between these published and our experimental data, we studied the recognition of the \(\text{D}^d\) molecule by Ly49A\textsuperscript{+} and Ly49A\textsuperscript{-} NK subsets in our syngeneic experimental system. We found that the Ly49A\textsuperscript{+} subset of NK cells
could not lyse syngeneic D\textsuperscript{d}-bearing ConA blasts pulsed with D\textsuperscript{d}-binding peptide (Fig 2-7), consistent with the Ly49A inhibitory receptor recognizing the D\textsuperscript{d} trimolecular complex and providing a dominant negative signal (Fig 2-7). In agreement with this, the targets were lysed when Ly49A was covered up by F(ab')\textsubscript{2} anti-Ly49A mAb. The Ly49A\textsuperscript{+} subset of NK cells killed the same syngeneic D\textsuperscript{d}-bearing ConA blasts pulsed with D\textsuperscript{d}-binding peptide, consistent with our hypothesis that there might be an as-yet unidentified inhibitory receptor (which may or may not be a member of the Ly49 family) that recognizes the empty form of the D\textsuperscript{d} molecule.

In support of our hypothesis that there are inhibitory receptors recognizing empty MHC-I molecules, a recent report (197, 303) concluded that cell-surface expression of human MHC-I molecules, in the absence of peptide, was both necessary and sufficient to inhibit HLA-specific human NK lines and clones. They transfected RMA-S cells with human HLA-C of two different allotypes along with human \(\beta_2m\). Culture of the cells at 26°C without exogenous peptide allowed for high expression of the transfected MHC-I and this persisted for at least 2h after the cells were transferred to 37°C. Presence of a particular empty HLA-C allotype was sufficient to inhibit lysis by an NK clone specifically inhibited by that allotype. Note that the inhibitory receptors involved in this study are most likely the members of NKIR (natural killer inhibitory receptor) family, structurally unrelated to the Ly49 family (160, 161).

Here, we propose a teeter-totter model for resistance vs. sensitivity to NK lysis. Correa et al. have shown that the Ly49A-D\textsuperscript{d} interaction is sufficient to inhibit all types of NK cell activation pathways that have been examined, but the contrary has been observed in this study (193). Our data showed that Ly49A\textsuperscript{+} NK cells could lyse D\textsuperscript{d}-expressing ConA blasts pulsed with Flu-NP-K\textsuperscript{d} peptide even if they were also pulsed with D\textsuperscript{d}-binding peptide (Fig 2-6c). This apparent discrepancy can be explained by the following model: (i) Individual NK cells have different inhibitory receptors that can recognize either empty or full MHC-I molecules. (ii) The strength of the inhibitory signal generated by a particular receptor is proportional to the number of MHC-I molecules it can recognize. (iii) For inhibition of lysis to occur, the summation of all inhibitory signals must exceed some critical threshold value.

Let us apply this model to all the data of Fig 7 using Ly49A\textsuperscript{+} NK cells (Table 2-3). We assume that 10\% (0.1) of K\textsuperscript{d} and D\textsuperscript{d} molecules on the ConA blasts used are empty, as has been reported (76, 186) for D\textsuperscript{b} molecules, but would reach the same conclusions for any value greater than zero and less than 1. For normal ConA blasts (line 1, Table 2-3), there is a total inhibitory
Table 2-3: Model for recognition by Ly49A⁺ NK cells including two additional inhibitory receptors.

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Lysis seen in Fig 6</th>
<th>D^d(f) *</th>
<th>D^d(e) *</th>
<th>K^d(e) *</th>
<th>Summation of inhibitory signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoblast</td>
<td>No</td>
<td>0.9 **</td>
<td>0.1</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Lymphoblast + D^d-peptide</td>
<td>no</td>
<td>1.0</td>
<td>0</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Lymphoblast + K^d-peptide</td>
<td>yes</td>
<td>0.9</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lymphoblast + K^d-peptide +D^d-peptide</td>
<td>yes</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lymphoblast + JR9-318 F(ab')_2</td>
<td>yes</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Lymphoblast + D^d-peptide + JR9-318 F(ab')_2</td>
<td>yes</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Lymphoblast + K^d-peptide + JR9-318 F(ab')_2</td>
<td>yes</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note: * D^d(f)- inhibitory signal from Ly49A receptor recognizing peptide-bound D^d
D^d(e)- inhibitory signal from receptors recognizing empty D^d
K^d(e)- inhibitory signal from receptors recognizing empty K^d
** The numbers are the inhibitory signal strengths assigned to each receptor involved in NK-recognition.
signal of 1.1 (0.9 (from Ly49A recognizing peptide-bound D^d) plus 0.1 (from a new receptor recognizing empty D^d) plus 0.1 (from a second new receptor recognizing empty K^d); no lysis is seen. When the ConA blasts are pulsed with D^d-specific peptide (line 2, Table 2-3), the total inhibitory signal remains as 1.1, because as the Ly49A signal goes up by 0.1, the inhibitory signal generated by the receptor recognizing empty D^d goes down by the same amount, 0.1, and again no lysis is seen. However, when they were pulsed with K^d-specific peptide (line 3) or with both K^d- and D^d- specific peptide (line 4), the total inhibitory signal falls to 1.0; lysis is now seen. In going down Table 2-3, one sees that lysis was observed whenever the summation of inhibitory signals was 1.0 or less. Comparison of lines 2 and 4 is particularly interesting in that pulsing ConA blasts with D^d-binding peptide alone does not block inhibition (line 2) but pulsing ConA blasts with both K^d- and D^d-binding peptide does (line 4).

The model implies that there is a critical balancing of activating and inhibitory signals leading either to sensitivity or resistance to lysis. It is much like a teeter-totter in a children's playground in which a given end is either fully up or fully down depending upon the balance of the forces last acting on the two ends. Whether there is a subset of B6 NK cells with an inhibitory receptor that recognizes peptide-bound K^d molecules cannot be determined from these data as inhibition or activation of such a subset is difficult to detect unless the subset is relatively pure. We could detect the effect of the Ly49A inhibitory receptor in our system only after purifying Ly49A^+ cells.

Most previous studies (for an exception, (304)) supporting the existence of negative-signaling NK receptors have involved the protection from lysis of allogeneic target cells recognized by inhibitory receptors. The data presented here provide direct evidence that negative-signaling receptors can also protect normal syngeneic target cells from lysis. They also suggest a possible explanation as to why some virus infected cells become targets for syngeneic NK cells: As a result of the virus infection very few empty MHC-I molecules are exported to the cell surface either because very large quantities of viral peptide inside the cell saturate MHC-I or because the virus greatly reduces overall MHC-I production such that few empties (albeit a higher percentage of all class I) reach the cell surface.
CHAPTER 3

LY49C$^{66}$ NK INHIBITORY RECEPTOR RECOGNIZES
"PEPTIDE-RECEPTIVE" H-2K$^{b}$. 

3.1 ABSTRACT:

NK-mediated cytotoxicity involves two families of receptors: activating receptors that trigger lysis of the target cells being recognized and inhibitory receptors specific primarily for MHC-I on the target cell surface that can override the activating signal. MHC-I molecules on the cell surface can be classified into molecules made stable by the binding of peptide with high affinity or unstable molecules potentially capable of binding high affinity peptide (hence “peptide receptive”) and being converted into stable molecules. It has been previously shown that the Ly49A inhibitory receptor recognizes stable D\textsuperscript{d} molecules. We show here that the inhibitory receptor Ly49C\textsuperscript{86} recognizes “peptide receptive” K\textsuperscript{b} molecules but does not recognize K\textsuperscript{b} molecules once they have bound high affinity peptide.
3.2 INTRODUCTION:

It is widely accepted that NK cells recognize and lyse target cells through the interplay of two families of receptors (25, 27, 126, 156, 281). Activating receptors can trigger lysis of the target cell being recognized. The activating signal, however, can be overridden by a negative signal from an inhibitory receptor when the latter interacts with its ligand (if present) on the target cells. The ligand(s) for activating receptors are not yet clearly defined, but the only ligands identified to date for inhibitory receptors are associated with MHC-I alleles (25, 27, 126, 156, 281). Ly49A has been shown to bind to H-2D<sup>d</sup> and D<sup>k</sup>, and can inhibit the lysis of target cells expressing these molecules (20, 305). The same appears true for Ly49C. Thus, COS-7 cells transfected with Ly49C receptor can bind to H-2<sup>b</sup>, H-2<sup>e</sup>, H-2<sup>d</sup> and H-2<sup>k</sup> cell lines in cell-cell adhesion assays (306) and the presence of Ly49C has been shown to be responsible for the resistance to lysis of K<sup>b</sup>-expressing target cells by 5E6<sup>+</sup> F1 (NZB x B6) NK cells in cytotoxicity assays (307).

Most MHC-I molecules on the normal cell surface exist as ternary complexes, each composed of a properly folded heavy chain (α) containing the peptide binding groove, a non-covalently associated β<sub>2</sub>- microglobulin (β<sub>2m</sub>), and a peptide (p) (34). The stability of the complex depends primarily on the binding affinity of the peptide. The ternary complex p<sub>H</sub>-α-β<sub>2m</sub> (associated with high-affinity peptide, p<sub>H</sub>) is most stable, with a half-life of 10 h or more (34). The complex p<sub>L</sub>-α-β<sub>2m</sub>, in which the peptide is either too long or lacks the proper binding motif and thus binds with low affinity (therefore, p<sub>L</sub>), is also present and is less stable (97). Three other forms of MHC-I, all unstable, αβ<sub>2m</sub>, p-α and α (perhaps in decreasing order of stability) can also be found on the cell surface (33, 34). Collectively, these have a half-life of 30 min or less (34).

MHC-I molecules capable of binding exogenously added peptides are present on the surface of both normal and TAP-deficient (e.g., RAM-S) cells, and are conventionally referred to as "empty" MHC-I molecules. It is essentially unknowable, however, to what extent these molecules are truly empty, i.e., contain solvent in their binding groove (e.g., α-β<sub>2m</sub> or α) versus a weakly bound peptide (e.g., p<sub>L</sub>-α-β<sub>2m</sub>) (74). Nonetheless, the exogenously added peptide is most likely to bind to α-β<sub>2m</sub> and/or displace the p<sub>L</sub> in the p<sub>L</sub>-α-β<sub>2m</sub> complex (after the p<sub>L</sub> dissociates), instead of α chain. Binding to α chain alone is
probably unlikely as it is thought to be highly unstable on the cell surface at 37°C (33, 34). We, here, refer to all forms of MHC-I that can bind exogenous peptide of high affinity as “peptide-receptive” MHC-I (PR-MHC-I) (74). This is an operational definition. PR-MHC-I molecules are most likely the αβ2m binary complex and the pL-αβ2m ternary complex, but may also include α alone. Approximately 10% of D<sup>b</sup> molecules expressed on the surface of EL-4 tumor cells are peptide receptive (76, 186).

Several studies have focused on defining the regions of MHC-I involved in the recognition by NK inhibitory receptors. By performing exon-shuffling and point-mutation experiments on human MHC-I, Storkus et al. showed that the α<sub>1</sub>-α<sub>2</sub> region of the α chain appears to be critical in determining the specificity of MHC-I (HLA-A and HLA-B) as an inhibitory ligand (36), and that the amino acids in the peptide binding groove of MHC-I are important in conferring NK resistance (308). For mouse MHC-I, Karlhofer et al. (20), and Sundbäck et al. (40) have mapped the determinant recognized by the inhibitory receptor Ly49A to the α2-region of the D<sup>d</sup> molecule.

Several studies have addressed the question of whether the presence of peptide in the peptide-binding groove within the α<sub>1</sub>-α<sub>2</sub> region is critical in recognition by an NK inhibitory receptor. Storkus et al., using C1R cells that are normally lysed by human NK cells, found that they could be protected from lysis by transfection of certain HLA-A or HLA-B MHC-I and that protection was reversed by the addition of peptide that could bind to the protective MHC-I (192). Chadwick and Miller (38), and Chadwick et al. (39) found that normal non-transformed lymphoblasts could be lysed by syngeneic mouse NK cells in the presence of peptide specific for their MHC-I. They tested 9 different peptides specific for K<sup>b</sup>, D<sup>b</sup>, K<sup>d</sup>, L<sup>d</sup>, or D<sup>d</sup>. All of these peptides could sensitize a normal lymphoblast to be lysed if they could bind to it. Using a similar system, Su et al (309) found that the acquisition of sensitivity to lysis correlated with the disappearance of “PR” MHC-I. When lymphoblasts made sensitive to NK lysis by being pulsed with peptide were incubated in the absence of peptide, they lost their sensitivity to lysis as they reacquired PR-MHC-I, a process which took about 90 min. When production of new peptide receptive MHC-I was inhibited (by inhibiting transport of new cell surface protein through the trans-Golgi) lymphoblasts remained sensitive to NK lysis. These experiments led to the hypothesis that there are NK inhibitory receptors that
recognize PR-MHC-I. The hypothesis would gain considerable credibility if one could actually identify an NK inhibitory receptor reactive against PR-MHC-I.

Ly49A is the best characterized NK inhibitory receptor. Results of Correa and Raulet (282) and Orihuela et al (41) show that Ly49A recognizes not the peptide receptive but the peptide bound Dd molecule. These groups transfected TAP-deficient RMA-S (282) or LKD8 (41) cells with Dd and showed that the protection of Dd-transfected cells from lysis mediated by Ly49A+ B6 NK cells requires peptide-binding to the Dd molecule. The extent of protection correlated with the extent to which the added peptide stabilized Dd expression. Both groups suggested that the role of peptide was to promote the assembly and cell surface expression of MHC-I and that there was no peptide specificity in Ly49A recognition of Dd. Su et al (309), using Ly49A+ and Ly49A- BALB/c (H-2d) NK cells, investigated the effect of pulsing BALB/c lymphoblasts with a high affinity Dd-binding peptide. The results obtained were consistent with those just summarized i.e. Ly49A+ cells did not lyse Dd+ normal lymphoblasts either before or after they were pulsed with a high affinity Dd-binding peptide. However, pulsing the lymphoblasts with high affinity Kd peptide sensitized these Dd+ normal lymphoblasts to be lysis by Ly49A+ cells, whether or not they were also pulsed with Dd peptide, thus implying the existence of an inhibitory receptor reactive against PR-Kd and also implying that it is the total balance of inhibitory and stimulatory signals that determines whether there is lysis.

In this report, we have investigated whether another well-characterized NK inhibitory receptor, Ly49C, recognizes peptide-bound or "peptide receptive" Kb. We have used both a syngeneic experimental system (309) and an in vitro hybrid-resistance model (38 in which inhibitory function of Ly49A and 5E6 antigens has been demonstrated (307)). 5E6 mAb stains B6 LAK cells expressing Ly49C, and/or Ly49I receptors (140). Most of our study has used 5E6+ and 5E6- B6 LAK sub-populations as effector cells, but we also show that Ly49C- I' (5E6"4L03311") B6 LAK cells are not inhibited in the presence of either "peptide receptive" or peptide-bound Kb. We conclude from this study that the Ly49C6 NK inhibitory receptor recognizes the "peptide-receptive" form of the Kb molecule.
3.3 MATERIALS AND METHODS:

3.3.1 Mice:
Normal C57BL/6 (B6, H-2^b), BALB/c (H-2^d), and F1 (BALB/cxB6, H-2^bd) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 (H-2^b) athymic nude mice were purchased from Taconic, Germantown, NY. F1 (BALB/cxB6, H-2^bd) athymic nude mice were purchased from the Jackson Laboratory. D^b/- B6 mice (D^b-K^b+) and K^b/- B6 mice (D^b+K^b-) have been previously described (310, 311). They were generous gifts from Dr. F. Lemonnier (Pasteur Institute, Paris, France), and were bred in our animal facility. All mice were kept in a specific pathogen free environment. In most experiments, 6-10 week old female mice were used (although either sex gave similar results).

3.3.2 NK generation:
The method used for producing activated NK cells (LAK cells) was identical to that used previously (38, 309, 312). Briefly, 2x10^6 nylon wool non-adherent spleen cells from B6 athymic nude mice were cultured at 37°C for 3-4 days in 5 ml α-MEM supplemented with 10% FCS, 50 μM 2-ME, and 10 mM HEPES buffer (hereafter referred to as 10% CM), containing 500 U/ml mouse rIL-2. In some experiments, as specified, F1 (BALB/cxB6) athymic nude mice were used. Mouse rIL-2 was obtained as a supernatant from a cell line transfected with the IL-2 gene (285). These cells were cultured in 25 cm^2 flasks at 37°C in a 10% CO_2 in air incubator. Yields typically exceeded 1200 U/ml of rIL-2.

3.3.3 Target cell generation:
Target cells were B6, BALB/c or F1(BALB/cxB6) Concanavalin A (ConA, ICN Pharmaceuticals Canada Ltd., Montreal, Que.) activated lymphoblasts (ConA blasts), produced by incubating 10^7 splenocytes for 3 days in 10 ml CM supplemented with ConA (2 μg/ml). On day 3, ConA blasts were harvested on Lympholyte M (Cedarlane Lab., Hornby, ON) and ^51Cr-labelled by incubating about 6x10^6 cells for 90 min at 37°C with 360 μCi Na^51CrO_4 (NEN Life Science, Boston, MA) in 150 μl of PBS containing 67% FCS. They were then washed 3 times with 1% CM (containing 1% FCS instead of 10% FCS), to remove non-incorporated Na^51CrO_4.
3.3.4 MHC-I binding Peptides:
The effect of MHC-I binding peptides on normal ConA blasts sensitivity to NK lysis was assessed by pulsing ConA blasts with the experimental peptide (at a concentration of 100 ng/ml in CM, unless stated otherwise) for 45 min at 4°C before the assay. Peptides utilized were K\textsuperscript{b}-restricted epitopes of Chicken Ovalbumin, SIINFEKL, (OVAp\textsubscript{258-265}) (287) and Vesicular Stomatitis Virus NP, RGYVYQGL, (VSVp\textsubscript{52-59}) (75), a D\textsuperscript{b}-restricted epitope of influenza nucleoprotein, ASNENMETM, (Flu-NP\textsubscript{366-374}) (286), and a D\textsuperscript{d}-restricted epitope of HIV gp160, RGPGRAFVTL, (HIVp\textsubscript{318-327}) (288). Chicken Ovalbumin, SIINFEKL(OVAp\textsubscript{258-265}), and its derivative, biotinylated Ovalbumin peptide, SIINFEK(bio)L were prepared by the Ontario Cancer Institute Biotechnology Laboratory, using an Applied Biosystems Peptide Synthesizer (Applied Biosystems, Foster City, CA). Both VSVp\textsubscript{52-59} and Flu-NP peptides (>90% purity) were generous gifts from Dr. B. H. Barber (University of Toronto). HIVp (>90% purity) was a gift from Dr. D. Williams (University of Toronto). OVAp, and VSVp peptides are natural ligands for K\textsuperscript{b} and bind to K\textsuperscript{b} with high affinities (286, 287, 289). HIVp peptide is a natural ligand for D\textsuperscript{d} and binds with high affinity (288).

3.3.5 Cytotoxicity Assay:
Methods for measuring lytic activity were identical to those used previously (39, 155, 309). After 3 washes, \textsuperscript{51}Cr -labeled ConA blasts were incubated with peptide in 3 ml of 10% CM for 45 min at 4°C and washed again before being used in a 4 h \textsuperscript{51}Cr release assay performed in 96-well V-bottom microtiter plates using 2000 targets/well, dispensed in 100 μl aliquots and effectors at an E:T ratio as indicated or at 30:1, also added in 100 μl aliquots. For experiments in which pre-incubation of NK cells with 5E6 mAb was required, the pre-incubation was done at 4 °C for 30-45 min while preparing target cells for the assay. For experiments in which pre-incubation of target cells with 5F1, Y-3, or 25D1.16 mAb (5 μg/ml/2x10\textsuperscript{5} cells) was required, the pre-incubation was done at 4 °C for 20-30 min. Prior to the addition of mAb to target/effecter cells, mAb were pre-incubated with soluble Protein A (2μg per 10μg of mAb used, Sigma, St. Louis, MO) and soluble ProteinA/G mix (2μg per 10μg of mAb used, ICN Biomedicals Inc., Aurora, OH) for 30 min on ice. Soluble Protein A and G bind to the Fc portion of antibody, block the association of antibody to FcRIII on NK cells, and thus, prevent antibody-dependent cellular cytotoxicity (ADCC). The mAb remained in the assay mixture
during the 4h $^{51}$Cr-release assay. Specific lysis was calculated as % specific lysis = $(E-S)/(T-S)$ x 100 where each value represents the mean ± S.E.M. of five replicates. E is the experimental mean of $^{51}$Cr released; S, the amount of $^{51}$Cr released when the target cells were cultured in medium alone; and T, the total amount of $^{51}$Cr released in the presence of 2% acetic acid. Dialyzed FCS (12 kDa cut off) was regularly used in place of regular FCS during the $^{51}$Cr labeling, pulsing and assay stages (186, 291).

3.3.6 Flow cytometry/FACS Analysis:
To measure the disappearance of PR- K$^b$ using OVAp-K$^b$, day 3 B6 ConA blasts were pre-pulsed with increasing concentrations of non-labeled OVAp for 30-45 min to convert PR- K$^b$ to peptide-bound K$^b$. Unbound OVAp was removed and the cells were then incubated at 4°C for 45 min with biotinylated OVAp peptide (OVAp$^b$,1 μg/ml). The binding of biotinylated OVAp was visualized with R-phycoerythrin conjugated streptavidin (SA-PE, Sigma) which binds to biotin, and analyzed using a FACScan flow cytometer and LYSIS II program (Becton Dickinson). The maximum level of PR- K$^b$ was measured by omitting the addition of OVAp during the peptide-pulsing procedure. The relative level of PR- K$^b$ was calculated as the fraction of the mean fluorescent intensity (MFI) detected when a particular OVAp concentration was used over the MFI of the maximum level of PR- K$^b$ and is plotted against the OVAp concentrations used. Alternatively, the presence of PR- K$^b$ molecule on the cell surface can be measured with OVAp-binding (Fig 3-2 B,C), which can then be visualized using FITC-conjugated 25D1.16 mAb (313). 25D1.16 mAb was purified from the supernatant of 25-D1.16 hybridoma (re-clone #21) culture, a generous gift from Dr. R.Germain (NIH, Bethesda, MD). 25D1.16 mAb binds specifically to OVAp-K$^b$ complex, and not VSVp-K$^b$ complex or any other peptide-K$^b$ complex (313). The level of PR- K$^b$ on the cell surface was titrated by pulsing the B6 ConA blasts with increasing concentrations of VSVp for 30-45 min and then washing away unbound VSVp. The remaining PR- K$^b$ molecules were detected by pulsing with OVAp (1 μg/ml/10$^6$ cells for 30-45 min) and FITC conjugated 25D1.16 mAb, which detects the OVAp-K$^b$ complexes. The maximum level of PR- K$^b$ was measured when no VSVp was used assuming that OVAp would bind to all the PR-K$^b$ molecules. In separate kinetics experiments (unpublished), it was found that 1 μg of OVAp/ml/10$^6$ cells produced half-maximum binding within 15 min and saturation binding
within 45 min. The relative level of PR-K\textsuperscript{b} is calculated as the fraction of the mean fluorescent intensity (MFI) detected when a particular VSV\textsubscript{p} concentration is used over the MFI of the maximum level of PR-K\textsuperscript{b} and is plotted against the concentrations of VSV\textsubscript{p} used.

### 3.3.7 Cell Sorting:

Day 3 or day 4 B6 LAK cultures were harvested and resuspended in 1% BSA/PBS (10\textsuperscript{7} cells/ml). The cells were then incubated with 5E6 mAb (4 µg per 10\textsuperscript{6} cells, Pharmingen), at 4°C on a rotator for 45 min. 5E6 mAb recognizes the NK inhibitory receptors, Ly49-C and I (140). Stained cells were washed with cold 1% BSA/PBS and then sorted (Coulter Epics V) into 5E6\textsuperscript{+} and 5E6\textsuperscript{-} sub-populations. Ly49C\textsuperscript{+} (5E6\textsuperscript{*}4LO3311\textsuperscript{*}) and Ly49CT\textsuperscript{+} (5E6\textsuperscript{*}4LO3311\textsuperscript{*}) B6 LAK sub-populations were isolated by sorting 5E6\textsuperscript{+} B6 LAK cells (day 5 or day 6) stained with biotinylated 4LO3311 mAb (140) (1µg/10\textsuperscript{6} cells, specific for Ly49C). F1 (BALB/cxB6) LAK cells were sorted into 5E6\textsuperscript{*}Ly49A\textsuperscript{*} and 5E6\textsuperscript{*}Ly49A\textsuperscript{-} sub-populations using FITC-labeled 5E6 (4 µg/10\textsuperscript{6} cells), and biotinylated JR9.318 mAb (4 µg/10\textsuperscript{6} cells). JR9-318 mAb binds specifically to Ly49A (146); the hybridoma was obtained from Dr. D. Raulet with permission of Dr. J. Roland (Pasteur Institute, Paris, France). Sorted cells were cultured in 5 ml CM containing 500 U/ml mouse rIL-2 (285) for an additional 2-3 days as above.

### 3.3.8 F\textsubscript{ab} Fragment Generation:

For F\textsubscript{ab} fragment generation, 8 mg of affinity purified anti-K\textsuperscript{b} mAb (5F1) was resuspended in 1 ml of digestion buffer (20 mM phosphate, 20 mM cystein-HCl, 10 mM EDTA-Na\textsubscript{4}, pH 7.0) and then digested with 0.5 ml of immobilized papain (Pierce, Rockford, IL) for 10 h in a shaker at 37°C at high speed. The reaction was stopped by adding 1.5 ml of 10 mM Tris-HCl (pH 7.5) to the digestion mixture as suggested by the manufacturer's protocol (Pierce, Rockford, IL). After a centrifugation at 10,000 rpm for 5 min, the supernatant was collected and mixed with Protein A/G-Sepharose beads (ICN Biomedicals Inc., Aurora, OH) to remove undigested Ab and Fc fragments. The purity and the binding activity of F\textsubscript{ab} fragments were checked by 10% SDS-PAGE and flow cytometry, respectively.
3.3.9 COS-7 Cell Expression and Cell-Cell Adhesion Assay:
Ly49C^B6 and Ly49f^B6 cDNAs were inserted into the multiple-cloning site in PCI-neo mammalian expression vectors (Promega, Madison, WI). Transfection of COS-7 cells (gift from Dr. M. B. Wheeler, University of Toronto) with Ly49 cDNA was carried out using Lipofectamine (Life Technologies, Baithersburg, MD), following the manufacturer's instructions. Briefly, one day prior to transfection, COS-7 cells were seeded in 6-well plates (2x10^5 cells in 2 ml of 10% CM per well) and incubated at 37°C for sufficient time (18-24 h) to reach 50-80% confluence. On the day of transfection, 2 μg of DNA were incubated with 10 μl of lipofectamine reagent diluted in 750 μl of DMEM (free of serum and antibiotics) at room temperature for 30 min. COS-7 cells were washed once with 2 ml of DMEM (Dulbecco's MEM H21, free of serum and antibiotics) prior to the addition of transfecting reagent (DNA-lipofectamine complexes). At the end of a 5 h incubation at 37°C, transfecting reagent was replaced with 2 ml DMEM supplemented with 20% FBS. One day following transfection, COS-7 cells were trypsinized and transferred to 24-well plates at 0.5~16 cells per well. Three days post-transfection, day 3 ConA blasts (6x10^6 cells) were labeled for 90 min at 37°C with 360 μCi Na^51CrO_4 (NEN Life Science Products, Boston) in 150 μl of PBS containing 67% FCS. They were then washed 4 times with 1% CM, to remove non-incorporated Na^51CrO_4. These target cells were incubated with OVA_p (1μg/ml/10^6 cells), mAb (5F1, or Y-3, 50μg/ml/10^6) or 1% CM alone on ice for 45 min and tested for adhesion to COS-7 cells by addition to the wells at 4x10^5 cells/well in 0.5 ml. As indicated in Figure 3C, COS-7 cells were incubated with 5E6 mAb (20μg/well/0.2 ml) at room temperature 30 min prior to the addition of target cells. Plates were centrifuged for 5 min at 200g and incubated for 2 h at 37°C in a CO_2 incubator. At the end of incubation, plates were washed 5 times with pre-warmed media and then photographed. Bound ConA blasts were lysed with 500μl/well of 10% Triton X-100 (Sigma Chemical Co. St. Louis, MO) and the radioactivity was determined by subjecting 250 μl/well of cell lysate to γ-counting. Cell binding was calculated as percent cells bound = (E / T) x 100 where each value represents the mean ± S.E.M. of quadruplicate wells. E is experimental mean of the radioactivity in the lysate; and T is the total amount of radioactivity in the 4x10^5 ConA blasts added to each well.
3.4 RESULTS:

3.4.1 NK-resistant B6 ConA blasts became susceptible to lysis by 5E6+ B6 LAK on binding either peptide or selected antibodies specific for K\(^b\).

The mAb 5E6 identifies an inhibitory receptor that recognizes H-2K\(^b\) (307). We performed experiments to identify which form of K\(^b\) this receptor was recognizing, in particular whether it recognizes PR-K\(^b\). IL-2 activated NK (LAK) cells from B6 nude mice (lack T-cells) or normal B6 mice were sorted into 5E6+ and 5E6- populations and used as effectors. Targets were ConA activated B6 blasts (B6 ConA blasts) either unpulsed or pulsed with CM containing MHC-I specific peptide before being used as target cells in a 4-h \(^{51}\)Cr-release assay. As shown in Table 3-1, B6 ConA blasts were resistant to lysis by either 5E6+ or 5E6- B6 LAK cells (lines 1, 8). They became significantly susceptible to lysis by 5E6+ B6 LAK cells when pulsed with K\(^b\)-specific peptides (OVAp or VSVp) (Table 3-1: lines 2, 3). Further, the masking of 5E6 antigens on 5E6+ B6 NK cells resulted in the lysis of unpulsed syngeneic ConA blasts (line 4). K\(^b\)-peptide pulsed B6 ConA blasts remained resistant to lysis by 5E6- B6 LAK cells (lines 9, 10) and the addition of 5E6 mAb had little effect (line 11). These results are consistent with there being an inhibitory receptor present in the 5E6+ population and absent in the 5E6- population that recognizes PR-K\(^b\).

As a further test of this hypothesis, we examined the effect of mAbs recognizing different forms of K\(^b\). The mAb Y-3 recognizes both PR and stable ternary complexes of K\(^b\) (314, 315). Addition of Y-3 mAb led to the lysis of B6 ConA blasts by 5E6+ B6 LAK cells but not by 5E6- B6 LAK cells (Table 3-1: lines 5,12). Soluble Protein A and Protein G were used to pre-coat the Fc portions of mAbs used in the assay to block ADCC. The mAb 5F1 recognizes primarily stable ternary K\(^b\) complexes (295). Addition of either intact or F\(_{ab}\) fragments of 5F1 mAb had little effect on the resistance of B6 ConA blasts to lysis by either 5E6+ or 5E6- B6 LAK cells (lines 6,7,13,14). Nor did the addition of isotype control antibody (IgG2b, anti-TNP mAb) have any effect on the resistance of B6 ConA blasts to lysis (lines 1,8). In separate control experiments, it was shown that both Y-3 and 5F1 mAbs can bind to K\(^b\) under the conditions used (data not shown).

None of the 5E6- groups showed lysis above background. This is consistent with there being no inhibitory receptor for PR-K\(^b\) in the 5E6- NK sub-population. To verify that
Table 3-1. NK-resistant B6 Con A blasts became susceptible to lysis by 5E6⁺ B6 LAK on binding Kᵇ-specific peptide.

<table>
<thead>
<tr>
<th>Line #</th>
<th>B6 LAK Subset</th>
<th>Ly49 Expression</th>
<th>Target Treatment</th>
<th>E/T= 30</th>
<th>10</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5E6⁺</td>
<td>C⁺, I⁺</td>
<td>IgG2b³, Prot. A/G</td>
<td>19.1±1.0</td>
<td>8.9±0.9</td>
<td>2.1±1.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>OVAp (Kᵇ)</td>
<td>45.1±2.7</td>
<td>30.1±2.1</td>
<td>8.2±0.9</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>VSVp (Kᵇ)</td>
<td>55.6±3.4</td>
<td>29.6±2.1</td>
<td>6.5±0.8</td>
</tr>
<tr>
<td>4</td>
<td>5E6ᵇ</td>
<td></td>
<td>Prot. A/G</td>
<td>44.1±3.1</td>
<td>32.3±2.1</td>
<td>18.7±1.7</td>
</tr>
<tr>
<td>5</td>
<td>Y-3³</td>
<td></td>
<td>Prot. A/G</td>
<td>41.5±1.9</td>
<td>35.2±0.7</td>
<td>29.7±0.9</td>
</tr>
<tr>
<td>6</td>
<td>Intact 5F1 mAb, Prot. A/G</td>
<td></td>
<td>7.1±1.6</td>
<td>4.8±1.5</td>
<td>5.1±1.9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Fab of 5F1 mAb, Prot. A/G</td>
<td></td>
<td>12.3±1.2</td>
<td>3.8±0.8</td>
<td>4.2±0.8</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>5E6⁻</td>
<td>C⁻, I⁻</td>
<td>IgG2b³, Prot. A/G</td>
<td>16.1±1.3</td>
<td>8.9±0.9</td>
<td>2.5±1.0</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>OVAp (Kᵇ)</td>
<td>4.0±0.7</td>
<td>2.5±0.6</td>
<td>1.7±1.0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>VSVp (Kᵇ)</td>
<td>4.1±1.0</td>
<td>1.7±0.8</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>11</td>
<td>5E6ᵇ</td>
<td></td>
<td>Prot. A/G</td>
<td>14.9±1.2</td>
<td>3.1±0.8</td>
<td>2.9±0.9</td>
</tr>
<tr>
<td>12</td>
<td>Y-3³</td>
<td></td>
<td>Prot. A/G</td>
<td>17.9±2.0</td>
<td>12.0±1.0</td>
<td>2.9±1.0</td>
</tr>
<tr>
<td>13</td>
<td>Intact 5F1 mAb, Prot. A/G</td>
<td></td>
<td>5.3±1.8</td>
<td>4.6±1.4</td>
<td>3.2±1.3</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Fab of 5F1 mAb, Prot. A/G</td>
<td></td>
<td>14.7±1.1</td>
<td>10.3±1.0</td>
<td>5.5±1.2</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-1.

a. IgG2b is an isotype control Ab for non-specific binding; it recognizes TNP which is not found on the target cell surface. Comparable percentages of specific lysis of target cells not pulsed with peptide were observed in the absence and presence of IgG2b. Hence, it is also used as the negative control for peptide-pulsing.

b. 5E6 is a mAb of isotype IgG2a. The observation that the presence of 5E6 did not enhance the lysis of target cells by 5E6 B6 LAK suggests that non-specific ADCC against the target cells did not occur.

c. Y-3 is a mAb of isotype IgG2b and has been shown to recognize both peptide-receptive and peptide-bound forms of K\(^b\) molecules.

Note: Lines 2, 3, 9 and 10 are representative of more than 10 independent experiments. Lines 4-7, and 11-14 are representative of 3 independent experiments.
the 5E6' LAK had normal cytotoxic function, we tested both 5E6+ and 5E6' B6 LAK cells for their ability to lyse Db-peptide (Flu-NP366-374) pulsed B6 ConA blasts. They were lysed by both sub-populations (Table 1: lines 15-18). This observation verifies that the 5E6' LAK cells are active and implies the presence of an undescribed inhibitory receptor present on at least some LAK cells in both the 5E6+ and 5E6' sub-populations, a receptor which recognizes PR-Db molecules (See Discussion).

In summary, reagents (mAb or peptide) that could bind to PR-Kb molecules or 5E6 antigen rendered B6 ConA blasts susceptible to lysis by 5E6+ B6 LAK cells but had no effect on their lysis by 5E6' B6 LAK cells.

3.4.2 The presence of "peptide-receptive" Kb molecules on B6 ConA blasts correlates with the resistance to lysis by 5E6' B6 LAK cells.

We have previously shown that PR-MHC-I molecules can be regenerated when peptide-pulsed ConA blasts are incubated for 90 min or longer at 37°C in CM that contains no free exogenous peptide (309). We therefore tested whether the reappearance of peptide receptive Kb molecules on the target cell surface would restore the resistance of these peptide-pulsed B6 ConA blasts to lysis by 5E6' B6 LAK cells. As shown in Figure 3-1A, B6 ConA blasts pulsed overnight with the Kb-specific peptide, OVAp, had no detectable PR-Kb molecules on the cell surface. When these OVAp-pulsed B6 ConA blasts were incubated at 37°C for 2 h in 10% CM that contained no free exogenous peptide, PR-Kb molecules could be detected (Fig 3-1B). However, no measurable PR-Kb-molecules were observed if OVAp was included in the culture during that 2-h incubation (Fig 3-1C). The cell-surface expression of PR-Kb molecule was measured using an OVAp peptide in which the lysine (K) at position 7 was biotinylated (OVAp-K-bio). This lysine side chain is known to be one of the CTL epitopes on OVAp (316, 317) and has been shown to protrude from the peptide-binding groove towards the solvent (102, 103). We found that this peptide binds specifically to Kb and can be readily detected by the addition of streptavidin-phycoerythrin (SA-PE) (309).

The target cells analyzed in Fig 3-1A-C were tested for their sensitivity to lysis by 5E6' B6 LAK cells in a 4h 51Cr-release assay. The OVAp-pulsed B6 ConA blasts after being cultured at 37°C for 2h in the absence of exogenous Kb-specific peptide were resistant to lysis by B6 5E6+ LAK cells (Table 3-2: line 1). The same OVAp-pulsed B6 ConA blasts
C) The regeneration of PR-Kp molecule on the cell surface was not detected when these same OVAp-pulsed B6 ConA blasts were incubated at 24°C in the presence of OVAp for 2h. The expression of PR-Kp on the cell surface detected when these OVAp-pulsed B6 ConA blasts were incubated for 2h at 24°C in the absence of Kp-specific peptide. The expression of PR-Kp on their cell surface. (A) B6 ConA blasts pulsed overnight with OVAp showed no staining of PR-Kp with an FITC (gray line). (B) B6 ConA blasts pulsed with FITC anti-OVAp conjugate stained with PR-Kp mAb. The staining control was done by omitting the addition of OVAp-Kp600 (FITC-SA) shown in the graph as dark. Fluorescence intensity on the cell surface and can be detected with OVAp-Kp600 binds specifically to PR-Kp. Publishing the cells with a high concentration of OVAp-Kp600 (14F/ml) cells. OVAp-Kp600 binds specifically to PR-Kp molecule on the cell surface can be detected by

FIGURE S-1: Detection of PR-Kp molecules on the cell surface. PR-Kp molecule on the cell surface can be detected by

(SAP) Log Fluorescence Intensity

Cell number
Table 3-2. The presence of peptide-receptive K<sup>b</sup> on B6 Con A blasts correlates with the resistance to lysis by 5E6<sup>+</sup> B6 LAK cells.

<table>
<thead>
<tr>
<th>Line #</th>
<th>B6 LAK Subset</th>
<th>Presence of Empty K&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Target Treatment (B6 Con A blasts)</th>
<th>% specific lysis E/T= 30</th>
<th>10</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5E6&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Yes</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, CM (2 hr)&lt;sup&gt;b&lt;/sup&gt; mlG1&lt;sup&gt;c&lt;/sup&gt;, Prot. A/G</td>
<td>25.0±0.9</td>
<td>16.2±1.3</td>
<td>0.7±0.9</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>No</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, OVAp (2 hr)&lt;sup&gt;b&lt;/sup&gt; mlG1&lt;sup&gt;c&lt;/sup&gt;, Prot. A/G</td>
<td>94.5±2.1</td>
<td>70.3±2.7</td>
<td>11.3±0.7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Yes</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, CM (2 hr)&lt;sup&gt;b&lt;/sup&gt; 25D1.16 mAb&lt;sup&gt;d&lt;/sup&gt;, Prot. A/G</td>
<td>32.1±1.2</td>
<td>23.5±1.6</td>
<td>6.2±0.9</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>No</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, OVAp (2 hr)&lt;sup&gt;b&lt;/sup&gt; 25D1.16 mAb&lt;sup&gt;d&lt;/sup&gt;, Prot. A/G</td>
<td>94.1±2.1</td>
<td>62.1±2.9</td>
<td>12.8±0.9</td>
</tr>
<tr>
<td>5</td>
<td>5E6&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Yes</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, CM (2 hr)&lt;sup&gt;b&lt;/sup&gt; mlG1&lt;sup&gt;c&lt;/sup&gt;, Prot. A/G</td>
<td>21.1±1.1</td>
<td>11.9±0.8</td>
<td>0.0±0.7</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>No</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, OVAp (2 hr)&lt;sup&gt;b&lt;/sup&gt; mlG1&lt;sup&gt;c&lt;/sup&gt;, Prot. A/G</td>
<td>39.4±1.5</td>
<td>27.4±1.6</td>
<td>12.1±0.9</td>
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<td>7</td>
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<td>Yes</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, CM (2 hr)&lt;sup&gt;b&lt;/sup&gt; 25D1.16 mAb&lt;sup&gt;d&lt;/sup&gt;, Prot. A/G</td>
<td>20.8±1.2</td>
<td>8.2±0.7</td>
<td>0.0±0.9</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>No</td>
<td>OVAp (O/N)&lt;sup&gt;a&lt;/sup&gt;, OVAp (2 hr)&lt;sup&gt;b&lt;/sup&gt; 25D1.16 mAb&lt;sup&gt;d&lt;/sup&gt;, Prot. A/G</td>
<td>43.5±1.0</td>
<td>31.0±1.6</td>
<td>12.3±0.6</td>
</tr>
</tbody>
</table>
Table 3-2

a. Day 2 B6 Con A blasts were cultured in the presence of OVAp (1μg/ml) overnight (16-18hr).
b. On Day 3, B6 Con A blasts cultured in the presence of OVAp were washed twice with 1% CM and then cultured in 10% CM either in the presence of the absence of OVAp (100ng/ml) for 2 hr at 37 °C.
c. mlgG1 is an isotype control Ab; it recognizes HLA-B which is not found on the target cell surface.
d. 25D1.16 mAb is IgG1. The observation that the presence of 25D1.16 mAg (line 3) did not significantly enhance the lysis of target cells by 5E6+ B6 LAK suggests that non-specific ADCC against the target cells did not occur.

Note: Results on this table are representative of 3 independent experiments.
after being cultured at 37°C for 2h in the presence of OVAp remained significantly susceptible to lysis by B6 5E6+ LAK cells (Table 3-2: line 2). Taken together, the presence of PR-Kb molecules on the target cell surface seems to correlate with the resistance of the target cell to lysis by 5E6+ B6 LAK cells.

To eliminate the possibility that peptide-pulsing created a target structure that served to activate 5E6+ B6 LAK cells, 25D1.16 mAb, which recognizes only OVAp-Kb complexes (not VSVp-Kb nor other peptide-Kb complexes, (313)) was used to mask the putative target structure. As in Figure 3-1, B6 ConA blasts were pulsed overnight with OVAp and stained with FITC-labeled 25D1.16 mAb to detect the presence of OVAp-Kb complex (Fig 3-2A). These OVAp-pulsed B6 ConA blasts were then incubated at 37°C for 2h in the presence or absence of OVAp. The B6 ConA blasts incubated in the absence of peptide have PR-Kb molecules on their cell surface (Fig 3-1B) and were shown to be resistant to lysis by B6 LAK cells (Table 3-2: line 1). The ConA blasts incubated in the presence of peptide have no PR-Kb molecule on the cell surface (Fig 3-1C), and were shown to be susceptible to lysis by 5E6+ B6 LAK cells (Table 3-2: line 2). Both groups of target cells were stained positive by 25D1.16 mAb (Figure 3-2B,C) suggesting that both have OVAp-Kb complexes on the cell surface. Furthermore, masking of the OVAp-Kb complexes using 25D1.16 mAb had no effect on the resistance or sensitivity of these two groups of target cells to lysis by 5E6+ B6 LAK cells (Table 3-2: lines 3,4). Hence, the sensitivity of these target cells to lysis mediated by 5E6+ B6 LAK cells does not depend on either the recognition of or presence of stable OVAp-Kb complexes on the cell surface.

To further examine how the level of PR-Kb expression on the target cell surface correlates with the sensitivity of the target cell to lysis by 5E6+ and 5E6- B6 LAK populations, B6 ConA blasts were pulsed with increasing concentrations of OVAp. The level of the remaining PR-Kb molecules on the cell surface after OVAp pulsing was measured by pulsing these cells with 1 μg/10⁶ cells/ml of OVAp-K-bio as described above. As shown in Figure 3-3A, the relative level of remaining peptide receptive Kb molecules decreased as the concentration of OVAp used in pulsing these cells was increased. The sensitivity of these OVAp-pulsed B6 ConA blasts to lysis mediated by 5E6+ B6 NK cells was also evaluated, and found to be inversely correlated with the relative level of PR-Kb molecules on the cell surface after the OVAp-pulsing procedure (Fig 3-3A). Thus, as the
Figure 3-2. Detection of OVAp-K^b complexes on the B6 ConA blasts using mAb 25D1.16, which specifically recognizes OVAp-K^b complexes on the cell surface. (A) B6 ConA blasts pulsed with OVAp (100ng/ml/10^6 cells) stained positive by mAb 25D1.16, suggesting that OVAp-K^b complexes are present. (B) These OVAp-pulsed B6 ConA blasts after being cultured at 37°C for 2h in the absence of OVAp were still stained positive by mAb 25D1.16. (C) Furthermore, the B6 ConA blasts when cultured at 37°C for 2h in the presence of OVAp (100ng/ml/10^6 cells) remained positive for mAb 25D1.16-staining.
Figure 3-3: Lysis of B6 ConA blasts pulsed with K\textsuperscript{b}-specific peptide correlated inversely with the presence of PR-K\textsubscript{Kb} molecules on the target cell surface. (A) The decrement in the expression of PR-K\textsubscript{Kb} on the target cell surface (squares, right y-axis) correlated with the increasing sensitivity of these target cells to lysis by 5E6\textsuperscript{+} B6 LAK cells (closed triangles, left y-axis). The presence of PR-K\textsubscript{Kb} molecule on the cell surface was measured by OVAp\textsubscript{-K-bio} binding, as described in Fig 3-1. The relative level of PR-K\textsubscript{Kb} was calculated as the fraction of the mean fluorescent intensity (MFI) detected when a particular OVAp concentration was used over the MFI detected when OVAp was omitted during the peptide-pulsing procedure (right y-axis). The specific lysis of these OVAp-pulsed target cells by 5E6\textsuperscript{+} B6 LAK cells (closed triangles) or 5E6\textsuperscript{-} B6 LAK cells (open triangles) was evaluated using a 4h $^{51}$Cr-release assay. (B) The specific lysis (left y-axis) of VSVp-pulsed ConA blasts by Ly49C\textsuperscript{+} B6 LAK cells (closed triangles) increased proportionally to the decrement of PR-K\textsubscript{Kb} on the target cell surface (closed squares, right y-axis). The disappearance of PR-K\textsubscript{Kb} from the cell surface had no effect on the resistance of these B6 ConA blasts to lysis by Ly49C\textsuperscript{+} B6 LAK cells (open triangles). The susceptibility of the VSVp-pulsed B6 ConA blasts to lysis by either Ly49C\textsuperscript{+} (closed triangles) or Ly49C\textsuperscript{-} B6 LAK (open triangles) was examined with the $^{51}$Cr release assay. The presence of PR-K\textsubscript{Kb} molecule on the cell surface was measured with OVAp- binding, as described in (C). (C) The level of PR-K\textsubscript{Kb} on the cell surface was titrated by pulsing the B6 ConA blasts with increasing concentrations of VSVp for 30-45 min and then washing away unbound VSVp. The remaining PR-K\textsubscript{Kb} was then measured by pulsing with OVAp and labeling with FITC conjugated mAb 25D1.16. The level of PR-K\textsubscript{Kb} that remained on the cell surface was inversely proportional to the amount of VSVp used in the pulsing procedure. The maximum level of PR-K\textsubscript{Kb} was measured when no VSVp was used, assuming that OVAp would bind to all the PR-K\textsubscript{Kb} molecules. The relative level of PR-K\textsubscript{Kb} was calculated as the fraction of the MFI detected when a particular VSVp concentration was used over MFI of the maximum level of PR-K\textsubscript{Kb} and is plotted in panel B (closed squares, right y-axis).
OVAp concentration used in pulsing the target cells was increased, the specific lysis of these target cells also increased reaching a plateau around 1 ng/ml of OVAp. The resistance of these target cells to lysis by 5E6 B6 LAK cells was independent of their exposure to K<sup>b</sup>-binding peptide (Figure 3-3A, open triangles). We conclude that the sensitivity of the target cells to lysis by 5E6<sup>+</sup> B6 LAK cells is inversely proportional to the expression of PR-K<sup>b</sup> molecule on the cell surface.

### 3.4.3 The Ly49C NK inhibitory receptor recognizes the PR-form of the K<sup>b</sup> molecule.

The mAb 5E6 recognizes two members of the Ly49 family, Ly49C and Ly49I (140), whereas the mAb 4LO3311 binds specifically to Ly49C (318). To determine whether Ly49C, Ly49I, or both recognize the peptide-receptive form of K<sup>b</sup>, we sorted Ly49C<sup>+</sup> (i.e. 5E6<sup>+</sup>4LO3311<sup>+</sup>) and Ly49CT<sup>+</sup> (i.e. 5E6<sup>+</sup>4LO3311<sup>+</sup>) B6 LAK populations and used them as effectors in <sup>51</sup>Cr-release assays. B6 ConA blasts pulsed with K<sup>b</sup>-specific peptide (OVAp or VSVp) became significantly susceptible to lysis by Ly49C<sup>+</sup> B6 LAK cells (Table 3-3: lines 2,3) but remained relatively resistant to lysis by Ly49CT<sup>+</sup> B6 LAK cells (lines 8,9). Masking of peptide-bound K<sup>b</sup> molecules on B6 ConA blasts with 5F1 F<sub>ab</sub> fragments in the presence of soluble Protein A and Protein G did not alter their resistance to lysis by either Ly49C<sup>+</sup> or Ly49CT<sup>+</sup> B6 LAK cells (lines 4,10). However, the use of 5E6 mAb to block Ly49C and Ly49I led to the lysis of B6 ConA blasts by Ly49C<sup>+</sup> but not by Ly49CT<sup>+</sup> B6 LAK cells (lines 6,12). Taken together, the results indicate that Ly49C recognizes the peptide-receptive form of K<sup>b</sup> molecules and not the stable ternary K<sup>b</sup> complex containing a high affinity peptide. The data, however, do not enable one to determine the ligand identified by Ly49I or even whether it functions as an inhibitory receptor.

Titration of VSVp peptide during the peptide-pulsing step showed that the sensitivity of the target cells to lysis by Ly49C<sup>+</sup> B6 LAK cells correlated directly with the increasing concentrations of VSVp used in the pulsing procedure, and correlated inversely with the level of PR-K<sup>b</sup> on the B6 ConA blasts (Fig 3-3B). The relative number of PR-K<sup>b</sup> molecules remaining on the cell surface after VSVp pulsing was measured by pulsing these cells with 1 µg/10<sup>6</sup> cells/ml of OVAp. The binding of OVAp was then visualized with FITC-conjugated 25D1.16 mAb (Fig 3-3C). Here, the level of staining by FITC labeled 25D1.16 mAb reflects
Ly49C<sup>B6</sup> NK inhibitory receptor recognizes the peptide-receptive form of K<sup>b</sup>.

<table>
<thead>
<tr>
<th>Line #</th>
<th>B6 LAK Subset</th>
<th>Ly49 Expression</th>
<th>Target Treatment (B6 Con A blasts)</th>
<th>% specific lysis</th>
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<td></td>
<td></td>
<td></td>
<td>E/T= 30 10 3</td>
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</tr>
<tr>
<td>1</td>
<td>5E6&lt;sup&gt;+&lt;/sup&gt;4LO3311&lt;sup&gt;+&lt;/sup&gt;</td>
<td>C&lt;sup&gt;+&lt;/sup&gt;</td>
<td>None</td>
<td>1.3±0.6 0.1±0.7 1.4±0.6</td>
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<tr>
<td>2</td>
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<td>OVAp (K&lt;sup&gt;b&lt;/sup&gt;)</td>
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<tr>
<td>3</td>
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<td></td>
<td>VSVp (K&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>72.5±1.3 39.2±1.2 8.3±0.6</td>
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<tr>
<td>4</td>
<td></td>
<td></td>
<td>5F1(F&lt;sub&gt;ab&lt;/sub&gt;), Prot. A/G</td>
<td>1.2±1.1 1.4±0.7 0.5±0.6</td>
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<tr>
<td>5</td>
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<td></td>
<td>IgG2b&lt;sup&gt;a&lt;/sup&gt;, Prot. A/G</td>
<td>2.2±1.2 2.8±0.9 1.6±0.8</td>
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<td>6</td>
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<td></td>
<td>5E6&lt;sup&gt;b&lt;/sup&gt;, Prot. A/G</td>
<td>30.9±1.0 17.3±0.6 6.7±0.9</td>
</tr>
<tr>
<td>7</td>
<td>5E6&lt;sup&gt;+&lt;/sup&gt;4LO3311&lt;sup&gt;+&lt;/sup&gt;</td>
<td>C&lt;sup&gt;+&lt;/sup&gt;, I&lt;sup&gt;+&lt;/sup&gt;</td>
<td>None</td>
<td>0.9±0.7 0.6±0.7 0.0±0.8</td>
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<tr>
<td>8</td>
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<td></td>
<td>OVAp (K&lt;sup&gt;b&lt;/sup&gt;)</td>
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<tr>
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<td>5F1(F&lt;sub&gt;ab&lt;/sub&gt;), Prot. A/G</td>
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<td>11</td>
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<td>IgG2b&lt;sup&gt;a&lt;/sup&gt;, Prot. A/G</td>
<td>1.8±0.8 2.4±1.1 1.1±0.6</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>5E6&lt;sup&gt;b&lt;/sup&gt;, Prot. A/G</td>
<td>8.1±1.1 6.0±0.9 2.3±0.9</td>
</tr>
</tbody>
</table>
Table 3-3

a. IgG2b is an isotype control Ab; it recognizes TNP which is not found on the target cell surface. Comparable percentages of specific lysis of target cells not pulsed with peptide were observed in the absence or the presence of IgG2b. Hence, it is also used as the negative control for peptide pulsing.

b. 5E6 is a mAb of isotype IgG2a. The observation that the presence of 5E6 did not enhance the lysis of target cells by 5E6- B6 LAK suggests that non-specific ADCC against the target cells did not occur.

Note: Results on this table are representative of 2 independent experiments.
the amount of PR-K\textsuperscript{b} molecule on the cell surface at the time of the cytotoxicity assay of Figure 3-3B. The specific lysis of these target cells by Ly49C\textsuperscript{+} B6 LAK cells increased as the level of PR-K\textsuperscript{b} expression declined, and reached a plateau when about 10 ng/ml of VSVp was used. At this point, the expression of PR-K\textsuperscript{b} had dropped to about half of its starting level. Again, the resistance of these target cells to lysis by Ly49CT\textsuperscript{+} B6 LAK cells was independent of the exposure to K\textsuperscript{b}-binding peptide (Figure 3-3B, open triangles).

3.4.4 Ly49A\textsuperscript{F1} recognizes peptide-bound D\textsuperscript{d} on BALB/c and F1 (BALB/cxB6) cells.

Our results indicate that Ly49C\textsuperscript{B6} recognizes the peptide-receptive form of the K\textsuperscript{b} molecule in a syngeneic system. Using a system of hybrid-resistance, Yu et al. have shown that the ligand for Ly49C on F1 (NZB x B6, H-2\textsuperscript{db}) NK cells is the K\textsuperscript{b} molecule expressed on B6 (H-2\textsuperscript{b}) ConA blasts, and the ligand for Ly49A on the same F1 NK cells is the D\textsuperscript{d} molecule expressed on BALB/c (H-2\textsuperscript{d}) ConA blasts (307). Here, we first confirm their findings and then test whether Ly49C and Ly49A are recognizing peptide-receptive or peptide-bound forms of K\textsuperscript{b} and D\textsuperscript{d} respectively. In concordance with Yu et al., B6 ConA blasts were resistant to lysis by F1 (B6 x BALB/c) 5E6\textsuperscript{+}Ly49A\textsuperscript{-} LAK cells (Table 3-4: line 1) but were susceptible to lysis by 5E6Ly49A\textsuperscript{+} F1 LAK cells (line 8). This was interpreted by Yu et al. as meaning that the presence of K\textsuperscript{b} on B6 ConA blasts "prevented" the lysis mediated by 5E6\textsuperscript{+}Ly49A\textsuperscript{-} F1 LAK cells, and the absence of D\textsuperscript{d} rendered B6 ConA blasts sensitive to lysis by 5E6Ly49A\textsuperscript{+} F1 LAK (307). On the other hand, BALB/c ConA blasts were found to be resistant to lysis by 5E6\textsuperscript{+}Ly49A\textsuperscript{+} F1 LAK cells (line 11) because the presence of the D\textsuperscript{d} molecule could inhibit Ly49A expressing LAK cells whereas the absence of the K\textsuperscript{b} molecule on BALB/c ConA blasts made them sensitive to lysis by 5E6\textsuperscript{+}Ly49A\textsuperscript{-} F1 LAK cells (line 4).

BALB/c or F1 (B6 x BALB/c) ConA blasts pulsed with HIVp (D\textsuperscript{d}-specific peptide) remained relatively resistant to lysis by 5E6\textsuperscript{+}Ly49A\textsuperscript{+} F1 LAK cells (lines 12,14), consistent with Ly49A recognizing the p\textsubscript{H}-D\textsuperscript{d} complex. However, B6 ConA blasts pulsed with K\textsuperscript{b}-specific peptide (OVAp or VSVp) became more susceptible to lysis by 5E6\textsuperscript{+}Ly49A\textsuperscript{-} F1 LAK cells (lines 2,3), consistent with Ly49C recognizing PR-K\textsuperscript{b} as in Tables 3-1, 3-2, and 3-3.

The D\textsuperscript{d}-specific peptide results are in agreement with published studies using D\textsuperscript{d}-transfected RMA-S cells and our previous study using the BALB/c syngeneic system showing that Ly49A recognizes peptide-bound D\textsuperscript{d} (41, 282, 309). The same HIVp-pulsed
Table 3-4. While Ly49C<sup>F1</sup> recognizes peptide-receptive K<sup>b</sup>, Ly49A<sup>F1</sup> recognizes peptide-bound D<sup>d</sup>.

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<th>Line #</th>
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<th>Treatment</th>
<th>% specific lysis</th>
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<td>HIVp (D&lt;sup&gt;d&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

a. Target cells were pulsed with CM, in the absence of exogenous peptide.

Note: Results in this table are representative of 3 independent experiments.
BALB/c or F1 ConA blasts became more susceptible to lysis by 5E6-Ly49A' F1 LAK (line 5,7) suggesting that there is an unidentified inhibitory receptor expressed on the Ly49A' NK sub-population that recognizes PR-D^d.

### 3.4.5 Ly49C^B6 binds to B6 ConA blasts expressing PR-K^b molecules.

Brennan et al. have shown that Ly49C^B6 expressed on COS cells (by transfection) mediates cell-cell adhesion by binding to H-2^b or H-2^d on the cell lines tested (306). Here, this same assay was used to test whether Ly49C^B6 binds to the PR-K^b molecule or the p_H-αβ2m K^b ternary complex. As shown in Figure 3-4A, COS-7 cells transfected with Ly49C^B6 bound ConA blasts from D^b+/K^b+/+ B6 mice which express both PR-K^b and the p_H-αβ2m K^b ternary complex (a). The same ConA blasts, pulsed with OVAp (K^b-specific peptide) to remove PR-K^b, bound poorly to COS-7 cells transfected with Ly49C^B6 (b). Exogenous OVAp was left in the wells throughout the whole assay. Only 7±1 % of OVAp-pulsed ConA blasts added bound to the Ly49C^B6, compared to 56±6 % of non-peptide pulsed ConA blasts (Fig 3-4B). ConA blasts from D^b+/K^b+/+ B6 mice (which express only H-2D^b) were used as a negative control and did not bind to COS-7 cells transfected with Ly49C^B6 whether or not OVAp was present (Fig 3-4A, c,d). It has been shown previously that Ly49I^B6 does not bind to cells expressing H-2^b molecules using this assay system. COS-7 cells transfected with Ly49I^B6 were therefore, used as a negative control for cell-cell adhesion (Fig 3-4A e-h). As shown on Figure 3-4A, COS-7 cells transfected with Ly49I^B6 were bound by significantly fewer D^b+/K^b+/+ B6 ConA blasts than Ly49C^B6-expressing COS-7 cells (a vs. e). The presence of OVAp did not have a major effect on the number of D^b+/K^b+/+ B6 ConA blasts binding to COS-7 cells expressing Ly49I^B6 (Fig 3-4A e,f). There was no visible binding of D^b+/+K^b+/+ B6 ConA blasts to Ly49I^B6-expressing COS-7 cells regardless of the presence or absence of OVAp (Fig 3-4A g,h). Furthermore, none of the B6 ConA blasts tested bound to COS-7 cells treated with the same transfecting reagent from which DNA was omitted (Fig 3-4A i-l).

The effect of various mAbs on cell-cell adhesion was tested in a separate experiment (Fig 3-4C). 5E6 mAb (recognizes Ly49C and Ly49I) and Y-3 mAb (recognizes both p_H-K^b and PR-K^b) reduced binding of D^b-/K^b+/+ ConA blasts to both Ly49C- and Ly49I- transfected COS-7 cells to background values. 5F1 mAb (recognizes mainly p_H-K^b, but little PR-K^b) reduced binding of D^b-/K^b+/+ ConA blasts to Ly49I-transfected COS-7 cells but had little
Figure 3-4

A. 

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<td>D(^{b-/K^{b+/+}}) (OVAp)</td>
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<td>D(^{b+/+K^{b-/-}})</td>
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<td>D(^{b+/+K^{b-/}}) (OVAp)</td>
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</tbody>
</table>

B. 

% Cell Bound

- □ Ly49\(C^{B6}\)
- □ Ly49\(I^{B6}\)
- □ No DNA

% Cell Bound
Figure 3-4. Cell-cell binding mediated by Ly49C\textsuperscript{B6}, and Ly49I\textsuperscript{B6}. (A) Ly49C\textsuperscript{B6} cDNA (a-d), Ly49I\textsuperscript{B6} (e-h) cDNA, or blank (i-l) were transiently expressed in COS-7 cells and tested for binding to \textsuperscript{51}Cr-labelled ConA blasts derived from D\textsuperscript{b+/-}K\textsuperscript{b+/-} B6 mice in the presence (b,f,j) or absence (a,e,i) of exogenous OVA\textsubscript{p}, or from D\textsuperscript{b+/+}K\textsuperscript{b/-} B6 mice in the presence (d,h,l) or absence (c,g,k) of OVA\textsubscript{p}. 4x10\textsuperscript{5} cells were incubated in each well (24-well plate) for 2h at 37°C. Unbound cells were removed and plates were photographed. (B) Cell binding was quantitated for individual plate wells by lysing bound cells with Triton-X 100. \textsuperscript{51}Cr radioactivity in the cell lysate was measured by \gamma-counting as described in Materials and Methods. Results are represented as the mean percent of input cells bound and the S.E.M. from quadruplicate wells. Results in (A) and (B) are from one experiment and are representative of 3 independent experiments. (C) Cell-cell adhesion could be prevented by the binding of mAb Y-3 or mAb 5E6 to targets or Ly49C\textsuperscript{B6} transfected COS-7 cells respectively. Results are from one experiment and are representative of 3 independent experiments.
effect on Ly49C-transfected cells, again implicating PR-K\textsuperscript{b} as the ligand for Ly49C\textsuperscript{B6}. We conclude from the cell-cell adhesion studies that Ly49C\textsuperscript{B6} binds to PR-K\textsuperscript{b} and that Ly49I\textsuperscript{B6} binds weakly to peptide-containing K\textsuperscript{b}.
Our results demonstrate that the presence of PR-K\textsuperscript{b} on the target cell surface prevents lysis mediated by 5E6\textsuperscript{+} B6 LAK cells (Table 3-1, 3-2). Binding of high affinity K\textsuperscript{b}-specific peptide converts most if not all PR-K\textsuperscript{b} molecules on the target cell surface to the form of p\textsubscript{H}-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} ternary complexes which are no longer peptide-receptive (or only slightly so). This prevents the inhibitory recognition mediated by 5E6\textsuperscript{+} B6 LAK cells. The disappearance of PR-K\textsuperscript{b} from the target cell surface can be measured and was found to correlate with the increased sensitivity of these target cells to lysis by 5E6\textsuperscript{+} B6 LAK cells (Fig 3-1, 3-2, and 3-3).

Furthermore, when PR-K\textsuperscript{b} molecules were masked by Y-3 mAb, which binds to both peptide receptive and p\textsubscript{H}-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} ternary complexes (314, 315), the target cells became susceptible to lysis by 5E6\textsuperscript{+} B6 LAK cells but remained resistant when only p\textsubscript{H}-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} ternary complexes were masked using 5F1 mAb. This argues against the involvement of p\textsubscript{H}-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} ternary complexes in inhibitory recognition (Table 3-1).

NK recognition involves both activation and inhibitory receptors. It is possible (although rendered unlikely by the results just summarized) that pulsing normal B6 ConA blasts with K\textsuperscript{b}-specific peptide creates a target structure that activates the 5E6\textsuperscript{+} B6 LAK cells to kill the target cells rather than masking an inhibitory ligand as proposed above. In this case, the susceptibility of the target cells to lysis by 5E6\textsuperscript{+} B6 LAK cells would directly correlate with the formation of peptide-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} ternary complexes. To directly test this possibility, target cells expressing both PR-K\textsuperscript{b} molecules and OVA\textsubscript{p}-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} complexes were used. Cells carrying large numbers of OVA\textsubscript{p}-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} complexes were generated by culturing B6 ConA blasts overnight with OVA\textsubscript{p}. PR-K\textsuperscript{b} molecules were regenerated on OVA\textsubscript{p}-pulsed B6 ConA blasts by culturing these cells at 37°C for 2h in the absence of exogenous peptide. The 2h time period should be sufficient to allow newly synthesized PR-K\textsuperscript{b} molecules to restore equilibrium values on the cell surface (309). Despite the presence of OVA\textsubscript{p}-\alpha-\beta\textsubscript{2m} K\textsuperscript{b} complexes on the cell surface, as shown by 25D1.16 mAb staining (Fig 3-2B,C), the cells remained resistant to lysis by 5E6\textsuperscript{+} B6 LAK cells if PR-K\textsuperscript{b} molecules were expressed on the cell surface (Table 3-2). Furthermore, the blocking of OVA\textsubscript{p}-K\textsuperscript{b} complexes with 25D1.16 mAb did not alter the resistance of the B6 ConA blasts that expressed PR-K\textsuperscript{b}
or the sensitivity of the B6 ConA blasts that expressed no PR-K\textsuperscript{b} to lysis by 5E6\textsuperscript{+} B6 LAK cells (Table 3-2). Taken together, these observations support our hypothesis that pulsing the B6 ConA blasts with OVAp masked the inhibitory ligand (the PR-K\textsuperscript{b}) from being recognized by 5E6 antigens on B6 LAK cells instead of creating a target structure.

The 5E6 mAb has been shown to bind to both Ly49C and Ly49I B6 NK inhibitory receptors (140). Our data (from both the functional and binding assays) show that the NK inhibitory receptor that recognizes the PR-K\textsuperscript{b} molecule is Ly49C\textsuperscript{B6}, and not Ly49I\textsuperscript{B6}. We have not yet examined whether Ly49C\textsuperscript{BALB/c} recognizes PR-K\textsuperscript{b}. The 4LO3311 mAb, specific for Ly49C, was used in this study to sort Ly49C\textsuperscript{+} (4LO3311\textsuperscript{+}5E6\textsuperscript{+}) and Ly49CT\textsuperscript{+} (4LO3311\textsuperscript{-}5E6\textsuperscript{+}) B6 LAK sub-populations, which were then used in peptide-pulsing experiments. B6 ConA blasts pulsed with K\textsuperscript{b}-specific peptide (OVAp or VSVp) were susceptible to lysis by Ly49\textsuperscript{C\textsuperscript{+}} B6 LAK cells either in the presence or the absence of 5F1 mAb, while B6 ConA blasts pulsed with media alone remained resistant under the same conditions (Table 3-3 lines 1-5). Furthermore, the presence of 5E6 mAb in the assay enhanced the lysis of B6 ConA blasts by Ly49C\textsuperscript{+} B6 LAK cells as expected (Table 3-3 lines 6). These observations suggest that PR-K\textsuperscript{b} is the ligand for Ly49C\textsuperscript{B6}, and that blockage of Ly49C-mediated inhibitory signal by masking Ly49C\textsuperscript{B6} with 5E6 mAb or masking PR-K\textsuperscript{b} with high affinity K\textsuperscript{b}-specific peptide or mAb leads to lysis of target cells as observed (Table 3-1, lines 4 and 5; Table 3-3, line 6). In support of our functional data, we showed that Ly49C\textsuperscript{B6} expressed on the surface of COS-7 cells can bind to D\textsuperscript{b/-}K\textsuperscript{b+/+} B6 ConA blasts and that such binding could be blocked by the presence of K\textsuperscript{b}-specific peptide, OVAp (Fig 3-4A a,b). Again, the binding data strongly suggest that PR-K\textsuperscript{b}, and not the p\textsubscript{H}-\alpha-β\textsubscript{2}m K\textsuperscript{b} ternary complex, is the ligand for Ly49C\textsuperscript{B6} in inhibitory recognition.

Based on the predicted amino acid sequence, Ly49I\textsuperscript{B6} contains an ITIM motif in its intracellular domain and is therefore predicted to be an NK inhibitory receptor (137, 140). We observed (Fig 3-4) weak binding of B6 ConA blasts to COS-7 cells transfected with Ly49I\textsuperscript{B6} that required the presence of both K\textsuperscript{b} (B6 D\textsuperscript{b/-}K\textsuperscript{b+/+} bound, B6 D\textsuperscript{b+/+}K\textsuperscript{b/-} did not) and Ly49I\textsuperscript{B6} (no binding to non-transfected controls). Further, binding was marginally higher after pulsing with high affinity K\textsuperscript{b}-specific peptide (OVAp). These data suggest that Ly49I\textsuperscript{B6} recognizes stable K\textsuperscript{b}-peptide complexes. However, this recognition did not lead to inhibition in our experimental system. Masking of either Ly49I\textsuperscript{B6} or p\textsubscript{H}-K\textsuperscript{b} with appropriate mAbs
(Table 3-3, lines 12 and 10 respectively) did not lead to lysis. It may be that the Ly49IB^6-K^b interaction is of too low affinity to trigger inhibition or that the mAbs tested do not effectively block the functional sites in this particular interaction. The role of Ly49IB^6 in NK recognition remains unclear.

Using the same adhesion assay and COS cells transfected with the Ly49C^B^6 construct, Ly49C^B^6 was shown to bind to cells expressing H-2^b, H-2^a, H-2^k or H-2^d molecules (306). Despite the binding of Ly49C^B^6, H-2^d cells that do not express K^b molecules are highly sensitive to lysis by 5E6^+ B6 LAK cells (307). A possible explanation may be that Ly49C^B^6 has different affinities for H-2^b, H-2^a, H-2^d and H-2^k. Kane (1994) showed that a higher surface density of D^k than D^d is required to have equivalent binding to Ly49A^B^6 (17). Furthermore, mAb against D^d or K^d cannot completely block the binding of Ly49C^B^6 (140).

Ly49J, Ly49K, and Ly49N are all encoded in the B6 genome, are closely related to Ly49C^B^6 (319), and may also recognize PR-K^b. From the predicted amino acid sequences, the presumed epitope for 4LO3311 mAb (Lemieux, unpublished) is absent, but the epitope for 5E6 mAb may be present on Ly49J, Ly49K and/or Ly49N. Therefore, it is possible that these Ly49 members are expressed on either or both the 5E6^+4LO3311^+ and 5E6^+4LO3311^- NK subsets. Ly49J has an ITIM motif and may be implicated in the lysis of B6 ConA blasts pulsed with K^b-specific peptide. However, further study is required to determine whether Ly49I, Ly49J, Ly49K and/or Ly49N recognize PR-MHC-I.

A caveat should be added regarding our use of anti-MHC-I mAbs in lysis-blocking studies. Neither 5F1 (recognizes mostly p^-K^b ternary complexes) nor 25D1.16 (recognizes OVAp-K^b) were able to block the delivery of an inhibitory signal to Ly49C^B^6. Our interpretation above was that these mAbs do not block because they do not recognize PR-K^b. It may be that neither mAb blocks the epitope on the K^b molecule recognized by Ly49C^B^6. Thus, the anti-D^d mAb 34-5-8S blocks D^d signaling to Ly49A but other anti-D^d mAbs that bind to different sites do not block (41). Further, the effect of using mAb to block recognition elements (e.g., 5E6 antigen, K^b molecule) was not always optimal, and appeared to vary with the state of activation of the LAK cultures used. As shown in Table 3-3 (line 6), the use of 5E6 mAb in this experiment resulted in sub-optimal blockage of 5E6 antigens, compared to the use of K^b-specific peptide (lines 2 and 3) in blocking inhibitory recognition. However, our argument for PR-K^b being the ligand for Ly49C^B^6 does not depend exclusively
on our interpretation of the mAbs blocking studies. In particular, binding of peptide to K\textsuperscript{b} with high affinity does block signaling.

Our data strongly support the conclusion that Ly49A\textsuperscript{F1} and Ly49C\textsuperscript{B6} are NK inhibitory receptors that recognize different forms of MHC-I (p\textsubscript{H}-D\textsuperscript{d} for Ly49A\textsuperscript{F1} and PR-K\textsuperscript{b} for Ly49C\textsuperscript{B6}). Our data of this study and a previous study (309) also provide direct evidence for the existence of three new NK inhibitory receptors that recognize PR-MHC-I: (i) Both 5E6\textsuperscript{+} and 5E6\textsuperscript{-} B6 NK cells lyse B6 ConA blasts pulsed with high affinity D\textsuperscript{b}-specific peptide (Table 3-1, lines 15-18), implying the existence of a novel inhibitory receptor recognizing PR-D\textsuperscript{b}. (ii) 5E6\textsuperscript{+}Ly49A\textsuperscript{-} F1 (B6xBALB/c) NK cells lyse both F1 and BALB/c ConA blasts pulsed with high affinity D\textsuperscript{d}-binding peptide (Table 3-4, lines 5 and 7), implying the existence of a novel inhibitory receptor recognizing PR-D\textsuperscript{d}. Su et al. found a similar receptor studying lysis of BALB/c ConA blasts by Ly49A\textsuperscript{-} BALB/c NK cells (309). (iii) BALB/c NK cells, either unfractionated or sorted into Ly49A\textsuperscript{+} and Ly49A\textsuperscript{-} sub-populations lyse BALB/c ConA blasts pulsed with high affinity K\textsuperscript{d}-binding peptide (309) implying the existence of a novel inhibitory receptor recognizing PR-K\textsuperscript{d}. Whether these unidentified receptors are members of the Ly49 family, mouse homologs of the KIR family (yet to be found) or some completely novel structure is unknown at the present time.

It appears that the presence of one NK inhibitory signal may not be sufficient to prevent lysis if a second is removed. Thus, the observation that B6 ConA blasts pulsed with D\textsuperscript{b}-specific peptide were sensitive to lysis by either 5E6\textsuperscript{+} or 5E6\textsuperscript{-} syngeneic NK subsets suggests that the inhibitory signal mediated by 5E6 antigens in recognition of PR-K\textsuperscript{b} is not dominant. It is in concordance with the hypothesis that the outcome of NK recognition is dependent on the balance of inhibitory and stimulatory signals (25, 27, 126, 156, 281). The binding of K\textsuperscript{b}-specific peptide can remove inhibitory ligand recognized by 5E6 antigens, resulting in the reduction of the total inhibitory signal. By the same token, the binding of D\textsuperscript{b}-specific peptide can also reduce the inhibitory signal in NK cells that bear receptor(s) recognizing PR-D\textsuperscript{b}, resulting in the activation of the NK cells. Single NK cells have been shown to co-express multiple Ly49 receptors in a stochastic manner; in particular 5E6\textsuperscript{+} and 5E6\textsuperscript{-} B6 NK subsets express similar profiles of other Ly49 members (320). Therefore, it is likely that both 5E6\textsuperscript{+} and 5E6\textsuperscript{-} NK subsets have an equal representation of NK inhibitory receptor(s) recognizing PR-D\textsuperscript{b}. Although NK inhibitory receptors recognizing Qa1 (i.e., the
mouse homologs of human NKG2 and CD94, (321)) might also be equally expressed on both 5E6+ and 5E6- NK subsets, the addition of Dβ-specific peptide having an effect on the recognition of Qa1 is unlikely as there is no evidence that a Dβ-specific peptide such as Flu-NP can bind to non-classical MHC-I, like Qa1 (75, 286, 287).

It is not clear, how an NK inhibitory receptor can distinguish between peptide-receptive and pαβ 2m MHC-I molecules. A 30-min pulse with high affinity peptide at a concentration of 1μg/ml/10⁶ cells (Fig 3-3) was sufficient to load all PR-MHC-I. We found previously (309) that if export of newly synthesized MHC-I was blocked, no detectable new PR-MHC-I appeared on the cell surface over at least 4h, implying that peptide-bound MHC-I is extremely stable. We hypothesize that during the peptide pulse, all “empty” MHC-I and all MHC-I containing low affinity peptide are converted to pαβ 2m ternary stable form. It is likely that the conformation of the stable, peptide-bound MHC-I molecule differs from that of the possible peptide-receptive precursors. It could well be that NK inhibitory receptors can distinguish between these conformations.

Peptide binding is known to influence the conformation of the surface of class I molecules as detected with mAbs and TCR (187, 189, 322). Using a system employing fluorescence resonance energy transfer, Catipovic et al. found that H-2Kβ αβ 2m heterodimers are in a relatively extended conformation, and that this conformation becomes more compact when peptide is bound (190). This is consistent with PR-MHC-I molecules (pLα-β 2m and/or αβ 2m) having conformation(s) different from that of a pαβ 2m ternary complex. Using computer-modeling analysis of MHC-I structures, Achour et al. postulated that peptide binding to Dd imposes a specific conformation, different from that of PR-Dd, a conformation required for recognition by Ly49A (323). This specific conformation is not found on molecules such as Dβ or Kβ, which are not ligands for Ly49A. It is also possible that pαβ 2m and PR-MHC-I molecules may differ in their ability to associate with each other or with other cell surface molecules and thus affect their ability to be recognized by NK inhibitory receptors.

PR-MHC-I on the cell surface has a half-life of less than 30 min at 37 °C (34). For this reason, the recognition of PR-MHC-I by NK inhibitory receptors poses a potential advantage for the organism. During a viral infection, very few PR-MHC-I are exported to the cell surface either because very large quantities of viral peptide inside the cell saturate
MHC-I or because the virus greatly reduces overall MHC-I production such that few PR-MHC-I (albeit perhaps a higher percentage of all MHC-I) reach the cell surface. In the latter case, the disappearance of PR-MHC-I may be detected earlier compared to the detection of the loss of p\textsubscript{H}-\textalpha-\textbeta\textsubscript{2m} ternary MHC-I since these have a half-life of more than 10h (34, 185). The process described here would allow NK cells to detect virally infected host cells many hours earlier than if detection required a complete loss of MHC-I and many days before the acquired immune system (B cells and T cells) is able to mount an effective response.
CHAPTER 4

STABILITY OF SURFACE H-2K\textsuperscript{b}, H-2D\textsuperscript{b}, AND "PEPTIDE-RECEPTIVE H-2K\textsuperscript{b} ON SPLENOCYTES."
4.1 ABSTRACT

We have studied the stability and peptide-binding capability of MHC-I on the surface of normal mouse spleen cells in a natural environment. Stable MHC-I molecules consist of 3 subunits: heavy chain (α), β2-microglobulin (β2m) and a peptide (p) non-covalently associated with the α1-α2 domain of the α subunit, although molecules missing β2m and/or p can also be found. We found that both surface K^b and D^b on B6 T lymphoblasts consist of two approximately equal sized sub-populations with very different half-lives: unstable (t_{1/2} \sim 1.1 \pm 0.1 h) and quasi-stable (t_{1/2} \sim 20 \pm 11 h). Our data further suggest that quasi-stable surface MHC-I are associated with high affinity peptide (p_H) whereas some of the unstable MHC-I are associated with either low affinity peptides (p_L) or no peptide and are potentially capable of being stabilized by binding to high affinity peptide. They are hence, referred to as "peptide-receptive" MHC-I (PR-MHC-I). PR-K^b also consists of two sub-populations: Quasi-stable PR-K^b (~26%) has a t_{1/2} of 4.2 \pm 2 h and unstable PR-K^b (~74%) has a t_{1/2} of 38 \pm 13 min. We provide evidence implying that quasi-stable PR-K^b are K^b associated with low or medium affinity peptide, consistent with the finding that binding of exogenous peptide to such PR-MHC-I is determined by the off-rate of pre-bound peptide. Unstable PR-K^b may be K^b molecules that are truly empty or associating with peptides of extremely low affinity. We have also measured the rate of PR-K^b accumulation on the cell surface (~2860 \pm 230 molecules per hour), and found it to be in agreement with its short t_{1/2}. 
4.2 INTRODUCTION

Stable MHC-I is a heterotrimer consisting of a polymorphic integral membrane glycoprotein (heavy chain, α) non-covalently associated with an invariant protein (β2m) and with a peptide of the correct length and motif to bind with high affinity. The α chain consists of 3 domains. The α3 domain associates with β2m; and the α1 and α2 domains constitute the antigen binding groove (54, 324), which can accommodate peptides of 8-10 residues with the right binding motif (324-327). Such peptide binds to α chain with high affinity and is here referred to as pH. Peptides that are longer or contain partial binding motif may also bind, but do so with lower affinity (89, 186), such peptides being here referred to as pL. MHC-I are constitutively synthesized in the ER and exported to the cell surface as heterotrimers containing peptides of varying affinity. Once on the cell surface, both the bound β2m and peptide can exchange freely and independently with β2m and peptide from the surroundings (76, 302, 328).

Since the most well studied function of MHC-I is its ability to present peptide antigen to CD8-expressing T lymphocytes, many studies have focused on examining the kinetics of exogenous peptide binding to MHC-I. Studies using biochemically purified and re-folded MHC-I subunits show that D b α binds to a D b -specific pH (Y-Flu-NP) with a t1/2 of 13h at 22°C (283). If β2m is also present, the D b α binds Y-Flu-NP with a t1/2 of 0.75h. The study suggests that β2m is critical in re-configuring the α to make it receptive to peptide. When pre-formed α-β2m heterodimers were used in the binding, Y-Flu-NP bound much more efficiently, with a t1/2 of less than 0.2h at 22°C. Since most surface MHC-I contain bound peptide, Hörig et al. examined the binding of exogenous peptides to pre-formed p-α-β2m heterotrimers (328). In a K b -β2m complex loaded with PolyI peptide (pH), the pH was replaced by K6 peptide (a medium-affinity peptide, pM) with a t1/2 of 6h; whereas in a K b -β2m complex loaded with VSVp (pM), the pM could be replaced by another pM (E6 peptide) with a t1/2 of 1.5h. This study suggested that the rate of binding of exogenous peptide to MHC-I is determined by the off-rate of the pre-bound peptide.

Exogenous peptide binding to MHC-I on the cell surface has also been examined. The half-time required for the pH, Y-Flu-NP, to bind to cell-surface D b is 9.3±1.1 min.
(peptide concentration, 1.68μM, (186)), very similar to results for Y-Flu-NP binding to purified α-β2m heterodimers (283). MHC-I that are capable of binding exogenously added peptide are referred to as "peptide-receptive" MHC-I (PR-MHC-I) in this study (74.). Luscher et al. and Christinck et al. showed that approximately 10% of surface Db on EL4 thymoma cells are available for binding by 125I-Y-Flu-NP peptide during a 2h incubation at 37°C (76, 186). It is still unknown whether these PR-Db molecules contain solvent in their peptide binding groove (i.e., α-β2m) or a weakly bound peptide (i.e., pL-α-β2m; pL denoting a low affinity peptide).

To increase the number of PR-MHC-I on the cell surface and so to facilitate the study of peptide binding, many investigators have used the murine mutant cell line RMA-S (32, 97). RMA-S cells are MHC-I-deficient somatic variants of the T-lymphoma cell line, RMA resulting from a defect in the transporter associated with antigen processing (TAP), necessary for providing processed peptides for binding to newly synthesized MHC-I (185, 329). PR-MHC-I can be stabilized on RMA-S cells by culturing cells at 26°C but are unstable at 37°C (t1/2 ~30-60min) (32, 74, 97). The binding of exogenous peptide, however, can stabilize the PR-Kb at 37°C and thus, facilitate the study of peptide binding kinetics.

Stability of MHC-I complexes has generally been studied by monitoring changes in α conformation that result in gain or loss of conformationally specific antibody-recognition epitopes on the α1, α2 and/or α3 domains. Burshtyn et al. examined the retention of α1α2 antigenic epitopes on biochemically purified MHC-I complexes with an α1α2-specific mAb (283). It was found that a pH-containing heterotrimer, Y-Flu-NP-Db-β2m, is relatively stable with a t1/2 ranging from 2.5-5h. At 37°C, Y-Flu-NP-Db is slightly less stable (t1/2~2h) than the heterotrimer but is much more stable than Db-β2m complexes (t1/2~0.2h). The stability of Y-Flu-NP-Db-β2m heterotrimers on the EL4 cell surface was investigated and was found to be significantly more stable than the purified form with a t1/2 greater than 10h at 37°C (76, 186).

In the presence of peptide, α-β2m complexes can be stabilized (i.e., can retain α1α2 antigenic epitopes) by binding to peptide (32, 330). In the absence of peptide in the surroundings, the α-β2m heterodimer quickly undergoes conformational changes leading to the loss of the α1α2 antigenic epitopes (thereby denoted as "α1α2-α-β2m") (32, 34, 74). The
half time for the transition from $\alpha_1\alpha_2^+$ state to $\alpha_1\alpha_2^-$ state was estimated to be ~30-60 min (34, 74). Antibodies that recognize specifically the $\alpha_3$ antigenic epitopes are often used to detect the presence of $\alpha_1\alpha_2^-$ MHC-I on the cell surface because the $\alpha_3$ domain is generally not affected by peptide or $\beta_2m$ dissociation (33, 34). The $\beta_2m$ exchange occurs independently of the peptide associated with the $\alpha$ (i.e., it occurs for $p_H$-\(\alpha\)-\(\beta_2m\) at the same rate as for $p_L$-\(\alpha\)-\(\beta_2m\) or \(\alpha\)-\(\beta_2m\) complexes) (76, 328). The dissociation of $\beta_2m$ from the $\alpha_1\alpha_2^-$ $\alpha$-$\beta_2m$ complexes induces conformation changes in the $\alpha$ leading to loss of the $\alpha_3$ epitope (33). $\alpha_3^-$ $K^b$ was detected on the cell surface by immunoprecipitation using a mAb recognizing an intracellular domain of H-2$K^b$ (\(\alpha p8\)) (314). These $\alpha_3^-$ $\alpha$ have a $t^{1/2}$ of ~1h on the cell surface. It was found that $1/3$ of surface $K^b$ $\alpha$ on EL4 or spleen cells are not associated with $\beta_2m$ and are $\alpha_1\alpha_2^-$ (314). Surface expression of MHC-I is also lost through internalization (34). Internalization of MHC-I occurs constitutively on activated lymphoid cells at a rate of ~10% of cell surface $\alpha_3^+$MHC-I per hour (331-333).

As is clear from the above, much is known about the stability and expression of MHC-I. However, most studies have used transformed tumour and mutant cell lines, and biochemically purified and reconstituted MHC-I complexes. In this study, we have examined more fully the generation, stability, and peptide-binding capacity of MHC-I located in a "natural" environment on the surface of normal mouse spleen cells.
4.3 MATERIALS AND METHODS

4.3.1 Cells and Culture:
Normal C57BL/6 (B6, H-2b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and kept in a specific pathogen free environment. In most experiments, 6-10 week old female mice were used (although either sex gave similar results). Spleens harvested from B6 mice were pressed through a wire mesh screen with a disposable syringe plunger into α-MEM medium (Gibco, BRL, Burlington, ON, Canada) supplemented with 10% FCS, 50μM 2-ME, and 10mM HEPES (10% CM). Splenocytes were washed once with 1% CM (supplemented with 1% FCS, instead of 10% FCS) and were resuspended in 5 ml 10% CM, underlaid with 5 ml Lympholyte M (Cedarlane Lab., Hornby, ON), and centrifuged at 500g for 20 min to remove red cells and dead cells. Concanavalin A activated lymphoblasts (ConA blasts) were generated by culturing approximately 5x10⁶ splenocytes in 5 ml of 10% CM supplemented with ConA (2μg/ml, ICN Pharmaceuticals Canada Ltd., Montreal, Que.). For most experiments (unless stated otherwise) day 1.5 ConA blast cells were used.

4.3.2 Antibodies:
The following antibodies were used: PE-labelled mAb AF6-88.5 (~1μg/10⁶cells/100μl) recognizing specifically the α₁α₂ region of H-2Kb (334, 335) and FITC-labelled mAb KH95 (~1μg/10⁶cells/100μl) recognizing specifically H-2Db (336) were purchased from Pharmingen (San Diego, CA). FITC labelled anti-biotin mAb was purchased from Sigma (St. Louis, MO). The mAb Y3 (IgG2a, ~1.5μg/10⁶ cells/100μl) recognizing the α₁α₂ domain of H-2Kb (189, 337), mAb 5F1 (IgG2b, ~1μg/10⁶ cells/100μl) recognizing the α₂ domain of H-2Kb (189), and mAb 25D1.16 (IgG2a, ~0.5μg/10⁶ cells/100μl) recognizing the SIINFEKL peptide associated with H-2Kb (313) were purified from hybridoma culture supernatants using Protein A (Sigma, St. Louis, MO) chromatography. Purified antibodies were labelled with FITC by adding 0.5-1 mg FITC-CELITE (Calbiochem, La Jolla, CA) to the antibody solution (1-2 mg of purified mAb in PBS, adjusted to pH 9.0 with 5% sodium carbonate solution). The mixture was incubated in the dark for 30-45 min at room temperature. The FITC-labeled antibody was recovered by centrifugation at 500g for 5 min and fractionation on a P10 Bio-Gel exclusion column (BioRad, Hercules, CA). The yellow mAb-containing fractions were collected, passed through a 0.2 μm filter (Gelman Sciences, Ann Arbor, Michigan), and stored at 4°C.
4.3.3 Flow cytometry and data analysis:

Fluorescence and light scatter properties of individual cells were measured on a FACScan analyzer (Becton-Dickinson) using logarithmic amplification of the fluorescence signals and linear amplification of the right angle/forward angle light scatter signals. Live splenocytes were gated (on the basis of right angle/forward scatter measurements) and analyzed for their fluorescence. Cells (5x10^5) in 50 μl of 0.5% BSA/PBS were first incubated with 4 μl of reconstituted normal mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 5 min. FITC- or PE-labelled mAbs were then added to the cells and incubated on ice, in the dark for 30-45 min. The cells were then washed in 0.5% BSA/PBS, resuspended in 0.3 ml 0.5% BSA/PBS and analyzed in a FACScan analyzer. The number of antibody bound (antibody binding capacity, ABC) (for AF6-88.5 mAb, KH95 mAb, 5F1 mAb, Y3 mAb, and 25D1.16 mAb) per cell was calculated using a Quantum Simply Cellular Kit (Sigma, St. Louis, MO). Quantum Simply Cellular Kit is a mixture of four populations of microbeads with different ABCs plus one non-binding microbead population. The ABC is derived from covalently bound Goat anti-mouse Ig on the microbeads. This Goat anti-mouse Ig has equivalent reactivity to each mouse isotype (IgG1, IgG2a and IgG2b). Quantum Simply Cellular microbeads were used in the experiments as an external calibrator. 10^5 microbeads in 50 μl of 0.5% BSA/PBS were stained with FITC- or PE-labelled mAbs in parallel with the cell samples. Staining of microbeads and cell samples was analyzed with the same instrumental setting except that light side scatter was set lower for microbeads. A regression calibration curve was constructed using the QuickCal program (provided by the manufacturer), which plots the mean fluorescent intensities (MFI) of the Quantum Simply Cellular microbeads against the ABC values predetermined for each microbead population. The ABC values for cell samples were calculated using the regression calibration curve and the MFI of mAb-stainings. In studying the stability of surface K^b and D^b, the percentage of stable K^b (or D^b) molecules remaining on the cell surface was calculated as (ABC of AF6-88.5 (or KH95) per cell at time, t)/(ABC of AF6-88.5 (or KH95) per cell at t=0). Since ABC is proportional to the number of antigens per cell, for other studies, the number of antigens per cell was used directly in plotting and statistically analysis.
4.3.4 MHC-I binding peptide:
Peptides utilized were $\text{K}^b$-restricted epitopes of Chicken Ovalbumin, SIINFEKL, (OVAp$_{258-265}$) (287) and Vesicular Stomatitis Virus NP, RGYVYQGL, (VSVp$_{52-59}$) (75), and a $\text{D}^b$-restricted epitope of influenza nucleoprotein, ASNENMETM, (Flu-NP$_{366-374}$) (286). Chicken Ovalbumin, SIINFEKL (OVAp$_{258-265}$) was prepared by the Ontario Cancer Institute Biotechnology Laboratory, using an Applied Biosystems Peptide Synthesizer (Applied Biosystems, Foster City, CA). A derivative of SIINFEKL, biotinylated Ovalbumin peptide (SIINFEKL(bio)L) was purchased from Alberta Peptide Institute, prepared by using an Applied Biosystems Model 430A Peptide Synthesizer. Both VSVp$_{52-59}$ and Flu-NP peptides (>90% purity) were generous gifts from Dr. B. H. Barber (University of Toronto). OVAp, and VSVp peptides are natural ligands for $\text{K}^b$ and bind to $\text{K}^b$ with high affinities (75, 287). Flu-NP peptide is a natural ligand for $\text{D}^b$ and binds with high affinity (286).

4.3.5 Measurement of the stability of MHC-I and PR-K$^b$:
ConA activated splenocytes were washed twice with 1% CM and the cell pellet was suspended in 10% CM (4x10$^6$ cells/ml) supplemented with 5$\mu$g/ml of Brefeldin A (BFA, Sigma-Aldrich Canada, Oakville, Canada). BFA is a fungal metabolite that has been shown to block protein transport to the cell surface (298). Cells were then cultured at 37°C in a 4ml polystyrene tube (10$^6$ cells / 250$\mu$l per tube) for various lengths of time as indicated. For the 24h time point, cells were spun down at $t=15$h, and re-cultured in 10% CM supplemented with 2$\mu$g/ml of ConA to preserve cell viability and 0.5$\mu$g/ml of BFA, which is sufficient to maintain the BFA effect without provoking cytotoxicity. At the end of incubation, cells were washed twice with 0.5% BSA in PBS. The expression of $\text{K}^b$ and $\text{D}^b$ was examined by staining the cells with antibodies (PE-AF6-88.5 mAb for $\text{K}^b$ and FITC-KH95 for $\text{D}^b$). The expression of PR-K$^b$ was studied by incubating the cells with OVAp (or OVAp$_{K}$ when cells were pre-pulsed with OVAp) (100ng/ml/10$^6$cells) on ice for 45 min, washing twice and then staining with FITC-25D1.16 mAb (or FITC-anti-biotin Ab when OVAp$_{K}$ was used). Note that mAb 25D1.16 is specific for $\text{K}^b$-OVAp (313).
4.3.6 Pre-pulsing of cells with high affinity peptides:
Day 1 ConA-activated splenocytes were washed once with 1% CM and then re-cultured in 10% CM supplemented with OVAp (or VSVp), Flu-NP peptides (1μg/ml) and ConA (2μg/ml) overnight (8-10h). The cells were then washed three times and used to study the stability of surface K^b and D^b or the re-generation of surface PR-K^b. In experiments studying the stability of surface PR-K^b or OVAp binding to PR-K^b, the pre-pulsed cells were re-cultured in 10% CM supplemented with ConA (2μg/ml) at 37°C for an additional 6h in the absence of exogenous peptide.

4.3.7 Measurement of OVAp binding:
Day 1.5 ConA-activated splenocytes were washed twice and resuspended at 4x10^6 cells/ml in 1% CM containing OVAp (1μg/ml, 100ng/ml, 10ng/ml or 1ng/ml) with or without BFA (5μg/ml). Cells were then cultured at room temperature in 4ml polystyrene tubes (10^6 cells/250μl per tube) for various lengths of time as indicated. At the end of incubation, cells were washed twice with 0.5% BSA/PBS. OVAp binding was detected with FITC-25D1.16 mAb staining.

4.3.8 Generation of PR-K^b:
Day 1.5 ConA-activated splenocytes, after being pre-pulsed with OVAp or VSVp (1μg/ml) overnight, were washed three times with 1% CM and re-cultured in 10% CM supplemented with ConA (2μg/ml) at 37°C for various lengths of time as indicated. 250μl (4x10^6 cells/ml) of pre-pulsed cells was cultured in a 4ml polystyrene tube for each time-point. At the end of incubation, the expression of surface PR-K^b was detected by incubating the cells with OVAp (or OVAp_{K-bio} when cells were pre-pulsed with OVAp) (100ng/ml/10^6 cells) on ice for 45 min, washing twice and then staining with FITC-25D1.16 mAb (or FITC-anti-biotin Ab when OVAp_{K-bio} was used).

4.3.9 Data Analysis:
Curve fitting was performed using the GraphPad Prism™ data analysis program (version 2.0, Intuitive Software for Science, San Diego, CA). The following formulae were used: For two-phase exponential decay, \( f(t) = A_1 \cdot \exp(-k_1 \cdot t) + A_2 \cdot \exp(-k_2 \cdot t) \). Here, \( f(t) \) is the remaining
fraction of surface MHC-I at time t; $A_1$ and $A_2$ represent the initial fractions (or numbers of ABCs) of the two MHC-I sub-populations; and $k_1$ and $k_2$ are the first order decay rate constants. For loss of surface MHC-I $t^{1/2}$ was calculated from the relation $t_{1/2} = \ln(2)/k$. For first order decay: $f(t) = A*\exp(-k*t)$. "A" represents the number of Ab binding sites per cell and $k$ is the first-order rate constant. Data from the study of surface PR-K$^b$ on VSVp-pulsed cells were fit to the equation $f(t) = A*\exp(-k*t) + p$, where $p$ is the plateau value for the expression of the quasi-stable PR-K$^b$ sub-population. For peptide binding, the receptor-ligand association relation is $f(t) = P*(1-\exp(-k*t))$. "P" represents the plateau value of OVAp binding. The rate of PR-K$^b$ generation on the cell surface is the balance of the rate of PR-K$^b$ export and the rate of PR-K$^b$ decay on the cell surface. Data from the study of PR-K$^b$ generation were fit to this relation $f(t) = \alpha/k*(1-\exp(-k*t))$. "$\alpha$" is the rate of PR-K$^b$ export, in molecules per hour and "$k$" is the rate of decay of cell surface PR-K$^b$. Note that the ratio, $\alpha/k$ gives the plateau value.
4.4 RESULTS

4.4.1 Stability of total MHC-I on the cell surface.
Stability of total surface MHC-I on B6 ConA blasts was monitored as a function of time after blocking the export of newly synthesized MHC-I by addition of BFA. Antibodies and peptides used in this study are summarized in Table 4-1. Optimal staining conditions were determined for each mAb used in staining 5x10^5 cells in 50μl volume (data not shown). Examples of K^b and D^b staining with mAb AF6-88.5 and KH95, respectively, are shown in Figure 4-1 (A, B). Since the loss of antibody recognition epitopes is thought to be rapidly followed by the decay of the molecule (34, 74, 314), in this study, the loss of antibody recognition epitopes is regarded as the loss of MHC-I.

The expression of surface MHC-I at indicated time (t) after the addition of BFA is presented as a fraction of total surface MHC-I at t=0 (i.e., K^b(1)/K^b(0) and D^b(1)/D^b(0)) and is plotted as a function of time (Fig. 4-2). The mean values of measurements from three independent experiments were fit to a two-phase exponential decay equation (see materials and methods). Figure 4-2 (circles) shows an initial rapid decline in the antigenic epitopes recognized by K^b- and D^b- specific antibodies, followed by a pseudo plateau with a much slower rate of decline, suggesting two sub-populations (an unstable sub-population, and a quasi-stable sub-population) with very different half-lives for both K^b and D^b.

The t^1/2 values and curve-fitting results are summarized in Table 4-2 (#1,2). The analysis indicates that approximately 47±14% (K^b) or 68±17% (D^b) of MHC-I were quasi-stable with the unstable molecules decaying with a t_{1/2} of ~1h (K^b) or 0.9h (D^b).

4.4.2 Stability of pH-K^b and pH-D^b complexes on the cell surface.
We next investigated the decay of the quasi-stable MHC-I. MHC-I containing high affinity peptides (pH) have been shown in vitro to be very stable (33, 74, 187, 328), leading us to hypothesize that such pH-K^b and pH-D^b are the quasi-stable populations observed in Fig 4-2 (circles) and that K^b and D^b populations not associated with pH are unstable. We thus examined how an increased expression of pH-K^b and pH-D^b on the cell surface would influence the t_{1/2} of surface K^b and D^b. ConA blasts were pre-pulsed with pH (the K^b-specific peptides, VSVp or OVAp, and the D^b-specific peptide, Flu-NP) overnight to maximize the
Table 4-1. Specificity of Antibodies and Peptides used

<table>
<thead>
<tr>
<th>mAb / peptide</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb AF6-88.5</td>
<td>$\alpha_1\alpha_2$ of $K^b$</td>
<td>334, 335</td>
</tr>
<tr>
<td>mAb Y3</td>
<td>$\alpha_1\alpha_2$ of $K^b$</td>
<td>189, 337</td>
</tr>
<tr>
<td>mAb 5F1</td>
<td>$\alpha_2$ of $K^b$</td>
<td>189</td>
</tr>
<tr>
<td>mAb KH95</td>
<td>$D^b$</td>
<td>336</td>
</tr>
<tr>
<td>OVAp</td>
<td>$K^b$</td>
<td>287</td>
</tr>
<tr>
<td>VSVp</td>
<td>$K^b$</td>
<td>75, 287</td>
</tr>
<tr>
<td>Flu-NP</td>
<td>$D^b$</td>
<td>286</td>
</tr>
<tr>
<td>mAb 25D1.16</td>
<td>OVAp-$K^b$</td>
<td>331</td>
</tr>
</tbody>
</table>
Figure 4-1. Examples of FACscan data of splenocytes stained with mAb AF6-88.5, mAb KH95, mAb Y3, mAb 5F1 or mAb 25D1.16. (A) B6 ConA blasts stained with PE-conjugated mAb AF6-88.5, and (B) FITC-labeled mAb KH95 (solid lines). ConA blasts stained with isotype control IgG were used as control (dashed lines). (C) B6 ConA blasts stained with FITC-conjugated mAb Y-3, or (D) FITC-labeled mAb 5F1. (E) To measure the number of PR-K\(^b\), B6 ConA blasts pulsed with OVA\(\text{p}\) (100ng/ml/10\(^6\) cells) were stained with FITC-conjugated mAb 25D1.16. To control for the specificity of staining, B6 ConA blasts pulsed with VSV\(\text{p}\) (100ng/ml/10\(^6\) cells), which should not be recognized by mAb 25D1.16 (331) were used (dashed line). (F) To measure re-expression of PR-K\(^b\) on cells pre-pulsed with p\(\text{H}\) (VSV\(\text{p}\)), cells were pulsed immediately (t=0, solid line) or after 4h at 37\(^\circ\)C (dashed line) with OVA\(\text{p}\) and stained with mAb 25D1.16 as in (E).
Figure 4.1
Figure 4-2. Stability of cell surface $D^b$ and $K^b$ on spleen cells in the presence of BFA. The expression of $K^b$ (closed symbols) and $D^b$ (open symbols) on the cell surface was monitored using mAb AF6-88.5 and mAb KH95 respectively. ConA blasts were cultured in the presence of BFA (5μg/ml), which blocks the export of newly synthesized MHC-I, for various lengths of time (as indicated). At the end of the incubation, the expression of $K^b$ and and $D^b$ was assessed (circles). The stability of $K^b$ (closed symbols) and $D^b$ (open symbols) expression on the ConA blasts pre-pulsed with high affinity peptides (OVAp or VSVp specific for $K^b$ and Flu-NP or p33 specific for $D^b$) was also studied (triangles). Peptide pre-pulsed Day 1.5 ConA blasts were washed free of unbound peptide and incubated in the presence of BFA for various lengths of time as indicated. Numbers of Ab-binding sites (ABC) were calculated for each experiment using a Quantum Simply Cellular Kit (Sigma, St. Louis, MO). At t=0, the number of ABC per cell ranged from 118900 to 135700 for $K^b$ and 68000 to 75200 for $D^b$; the number of ABC per cell (pre-pulsed with VSVp) ranged from 116000 to 120000 for $K^b$ and 56000 to 76000 for $D^b$. The results are presented here as fractions of $K^b$ and $D^b$ molecules remaining on the cell surface at each time point after the start of incubation. The data plotted are the mean values of 3 independent experiments and were fit to a two-phase exponential decay relation. The fitting parameters were calculated and are summarized in Table 2: #1,2.
Table 4-2. Stability of surface MHC-I.

<table>
<thead>
<tr>
<th>Line #</th>
<th>$A_1$</th>
<th>$k_1 (h^{-1})$</th>
<th>$t'/2 (1)^a$</th>
<th>$A_2$</th>
<th>$k_2 (h^{-1})$</th>
<th>$t'/2 (2)^a$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>$K^b$</td>
<td>0.53 ± 0.1</td>
<td>0.63 ± 0.2</td>
<td>1.1 ± 0.1 h</td>
<td>0.47 ± 0.08</td>
<td>0.04 ± 0.04</td>
<td>20 ± 11 h</td>
</tr>
<tr>
<td>2.</td>
<td>$D^b$</td>
<td>0.34 ± 0.2</td>
<td>0.78 ± 0.6</td>
<td>0.9 ± 0.6 h</td>
<td>0.68 ± 0.12</td>
<td>0.03 ± 0.04</td>
<td>21 ± 6 h</td>
</tr>
<tr>
<td>3.</td>
<td>$K^b$</td>
<td>0.94 ± 0.03</td>
<td>0.008 ± 0.005</td>
<td>83 ± 37 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>$D^b$</td>
<td>1.00 ± 0.04</td>
<td>0.033 ± 0.008</td>
<td>21 ± 14 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>OVAp-$K^b$</td>
<td>7.3 ± 0.1 x10^4</td>
<td>0.015 ± 0.004</td>
<td>45 ± 3 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>OVAp-$K^b$</td>
<td>7.4 ± 0.1 x10^4</td>
<td>0.051 ± 0.002</td>
<td>14 ± 1 h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>PR-$K^b$</td>
<td>9373 ± 366</td>
<td>1.08 ± 0.10</td>
<td>0.63 ± 0.22 h</td>
<td>3215 ± 25</td>
<td>0.12 ± 0.04</td>
<td>4 ± 2 h</td>
</tr>
<tr>
<td>8.</td>
<td>PR-$K^b$</td>
<td>13000 ± 2100</td>
<td>1.41 ± 0.28</td>
<td>0.50 ± 0.06 h</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$K^b$ and $D^b$ on B6 ConA blasts

$K^b$ and $D^b$ on B6 ConA blasts pre-pulsed with VSVp (or OVAp) and Flu-NP

OVAp-$K^b$ complex in the presence of BFA

OVAp-$K^b$ complex in the absence of BFA

PR-$K^b$ on B6 ConA blasts

PR-$K^b$ on B6 ConA blasts pre-pulsed with VSVp
Table 4-2

Note: a. The SD value for the $t^{1/2}$ represents the variation between the $t^{1/2}$ values from independent experiments that were calculated independently.

b. The mean values of three independent experiments are plotted as a function of time (Fig 4-2, circles) and fitted to a two-phase exponential decay relation: $f(t)=A_1\exp(-k_1*t)+A_2\exp(-k_2*t)$. $f(t)$ is the remaining fraction of surface MHC-I at time, t; $A_1$ and $A_2$ represent the fractions of the two MHC-I sub-populations; and $k_1$ and $k_2$ are the first order rate constants. The $t^{1/2}$ for loss of surface MHC-I was calculated from the relation $t_{1/2}=\ln(2)/k$.

c. The mean values of three independent experiments are plotted as a function of time (Fig 4-2, triangles) and fitted to a first order exponential decay relation: $f(t)=A\exp(-k*t)$. "A" represents numbers of Ab binding sites per cell and $k$ is the first-order rate constant. The rather poor fit (see $r^2$ values) may be explained by heterogeneity in the actual $k$ value.

d. The mean values of two independent experiments are plotted as a function of time (Fig 4-4) and fitted to a first order exponential decay relation.

e. The mean values of three independent experiments are plotted as a function of time (Fig 4-5, open symbols). They were fitted to a two-phase exponential decay relation (as in a).

f. The mean values of two independent experiments are plotted as a function of time (Fig 4-5, closed symbols). They were fitted to a first-order exponential decay relation. The plateau value obtained from the fitting program was 370±160 molecules.
expression of $p_H$-bound $K^b$ and $D^b$ and hence minimize the expression of $K^b$ and $D^b$ not associated with $p_H$ (74, 328). The cells were washed free of exogenous peptide, and BFA was immediately added to prevent export of newly synthesized MHC-I. Surface $K^b$ and $D^b$ expression was then followed for the next 10h (Fig 4-2. triangles). The expression of surface $K^b$ and $D^b$ at various time points after the addition of BFA is presented as a fraction of total surface $K^b$ and $D^b$ at $t=0$, respectively. The mean values of measurements from three independent experiments were plotted as a function of time and were fitted to a first order decay equation (see materials and methods). The results of curve fitting are summarized in Table 4-2 (#3,4). The data did not fit the first order decay equation with a single $k$ value particularly well (see $r^2$ values), suggesting heterogeneity in the $k$ values. With this reservation, the $t_{1/2}$ value for the loss of surface $K^b$ was $83\pm37h$, and for surface $D^b$ was $21\pm14h$ (Table 4-2).

In comparison with the results obtained without pre-pulsing with $p_H$ (Fig 4-2. circles), there was no early drop in $K^b$ or $D^b$ expression and therefore, unstable sub-populations of $D^b$ and $K^b$ (with $t_{1/2} \sim 1h$) were not present on these $p_H$-pulsed cells (Fig 4-2. triangles). The data suggest that $p_H$-$K^b$ and $p_H$-$D^b$ are quasi-stable and that the unstable populations are not associated with $p_H$, and that their expression is minimized by pre-pulsing with $p_H$.

Two other mAbs, 5F1 and Y3, were also used to examine the stability of surface $K^b$ in the presence of BFA as a function of time (Fig 4-3). MAb 5F1 has been shown to recognize mostly peptide associated $K^b$ (189) whereas mAb Y3 was shown to recognize both PR-$K^b$ and peptide-bound $K^b$ (189, 337). Examples of $K^b$ staining with mAb Y3 and mAb 5F1 at $t=0$ are shown in Figure 4-1 (C, D). The staining pattern of $K^b$ with Y3 mAb and AF6-88.5 mAb were very similar (Fig 4-3). Both mAb Y3 and mAb AF6-88.5 have comparable binding sites on each cell, while mAb 5F1 seems to have fewer binding sites per cell. In the presence of BFA, the $K^b$ that are recognized by mAb 5F1 seemed to remain stable on the cell surface (Fig 4-3A). In comparison, the $K^b$ molecules that were recognized by mAb Y3 represent two sub-populations: unstable and quasi-stable, as observed with mAb AF6-88.5. When the stability of $K^b$ on cells pre-pulsed with $p_H$ was examined, there was no difference in the decay of $K^b$ monitored by either mAb (Fig 4-3B), suggesting that the quasi-stable $K^b$ are recognized by mAb 5F1 and are the peptide associated $K^b$. Furthermore, the unstable $K^b$ sub-population was not obviously detectable by mAb 5F1 (Fig 4-3A), suggesting
that unstable K\textsuperscript{b} do not contain a peptide that could stabilize the K\textsuperscript{b} \alpha chain for recognition by mAb 5F1.

![Graph](image.png)

**Figure 4-3.** Stability of cell surface K\textsuperscript{b} assessed with mAbs AF6-88.5, Y3 and 5F1. (A) B6 ConA blasts were cultured in the presence of BFA (5\mu g/ml), for various lengths of time (as indicated) and the expression of K\textsuperscript{b} was assessed using PE-conjugated mAb AF6-88.5 (squares), FITC-labeled mAb Y3 (circles) and FTTC-labeled mAb 5F1 (triangles). This is representative of two independent experiments. (B) Expression of K\textsuperscript{b} on ConA blasts pre-pulsed with OVAp (closed symbols) were also examined as described in Fig 4-2, with mAb AF6-88.5 (squares), mAb Y3 (circles) and mAb 5F1 (triangles). This is representative of two independent experiments. For each experiment, the number of ABC per cell was calculated using a Quantum Simply Cellular Kit (Sigma, St. Louis, MO). The data plotted are number of ABC per cell against time and fit to an exponential decay relation, \( f(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) \) for measurements made in (A) and a one-phase decay relation, \( f(t) = A_1 \exp(-k_1 t) \) for measurements made in (B).
4.4.3 **Half-life of the OVAp-K^b complex on cell surface.**

We next examined whether the decay of the OVAp-K^b complex is similar to the decay of total K^b, as monitored with mAb AF6-88.5. ConA blasts were pre-pulsed with OVAp overnight, washed free of unbound exogenous OVAp, and recultured immediately with BFA. The expression of OVAp-K^b complexes and of total K^b were monitored using FITC-labeled 25D1.16 mAb (313) and PE-labeled AF6.88.5 mAb, respectively (Fig 4-4A). The data were again successfully fit to a first order decay model; the t_{1/2} value for OVAp-K^b complexes was calculated to be 45±3h (Table 4-2: #5) and for total surface K^b was 29±9h. In comparison to quasi-stable K^b, OVAp-K^b seems to be more stable with a longer t_{1/2}. As shown in Figure 4-4A, only a very slight decline in the OVAp-K^b expression was observed over 24h but only approximately 65% of surface K^b remained detectable by mAb AF6-88.5, 24h after the BFA addition (Fig. 4-4A). Changes in total surface K^b expression during the first 10h post BFA addition are in agreement with what was observed in Fig 4-2 (triangles).

The t_{1/2} for OVAp-K^b was also measured in the absence of BFA and, to our surprise, it was 14h, much shorter than that in the presence of BFA (Fig 4-4B, Table 4-2: #6). This suggests that BFA increases the cell surface persistence of MHC-I, perhaps by preventing its internalization. This would be a previously undescribed property of BFA and would imply that the recycling mechanism for MHC-I has a t_{1/2} of about 14h. There was very little change in total surface K^b expression (Fig. 4-4B), certainly due to it being continuously replaced by new synthesis.

4.4.4 **Stability of PR-K^b on cell surface.**

Besides pH-MHC-I, a population of MHC-I called "peptide-receptive" has been documented, these being MHC-I on the cell surface that can bind appropriate exogenous peptides (74, 186, 328). We next studied the stability of these "peptide-receptive" (PR) K^b on the cell surface. In the presence of BFA, the surface expression of PR-K^b was monitored for 6h using OVAp and FITC-labeled 25D1.16 mAb as described previously (313, 338). An example of PR-K^b staining at t=0 is shown on Fig 4-1E. The mean values of measurements from three independent experiments were plotted as a function of time. The data suggest the existence of two sub-populations of PR-K^b with very different half-lives and were fitted successfully to a two-phase exponential decay equation (Fig 4-5, Table 2, #7). The less stable subpopulation
Figure 4-4. Stability of cell surface OVAp-K\textsuperscript{b} complexes in the presence or absence of BFA. Day 1 B6 ConA blasts were pre-pulsed with OVAp (1\mu g/ml) overnight and washed free of unbound peptide before being re-cultured in 10% CM with (A) or without (B) BFA for various lengths of time (as indicated). At the end of incubation, the expression of OVAp-bound K\textsuperscript{b} complexes (solid circles) and total surface K\textsuperscript{b} molecules (open circles) were examined using 25D1.16 mAb and AF6-88.5 mAb respectively. For each experiment, the number of ABC per cell was calculated. The data plotted are number of ABC per cell against time and fit to an exponential decay relation, \( f(t) = A \cdot \exp(-k \cdot t) \). The results of the curve fitting are summarized in Table 2: #5,6.
(-74% of total, calculated from $A_1/(A_1+A_2)$) has a $t_{1/2}$ of 0.6±0.2h and the quasi-stable subpopulation (~26% of total, calculated from $A_2/(A_1+A_2)$) has a $t_{1/2}$ of 4.2±2h (Table 4-2: #7). As seen in Figure 4-5 (open circles), the surface expression of PR-K<sup>b</sup> decreased drastically during the first 2h (~24±1% PR-K<sup>b</sup> remained) followed by a much slower rate of decline, such that by 6h, about 10±2% PR-K<sup>b</sup> remained on the cell surface, implying that PR-K<sup>b</sup> are included in the unstable K<sup>b</sup> observed in Fig 4-2 (circles).

We next studied the decay of PR-K<sup>b</sup> on cells pre-pulsed with a high-affinity peptide (p<sub>H</sub>). ConA blasts pre-pulsed with VSVP (p<sub>H</sub>) were washed three times and then re-cultured at 37°C for 6h to allow the constitutive export and accumulation of newly synthesized PR-K<sup>b</sup> on the cell surface. At the end of the incubation, BFA was added and surface PR-K<sup>b</sup> expression was monitored for 6h. The mean values of measurements from two independent experiments were plotted as a function of time (Fig 4-5 closed circles). There was a rapid decay of surface PR-K<sup>b</sup> in the first 2h (~5.3±0.1% PR-K<sup>b</sup> remained) followed by a pseudo plateau with a much slower decline rate. The data were successfully fitted to a first order decay equation. The $t_{1/2}$ of the PR-K<sup>b</sup> on VSVP-pre-pulsed cells was calculated to be 0.5±0.1h (Table 4-2: #8). By 6h, there were only 4.1±0.1% PR-K<sup>b</sup> remaining on the cell surface, suggesting a small population of quasi-stable PR-K<sup>b</sup> on VSVP pre-pulsed cells in comparison to non- pre-pulsed cells (~24±1%) and hence, implying that quasi-stable PR-K<sup>b</sup> might be K<sup>b</sup> associated with p<sub>M</sub> or p<sub>L</sub>, the expression of which was greatly reduced on VSVP pre-pulsed cell.

4.4.5 OVAp binding to PR-K<sup>b</sup>

We next studied the kinetics of p<sub>H</sub> binding to surface PR-K<sup>b</sup>. ConA blasts were pulsed with OVAp peptide for various lengths of time and the formation of OVAp-K<sup>b</sup> complex was detected using FITC-labeled 25D1.16 mAb. It was found that OVAp binding to H-2K<sup>b</sup> followed biphasic kinetics with a pseudo plateau being reached by ~1h (Fig. 4-6A). The initial binding appeared to follow first order kinetics ($t_{1/2}$ of ~15-16 min), while the latter binding occurred at a slower steady rate ($t_{1/2}$ of ~45-53 min). The half times for OVAp binding were derived by fitting the data to a receptor-ligand association equation (see Materials and Methods) and are summarized in Table 4-3. A plateau for OVAp binding was not observed during the 2h pulse, perhaps because newly synthesized PR-K<sup>b</sup> were being
Figure 4-5. Stability of PR-K\(^b\) on spleen cells. The expression of PR-K\(^b\) on day 1.5 B6 ConA blasts after being cultured in the presence of BFA (open circles) was examined as previously described. Briefly, at each time point, cells were pulsed with OVA\(_p\) (1\(\mu\)g/ml) for 45 min, and then stained with 25D1.16 mAb. The expression of PR-K\(^b\) on ConA blasts pre-pulsed with VSV\(_p\) overnight was also examined (closed circles). Day 1 ConA blasts were pre-pulsed overnight with VSV\(_p\) (1\(\mu\)g/ml), washed three times with 1\% CM, re-cultured in 10\% CM for 6h, and then re-cultured again in 10\% CM supplemented with BFA (5\(\mu\)g/ml) for various lengths of time as indicated. The expression of PR-K\(^b\) was then examined. The data for no pre-pulse were fit to a two-phase exponential decay model, \(f(t)=A_1*\exp(-k_1*t)+A_2*\exp(-k_2*t)\) (open circles) and for VSV\(_p\) pre-pulse to an one-phase exponential model, \(f(t)= A*\exp(-k*t)+p\) where p is a plateau value (closed circles). The results of the curve-fittings are summarized in Table 4-2: #7,8.
Figure 4-6. Kinetics of OVAp binding to surface PR-K\textsuperscript{b} on spleen cells. (A) Binding of OVAp to PR-K\textsuperscript{b} on day 1.5 ConA blasts was examined by pulsing the cells with OVAp (100ng/ml) for various lengths of time as indicated. The OVAp-K\textsuperscript{b} complexes were detected using 25D1.16 mAb. This is representative of 2 independent experiments. (B) Binding of OVAp to PR-K\textsuperscript{b} on ConA blasts was repeated in the presence of BFA, which blocks the export of newly synthesized proteins. Four different concentrations of OVAp were used in the assay: 1μg/ml (not shown in the graph, see Table 4-3), 100ng/ml (open circles), 10ng/ml (closed triangles) and 1ng/ml (open squares). ABCs were calculated, plotted against time and fitted to a binding equation, \( f(t)=p*(1-exp^\lambda(-kt)) \) where p is the plateau value and k is the rate constant. The results of curve fitting are summarized in Table 4-3.
continuously exported to the cell surface. We therefore repeated the experiment in the presence of BFA (Fig 4-6B).

In the presence of BFA, a rapid increase in OVAp (100ng/ml) binding was observed in the initial phase, with a half-time of ~11 min (Table 4-3: #3), followed by a pseudo plateau with a much slower rate of OVAp-binding (t"/2 of 2.2h) (Fig 4-6B). The presence of BFA greatly reduced the rate of binding in the latter phase, in concordance with our hypothesis that the export of newly synthesized PR-Kb might be the major source of surface PR-Kb for OVAp binding in the latter phase observed in Figure 4-6A. Another source of PR-Kb might be from the dissociation of peptide from Kb containing pM. The quasi-stable PR-Kb sub-population observed in Fig 4-5A (~26% of total PR-Kb) very likely consists of pM-Kb-β2m, which is more stable on the cell surface than pL-Kb-β2m and becomes peptide-receptive upon the dissociation of pM.

We also examined OVAp binding to surface PR-Kb using various concentrations of OVAp peptide and found that the rates of OVAp-binding (during the initial phase of binding) are logarithmically proportional to the concentrations of OVAp used (Fig 4-6B). On average, ~11 min was required for either 1μg/ml or 100ng/ml of OVAp to saturate half of the surface PR-Kb while ~17.5 min was required for 10ng/ml of OVAp and ~29 min was required for 1ng/ml of OVAp (Table 4-3). In addition, we found that the OVAp concentration used appeared to have very little effect on the latter binding phase. Although different amounts of time were required for different concentrations of OVAp to reach a plateau of binding, there was no significant variation in the plateau values. The plateau values for OVAp-Kb formation were ~15000 molecules calculated for Fig 4-6B independent of the OVAp concentration (1μg/ml, 100ng/ml and 10ng/ml) used in the study (Table 4-3).

Taken together, these observations suggest that the concentration of OVAp used is the rate-limiting factor during the initial phase of binding but not during the latter phase (pseudo plateau).

4.4.6 Rate of PR-Kb generation.

We next measured how fast new surface PR-Kb were generated and accumulated on the cell surface to keep the PR-Kb expression relatively constant on resting cells. In this experiment, a zero baseline of PR-Kb expression was established by pre-pulsing ConA blasts overnight.
with VSVp and then washing the cells 3 times. PR-K\textsuperscript{b} generation was monitored using OVAp and FITC-labeled 25D1.16 mAb. The measurements were fit to the equation, 
\[ f(t) = \alpha/k*(1-exp^(-k*t)), \]
where \( \alpha \) is the number of PR-K\textsuperscript{b} exported to the cell surface per unit of time and \( k \) is the exponential decay constant for those that have reached the surface. Note that the ratio \( \alpha/k \) defines the plateau value reached. The fitting parameters are summarized in Table 4-4. The mean values of two independent experiments presented in Figure 4-7 depict the kinetics of PR-K\textsuperscript{b} generation at 37°C. There was a steady increase in PR-K\textsuperscript{b} expression within 4h of incubation, followed by a plateau of \(-17700\) PR-K\textsuperscript{b} molecules per cell (Fig. 4-7, Table 4-4). The plateau value was a bit higher than the number of surface PR-K\textsuperscript{b} on unmanipulated cells (\(-12700\) PR-K\textsuperscript{b} molecules per cell, Table 4-2: #7, \( A_1+A_2 \)).
Table 4-3.  OVAp binding to surface PR-K$^b$$^a$

<table>
<thead>
<tr>
<th>Line #</th>
<th>[OVAp] (ng/ml)</th>
<th>Overall $t^{1/2}$ (min)</th>
<th>Pseudo-plateau ($\times 10^3$)</th>
<th>$t^{1/2}$ (1) (min)$^b$</th>
<th>$t^{1/2}$ (2) (h)$^c$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>100</td>
<td>55</td>
<td>16 ±3</td>
<td>16</td>
<td>0.9</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In the presence of BFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>1000</td>
<td>32</td>
<td>15 ±1</td>
<td>11</td>
<td>2.6</td>
<td>0.97</td>
</tr>
<tr>
<td>3.</td>
<td>100</td>
<td>30</td>
<td>15 ±1</td>
<td>11</td>
<td>2.2</td>
<td>0.97</td>
</tr>
<tr>
<td>4.</td>
<td>10</td>
<td>54</td>
<td>15 ±1</td>
<td>17</td>
<td>2.9</td>
<td>0.99</td>
</tr>
<tr>
<td>5. $^d$</td>
<td>1</td>
<td>83</td>
<td>-</td>
<td>29</td>
<td>na</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table 4-3
Note:  
$^a$ This is representative of two independent experiments. The measurements were plotted against time (Fig 6) and were fitted to the receptor-ligand association relation, \( f(t) = P*(1 - \exp^(-k*t)) \). “P” represents the plateau value of OVAp binding and “k” represents the binding constant.

$^b$ The measurements of OVAp binding within the first hour after the addition of OVAp were fitted to the receptor-ligand association relation with fixed “P” value (from the curve fitting results using the whole set of data).

$^c$ The measurements of OVAp binding during the second hour after the addition of OVAp were fitted to the receptor-ligand association relation with fixed “P” value (from the curve fitting results using the whole set of data).

$^d$ OVAp (1ng/ml) binding did not reach a plateau during the 2h-experiment, therefore the pseudo-plateau value could not be calculated.
Table 4-4. Re-generation of surface PR-K^b

<table>
<thead>
<tr>
<th>( \alpha ) (per hour)</th>
<th>k (decay, h(^{-1}))</th>
<th>( t^{1/2} ) (decay) (h)</th>
<th>Plateau (x10(^3))</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2861 ± 233</td>
<td>0.159 ± 0.03</td>
<td>4.4 ± 0.04</td>
<td>17970 ± 162</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Figure 4-7. Generation of PR-K^b on spleen cells. The accumulation of surface PR-K^b on ConA blasts was studied by pre-pulsing day 1 ConA blasts with VSVp (1\( \mu \)g/ml) overnight, and then re-culturing the cells at 37°C for various lengths of time (as indicated). At each time point, the expression of surface PR-K^b was examined by pulsing cells with OVAp (1\( \mu \)g/ml) for 45min and then staining the OVAp-K^b complexes with 25D1.16 mAb, as described in Fig. 4-3A. The data were plotted as the mean values (n=2) of the number of 25D1.16 binding sites per cell against time. The results of curve fitting are summarized in Table 4-4.
4.5 DISCUSSION

In this study, we have used conformationally sensitive antibodies to examine the stability of surface MHC-I on spleen cells. AF6-88.5 mAb recognizes a conformationally specific epitope on the $\alpha_1\alpha_2$ domain of the K$^b$ molecule (334, 335); KH95 mAb recognizes a conformationally dependent epitope on the $\alpha_2$ domain of D$^b$ (336) (Table 4-1). Note that loss of the conformational epitope of the $\alpha$ subunit is thought to be followed by rapid degradation/ internalization of the molecule (34, 74, 314). Our data suggest that K$^b$ and D$^b$ both exist on the cell surface as two sub-populations with very different half-lives (Fig. 4-1 & Table 4-2: #1,2): unstable and quasi-stable. Approximately 50% of surface K$^b$ molecules were quasi-stable while the other 50% were unstable and were lost with a $t_{1/2}$ of $-1.1\pm0.1h$. Similar observations were made for surface D$^b$: ~68% of surface D$^b$ were quasi-stable.

Quasi-stable K$^b$ and D$^b$ sub-populations appeared to be MHC-I molecules associated with p$_M$ or p$_H$ because MHC-I associated with p$_H$ (e.g., OVAp-K$^b$) were more stable and had a longer $t_{1/2}$ (Fig 4-4A; Table 4-2: #5; $t_{1/2} = 45\pm3h$) than the MHC-I associated with p$_M$ or p$_L$ (328, 339). In support of this hypothesis, surface K$^b$ on cells pre-pulsed with p$_H$ also had a longer $t_{1/2}$ (~83±37h) than that of the K$^b$ on non-pre-pulsed cells (~20±11h) (Fig 4-2 triangles, Table 4-2: #3,4). We assume that when cells are pre-pulsed with a high concentration of p$_H$ overnight, pre-bound p$_M$ and p$_L$ are replaced by p$_H$ as they dissociate during the incubation. Thus, pre-pulsing cells with p$_H$ increases the percentage of p$_H$-MHC-I and decreases the amount of p$_M$-/p$_L$-MHC-I on the cell surface. Figure 2 depicted a strong correlation between the increased percentage of p$_H$-K$^b$/D$^b$ and increased sizes of quasi-stable K$^b$ and D$^b$ sub-populations. Note that unstable K$^b$ and D$^b$ sub-populations were not detectable on cells pre-pulsed with p$_H$ when export of newly synthesized MHC-I was blocked (Fig 2).

Peptide receptive K$^b$ was not detectable on cells pre-pulsed with high concentrations of p$_H$ if the continuous export of new MHC-I from the ER was blocked by BFA (Fig 4-1F) (309), suggesting that PR-K$^b$ constituted at least part of the unstable K$^b$ sub-population. Surface PR-K$^b$ have been reported (32, 34, 74) and are confirmed here by us to be very unstable (Fig. 4-5, Table 4-2: #7,8). In studying the stability of PR-K$^b$, we found that there were again two sub-populations. Approximately 74% PR-K$^b$ had short $t_{1/2}$, 0.6±0.2 h (Fig 4-
5, Table 4-2: #7) and could not be detected after 2h of incubation in the presence of BFA. The quasi-stable PR-Kb sub-population had a longer $t^{1/2}$, 4.2±2 h (Table 4-2: #7). PR-Kb reappear (at a rate of 2860±230 ABCs per h, Table 4-4) on the cell surface pre-pulsed with pH in the absence of exogenous pH via the continuous export and accumulation of new Kb (Fig 4-7; (338)). Pre-pulsing cells with high concentrations of pH decreased surface pL and pM associated Kb suggesting that the quasi-stable PR-Kb might be Kb associated with pL or pM. Table 4-2 (#8) and Fig 4-5 show that the size of the quasi-stable PR-Kb sub-population was substantially reduced on cells pre-pulsed with pH.

Binding of exogenous peptide to PR-MHC-I is determined by the off-rate of pre-bound peptide (302, 328); therefore, a Kb molecule is truly peptide-receptive when the pre-bound peptide has already dissociated and before the molecule decays. Consequently, the unstable PR-Kb sub-population may contain no peptide or peptides of extremely low affinity in their binding groove and the PR-Kb quasi-stable populations may be the Kb bound with pL or pM. The release of pL and pM can occur later during the incubation, resulting in the formation of PR-Kb. The reduction in the size of the quasi-stable PR-Kb sub-population on cells pre-pulsed with pH implicates that less quasi-stable PR-Kb was exported to the cell surface during the 6h post-pulsing incubation, in comparison to unstable PR-Kb. Such observation makes logical sense in the maintenance of surface Kb expression: unstable PR-Kb has a much shorter $t^{1/2}$ and therefore, more has to be made to balance the rapid loss. The quasi-stable PR-Kb has a longer $t^{1/2}$ and therefore, a slower rate of generation will avoid over-accumulation of quasi-stable PR-Kb on cell surface. In agreement with this speculation, Christinck et al. and Luscher et al. showed that ~10% of total Db on EL4 cells are peptide receptive (76, 186). Here, we also showed that ~10% (~12600 binding sites for 25D1.16 mAb per cell, Fig 4-5, t=0) of surface Kb (~124000 binding sites for AF6-88.5 mAb per cell) are peptide receptive. At equilibrium, the number of molecules on the cell surface with a particular half-life will be given by the expression $\alpha/k$ (see Fig 4-7) where $\alpha$ is the rate of export (molecules per unit time) and $k$ is the decay constant (unit time$^{-1}$). Using the data obtained in this study, we have calculated that for every twenty Kb exported to the cell surface, only one Kb is associated with a pH (see footnote for calculation)\textsuperscript{1}.

\textsuperscript{1} Change in surface MHC-I (dN) can be calculated as the difference between the number of MHC-I generated and the number of MHC-I lost. That is $dN = \alpha*dt - k*N(t)*dt$. Here, $N(t)$ represents the number of molecules
The kinetics of OVAp binding to PR-K^b was examined in this study. OVAp binding was biphasic with a rapid increase whose rate was dependent upon peptide concentration, followed by a much slower increase whose rate was independent of peptide concentration (Fig 4-6A). We hypothesize that the initial rapid increase in OVAp binding depicts the stabilization of unstable PR-K^b containing either no peptide or p_L of extremely low affinity, and the much slower rate represents OVAp binding to the quasi-stable PR-K^b which became available for peptide binding on dissociation of the p_M they contain. In comparing the rate of binding using different concentrations of p_H, we observed that a higher concentration of p_H (100ng/ml) had a more rapid binding rate ([1/2] ~30 min) and a lower concentration of p_H (1ng/ml) had a slower binding rate ([1/2] ~83 min). These observations suggest that peptide concentration is the rate limiting factor during the initial phase of binding and that the number of PR-K^b is finite. In addition, since the peptide concentrations used did not affect the binding rate or plateau values during the pseudo-plateau period peptide concentration may not be a limiting factor.

The equilibrium constant (k_d) for dissociation was calculated to be ~1.1 nM at 24°C in the presence of BFA, assuming a reaction of the form, (peptide + K^b) \xrightarrow{k_{ass}} K^b-peptide, where 0.104μM of OVAp (equivalent to 100ng/ml) was used, and k_d= k_{dis}/k_{ass}; k_{dis} values are from Table 4-2: #5,6; k_{ass}= k_{obs}/0.104; k_{obs} values are from Table 4-3:#1, #7). The k_d calculated in the absence of BFA is ~7.3nM, suggesting that OVAp will start to dissociate from K^b when the exogenous OVAp concentration falls below 7.3nM. It might be an over-estimation because in the absence of BFA, PR-K^b is continuously exported to the surface and therefore, the starting number of K^b is not fixed in the equation and over time, more peptide is required to favor the formation of K^b-peptide complex. In the presence of BFA, the initial number of peptide and K^b are fixed and therefore, the k_d value may be more valid.

Although this study has revealed some insights into the expression and stability of surface K^b, D^b and PR-K^b in particular, it has also raised a few questions. Approximately 50% of total surface K^b molecules were unstable. This includes PR-K^b. However PR-K^b

at time t, “k” is the decay constant and is calculated as ln(2)/[1/2] and “α” represents the generation constant. The number of total MHC-I on the cell surface is constant. Hence, dN=0 and the α values for quasi-stable (α_q) and unstable (α_u) K^b sub-populations can be calculated. α = k*N(t); α_q = (124000 * 0.47)*(ln(2)/20)=2020 molecules/h; α_u = -(124000* 0.53) * (-ln(2)/1.1) = 41412 molecules per h. α_q/(α_q+α_u)= ~ 1/20.
only constitutes ~10% of total surface Kb; even in the presence of continuous Kb export, the maximum number of PR-Kb that can be stabilized by OVAp binding within the first 2h of incubation is approximately $13 \pm 1 \times 10^3$ molecules, (~10±2% of AF6-88.5 positive surface Kb). Then what constitutes the other approximately 4/5 of unstable surface Kb? Although this study has revealed some characteristics of this 4/5 of unstable surface Kb, its molecular identity awaits to be revealed. Our data showed that it has a $t_{1/2}$ of ~1.1h and that it disappeared during the overnight pre-pulse with pH because the unstable Kb sub-population was not observed on pre-pulsed cells (Fig 4-2, triangles). Figure 4-3 showed that the unstable Kb sub-population was not obviously detectable by mAb 5F1, which recognizes mainly peptide associated Kb and thus suggesting it lacks peptide. Moreover, studies with purified Kb complexes and Kb in the cell lysates suggest that some a2m, a, and p-a do not contain the $\alpha_1\alpha_2$ antigenic epitope and are not stable on the cell surface (33, 34, 74, 97, 314, 340). These could all very well be the candidates for the 4/5 of the unstable sub-population of Kb. There is still much to be discovered about the expression, structure and stability of MHC-I.

It has been widely assumed that the functional MHC-I conformation is the peptide-associated heterotrimer. Previous work from this laboratory suggests that NK cells express inhibitory receptor(s) recognizing the peptide-receptive form of MHC-I (309, 338) and not the stable heterotrimer. While T cells recognize the peptide residues presented in the peptide binding groove of MHC-I as well as part of the MHC-I molecules itself (316), NK cells seemed to “see” conformationally specific MHC-I (194). The prototypic mouse NK inhibitory receptor, Ly49A, was shown to recognize MHC-I associated with peptide (41, 282). Analysis of the crystal structure of the extracellular domain of Ly49A and its ligand, Dd, suggests that the peptide is not directly involved in the recognition (194). It is the conformation of Dd, shaped by peptide binding, that is important for Ly49A recognition. In support of this, Chung et al. showed that the Dd-peptide complex (transgenically expressed in a β2m-deficient background) could stimulate T cell activation but failed to interact with NK cells expressing Ly49A, suggesting, again that NK and T cells recognize MHC-I differently (341). Furthermore, another NK inhibitory receptor, Ly49C seemed to recognize PR-Kb and not Kb associated with a high affinity peptide; binding of Ly49C to Kb was prevented by loading surface PR-Kb with a high affinity peptide (338). The important physiological roles
of MHC-I in both NK and T recognition highlight the necessity in studying the structural conformations and stability of cell surface MHC-I.
A NOVEL MECHANISM FOR RECOGNITION OF ADENOVIRUS TYPE V INFECTED CELLS BY NATURAL KILLER CELLS
5.1 ABSTRACT

NK specificity is primarily determined by inhibitory receptors that recognize particular MHC-I. We have found that these include inhibitory receptors that recognize only MHC-I capable of binding exogenous peptide. Thus, the mouse inhibitory receptor Ly49C\textsuperscript{B6} recognizes only the peptide-receptive form of K\textsuperscript{b} (PR-K\textsuperscript{b}). Following Adenovirus type V infection of a cell, total K\textsuperscript{b} expression was little affected but PR-K\textsuperscript{b} disappeared between 6 and 9h post infection. This correlated directly with acquisition of sensitivity to NK lysis and the stimulation of IFN-\gamma synthesis. Adenovirus type XII infected cells did not down-regulate either total or PR-K\textsuperscript{b} and did not become sensitive to NK cells. The data suggest an extension of the "missing self" hypothesis to include different forms of MHC-I.
5.2 **INTRODUCTION**

Natural killer (NK) cells play a critical role in the control of viral infection, both by their cytotoxic potential and by their capacity to secrete anti-viral cytokines (259, 342, 343). However, target structures important for NK cell recognition of virally infected cells are not well defined. NK cells stimulated by viral infections *in vivo* were shown in the 1970s to lyse targets expressing reduced/no Class-I major histocompatibility complex (MHC-I) (257, 344, 345). This later became a critical criterion in formulating the “missing-self” hypothesis (11). It was hypothesised that NK cells look for the absence of a self-marker on the target cell surface, and that this self-marker is surface MHC-I. Evidence continues to accumulate in support of this hypothesis (346). However, down-regulation of total surface MHC-I is not always observed on virally infected cells that are sensitive to NK lysis and down-regulation of total MHC-I does not always correlate with the sensitivity of the infected cells to NK lysis. For example, cells infected by HSV (347) or transformed by adenovirus type V (Ad5) (267, 268, 348, 349) become susceptible to NK lysis but do not have reduced total surface MHC-I expression. Further, cells transformed by adenovirus type XII (Ad12) have reduced expression of total surface MHC-I but remain resistant to NK lysis (348, 350, 351). These observations suggest that the “missing-self” hypothesis may be incomplete in depicting the mechanisms of NK recognition. Perhaps, our understanding of MHC-I as a self-marker in NK recognition is incomplete.

Several NK receptors involved in activating and inhibiting NK function have been identified and characterized (23-25); the current model of NK-recognition suggests that NK recognition and lysis of targets is mediated by activation receptor(s) whose effects may be overridden by inhibitory receptor(s) recognizing MHC-I (126, 168). Viral modulation of ligands recognized by NK activating receptors is yet to be defined (256, 261, 271). However, there are two major sub-populations of surface MHC-I (peptide-receptive MHC-I or “PR-MHC-I”, and non-peptide-receptive MHC-I associated with high affinity peptides (pH)) (74, 328) that have been implicated in recognition by NK inhibitory receptors (41, 282, 338) and may be differentially regulated by viral proteins.
Viral proteins that are involved in interfering with the processing pathway of MHC-I have been identified (48, 66, 256). Examples of viral strategies in shutting down surface MHC-I expression are global disruption of protein synthesis, prevention of peptide binding to MHC-I in the ER, and transport of MHC-I to the cytosol instead of the cell surface (48, 66, 256, 352). Surface PR-K^b molecules on a TAP-deficient cell line (RMA-S, which lacks proper peptide-loading machinery) have been shown to be unstable, with a t^{1/2} of less than 0.5h, whereas surface MHC-I associated with p_H have a t^{1/2} of greater than 10h on EL-4 cells. Thus, when export of MHC-I to the cell surface is disrupted during a viral infection, the virus-infected cells will be detected first by NK cells expressing inhibitory receptors for PR-MHC-I, perhaps within 1-2h after disruption of MHC-I synthesis and/or transport, and then later by NK cells expressing inhibitory receptors for p_H-bound MHC-I, perhaps 20h later.

No monoclonal antibody is yet available for staining surface PR-MHC-I. They can only be detected by measuring the binding of MHC-I specific peptide and have only recently been shown to be a ligand for NK inhibitory receptor(s) (309, 338). In most studies of viral infection, only the changes in total MHC-I expression have been examined (267, 346, 347). Since PR-MHC-I appears to constitute only ~10% of total surface MHC-I (shown with PR-K^b and PR-D^b) (76, 186) (Su & Miller, in preparation), changes in PR-MHC-I expression on virally infected cells might only reflect a slight change in total surface MHC-I expression. Consequently, the loss of PR-MHC-I on virally infected cells might not be detected with mAb against total cell surface MHC-I, but could be detected by NK cells expressing inhibitory receptors recognizing PR-MHC-I. In this study, changes in surface PR-K^b and total surface MHC-I on B6 spleen cells infected with Ad5 or Ad12 have been studied during the first 24h after viral infection.
5.3 MATERIALS AND METHODS

5.3.1 Mice and viruses:
Normal C57BL/6 (B6, H-2b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 (H-2b) athymic nude mice were purchased from Taconic (Germantown, NY). All mice were kept in a specific pathogen free environment. In most experiments, 6-10 week old female mice were used (although either sex gave similar results). The human Adenovirus type 5 (Ad5) and type 12 (Ad12) used have been previously described (353, 354).

5.3.2 Cells:
Splenocytes were prepared by pressing spleens through a wire mesh screen with a disposable plunger into α-MEM (Gibco, BRL, Burlington, ON, Canada) supplemented with 10% FCS (Gibco, BRL), 50 μM 2-ME, and 10 mM HEPES (10% complete medium, CM). Splenocytes were resuspended in 5ml CM, underlaid with 5ml lympholyte-M (Cedarlane Laboratories, Hornby, ON, Canada), and centrifuged at 500x g for 20 min to remove red cells and dead cells. After two washes in CM, 5x10^6 splenocytes were re-suspended and cultured overnight in 5ml CM supplemented with Concanavalin A (ConA, 2μg/ml, ICN Pharmaceuticals Canada Ltd., Montreal, Que.). Day 1 ConA-activated blasts (ConA blasts) were used in the virus infection experiments. A cell population enriched in dendritic cells was obtained by plating splenocytes from B6 mice on polystyrene petri dishes at 10^7 cells per ml and culturing at 37°C for 3h. At the end of incubation, non-adherent cells were washed off gently with warm CM. The adherent cells were re-cultured at 37°C for an additional 1.5h and washed twice with warm CM. The resulting adherent cells are enriched in dendritic cells (355). These detached from the petri dish on being cultured overnight at 37°C and could then be harvested by gentle washes with warm CM. A cell population enriched in dendritic cells was used as antigen presenting cells for raising virus-specific cytotoxic T lymphocytes (CTL).

5.3.3 Virus infection:
Day 1 ConA blasts were pre-washed twice with 1% CM (supplemented with 1% FCS instead of 10% FCS) and co-cultured with Ad5 or Ad12 virus (100 pfu/cell) in PBS for 2h at 37°C in
a 7% CO₂ incubator (~4×10⁶ cells in 0.2ml PBS per well of a 24-well plate) for absorption. At the end of incubation, ConA blasts were washed once with 1% CM to wash away unabsorbed viruses and re-cultured in 2ml diluted supernatant from ConA blasts culture (50% dilution with 10% CM), at a density of 10⁶ cells per 0.5ml per well of a 24-well plate. A cell population enriched in dendritic cells was also infected with Ad12 (100 pfu/cell in PBS) in a similar manner and used for raising Ad12-specific T cells. At the end of co-incubation with viruses, the dendritic cell enriched population was washed twice with 1% CM and re-cultured in 1 ml 10% CM in 25 cm² flasks (in up-right position) in a 7% CO₂ incubator until the addition of T cells.

5.3.4 Surface MHC-I expression on virus-infected cells:
Surface Kᵇ and Dᵇ expression were examined using PE-labeled AF6-88.5 mAb and FITC-labeled KH95 respectively (1μg/10⁶ cells/0.1ml, purchased from Pharmingen, San Diego, CA). As described previously (338), the expression of surface PR-Kᵇ was detected by incubating the cells with OVAp (100ng/ml/10⁶ cells) on ice for 45 min, washing twice and then staining with FITC-labeled 25D1.16 mAb. 25D1.16 mAb recognizes specifically the OVAp-PR-Kᵇ complex (313). To stain cells with mAb, 2×10⁵ cells in 100 μl of 0.5% BSA/PBS were first incubated with 4 μl of reconstituted normal mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 5 min. Fluorescently-labeled mAbs were then added to the cells and incubated on ice, in the dark for 30-45 min. The cells were then washed in 0.5% BSA/PBS, resuspended in 0.3 ml of 0.1% paraformaldehyde and 0.25% BSA in PBS and analyzed by flow cytometry. The fluorescence and light scatter properties of individual cells were measured on a FACScan analyzer (Becton-Dickinson), using logarithmic amplification of the fluorescence signals and linear amplification of the right angle/forward angle light scatter signals. Live splenocytes were gated (on the basis of right angle/forward scatter measurement) and analyzed for their fluorescence. The number of antibody binding sites (ABC) (for AF6-88.5 mAb, KH95 mAb, and 25D1.16 mAb) per cell could be calculated using a Quantum Simply Cellular Kit (Sigma, St. Louis, MO). Quantum Simply Cellular Kit is a mixture of four populations of microbeads with different ABCs plus one non-binding microbead population. The ABC is derived from covalently bound Goat anti-mouse Ig on the microbeads. This Goat anti-mouse Ig has equivalent reactivity to each mouse isotype (IgG1, IgG2a and IgG2b).
Quantum Simply Cellular microbeads were used in the experiments as an external calibrator. 10^5 microbeads in 50μl of 0.5% BSA/PBS were stained with FITC- or PE-labelled mAbs in parallel with the cell samples. Staining of microbeads and cell samples was analyzed with the same instrument settings except for side light scatter (much lower for microbeads). A regression calibration curve was constructed using the QuickCal program (provided by the manufacture), which plots the mean fluorescent intensities (MFI) of the Quantum Simply Cellular microbeads against the ABC values predetermined for each microbead population. The ABC values for cell samples were calculated using the regression calibration curve and the MFI of mAb-staining. In this study, the number of ABC was equated to the number of antigenic molecules on the cell surface.

5.3.5 NK and CTL generation:
The method used for producing activated NK cells (LAK cells) was similar to that used previously (38, 309, 312). Briefly, 2x10^6 nylon wool non-adherent spleen cells from B6 athymic nude mice were cultured at 37°C in 5 ml 10% CM supplemented with 5000 U/ml human IL-2 (Chiron Corp. Emeryville, CA). Day 3-4 NK cells were used in cytotoxicity assays. Day 0.5 NK cells were used in IFN-γ assays. Ad12-specific CTL were generated in vitro using Ad12-infected dendritic cells (within 24h post-infection) as stimulators and lymphocytes from normal C57BL/6 (B6) mice, depleted of B cells by passage through nylon wool, as a source of T cells. For primary stimulation, 10^7 T cells were stimulated with 5x10^5 Ad12-infected dendritic cells in 10ml of 10% CM supplemented with 5u/ml of mouse rIL-2 in 25cm² flask, cultured in an up-right position at 37°C in a 7% CO₂ incubator. To maintain a CTL line, CTL were harvested after 7-10 days of culture (or 12-14 days after the primary stimulation) on Lympholyte M (Cedarlane Lab., Hornby, ON) and re-cultured with 1x10^6 Ad12-infected dendritic cells as in the primary stimulation. For cytotoxicity assays, day 5 CTL were harvested on Lympholyte M and used. Mouse rIL-2 was obtained as a supernatant from a cell line transfected with the IL-2 gene (285).

5.3.6 ⁵¹Cr-release assay
Methods for measuring lytic activity were identical to those used previously (39, 155, 309, 338). Ad5- and Ad12-infected ConA blasts were ⁵¹Cr-labelled by incubating about 1x10^6 cells for
90 min at 37°C with 180 μCi Na\textsuperscript{51}CrO\textsubscript{4} (NEN Life Science, Boston, MA) in 75 μl of PBS containing 67% FCS. They were then washed 3 times with 1% CM, to remove non-incorporated Na\textsuperscript{51}CrO\textsubscript{4} before being used in a 4 hr (unless stated otherwise) \textsuperscript{51}Cr release assay performed in 96-well V-bottom microtiter plates using 2000 targets/well, dispensed in 100 μl aliquots. NK cells or CTL (at E/T ratios, 30, 10 and 3) were also added in 100 μl aliquots. RMA-S cells (~10\textsuperscript{6}) were \textsuperscript{51}Cr-labelled for 60 min at 37°C or for 90 min at 26°C and then prepared in the same way as ConA blasts for the assay. For experiments in which pre-incubation of NK cells with soluble NK1.1 mAb or 5E6 mAb bound to Dynabeads was required, the pre-incubation was done at 4°C for 30-45 min while preparing target cells for the assay. Prior to the addition of NK1.1 mAb to NK cells, NK1.1 mAb was pre-incubated on ice for 30 min with soluble Protein A (2μg per 10μg of mAb used, Sigma, St. Louis, MO) and soluble Protein A/G mix (2μg per 10μg of mAb used, ICN Biomedicals Inc., Aurora, OH). Soluble Protein A and G bind to the Fc portion of antibody preventing antibody from binding to the Fc receptor on NK cells and thus, prevent antibody-directed cellular cytotoxicity (ADCC). The mAb remained in the assay mixture during the 4h \textsuperscript{51}Cr-release assay. For experiments in which pre-incubation of target cells with OVA\textsubscript{p} (100ng/ml/10\textsuperscript{6}, (287)) was required, the pre-incubation was done at room temperature for 30-45 min. The target cells were then washed twice before being used in the assay. Specific lysis was calculated as % specific lysis = (E-S)/(T-S) x 100 where each value represents the mean ± S.E.M. of five replicates. E is the experimental mean of \textsuperscript{51}Cr released; S, the amount of \textsuperscript{51}Cr released when the target cells were cultured in medium alone; and T, the total amount of \textsuperscript{51}Cr released in the presence of 2% acetic acid.

5.3.7 IFN-γ production assay

NK cells used in this study were nylon wool non-adherent spleen cells from B6 athymic nude mice cultured at 37°C in 5 ml 10% CM supplemented with 2200 U/ml human IL-2 for 10-12h. On the day of assay, NK cells were washed twice with 1% CM and kept on ice in 10% CM before being added to the infected cells. Ad5- and Ad12- infected ConA blasts were co-cultured with NK cells (at least one NK cell per three infected ConA blasts) for 2h at 37°C, allowing time for NK activation. Brefeldin A (BFA, 5μg/ml, Sigma-Aldrich Canada, Oakville, Canada), a fungal metabolite that blocks protein export to the cell surface and thereby stops
IFN-γ secretion, was added to the "co-culture" containing NK cells and virus-infected cells, 2h after the addition of NK cells. The "co-culture" was then incubated at 37°C for an additional 4h allowing time for IFN-γ to accumulate in the cytosol. IFN-γ synthesis was assayed by flow cytometry. Cells were washed once in Mg²⁺- and Ca²⁺-free PBS (PBS⁻), re-suspended in 100 µl of PBS⁻ and then incubated with 4 µl of reconstituted normal mouse serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 8µl of the supernatant from hybridoma (2.4G2) at room temperature for 10 min. MAb 2.4G2 binds to the FcRIII on NK cells and thus, prevents the subsequently added mAb from binding to FcRIII non-specifically. PE-labeled NK1.1 mAbs were then added to the cells and incubated at room temperature for 20 min. The cells were then washed with PBS⁻, fixed and permeablized using the Cytofix/Cytoperm kit purchased from Pharmingen (San Diego, CA). Fixed cells were incubated with anti-mouse IFN-γ mAb (0.5µg/10⁶ cells per 100µl, clone XMG1.2, Pharmingen, San Diego, CA) on ice for 30 min. At the end of incubation, cells were washed twice and re-suspended in 0.3 ml of washing buffer for FACScan analysis.
5.4 RESULTS

5.4.1 Changes in host MHC-I expression during adenovirus infection.

Adenovirus type V (Ad5) and type XII (Ad12) have been shown to have different effects on transformed cell lines (267, 268, 348, 349, 352, 356). We measured whether there were differential effects on host MHC-I expression during the early phase of viral infection. Figure 5-1 shows the staining of K\(^b\), D\(^b\) and PR-K\(^b\) on cells infected with Ad5 or Ad12, 7h and 10h post infection while Figure 5-2 shows changes of MHC-I expression over time. A small but significant increase in surface K\(^b\) expression was observed on the ConA blasts infected with either Ad5 or Ad12 by 18h post-infection (student t-test, \(p < 0.01\)) (Fig 5-2A). Surface D\(^b\) expression was also slightly up-regulated on Ad12-infected cells by 18h post-infection (\(p < 0.02\)), but not on Ad5-infected cells (Fig 5-2B). Down-regulation of either K\(^b\) or D\(^b\) surface expression was not observed over the first 42h following infection.

Since changes in surface peptide-receptive K\(^b\) (PR-K\(^b\)) expression might affect the susceptibility of the infected cells to NK lysis (309, 338), we also examined whether Ad5- and Ad12-infection had an effect on surface PR-K\(^b\) expression, something not previously examined. Surface expression of PR-K\(^b\) remained unchanged during the first 6h after Ad5- and Ad12-infection (Fig 5-2C). A drastic drop in PR-K\(^b\) expression on Ad5-infected ConA blasts occurred between 6h and 9h post-infection such that there was very little or no detectable PR-K\(^b\) expression between 9h and 42h post-infection. On the contrary, Ad12-infected ConA blasts expressed up-regulated PR-K\(^b\) on their surface by 18h post-infection (\(p < 0.01\)). No decrease in surface PR-K\(^b\) expression was observed on Ad12-infected cells (Fig 5-2C) over the 42 hours.

In summary, Ad5-infection down-regulated surface PR-K\(^b\) expression between 6h and 9h post-infection and up-regulated K\(^b\) expression by 18h post-infection, without affecting D\(^b\) expression. In contrast, Ad12-infection increased surface K\(^b\), D\(^b\) and PR-K\(^b\) expression by 18h post-infection.
Figure 5-1. Surface K^b, D^b and PR-K^b expression on Ad5- and Ad12-infected cells at 7h and 10h post-infection. B6 ConA blasts infected by Ad5 (a,d,g), Ad12 (b,e,h) or mock-infected (c,f,i) were examined for surface K^b (a,b,c), D^b (d,e,f) or PR-K^b (g,h,i) expression at 7h (black lines) and 10h (grey lines) post-infection. The data shown are representative of data used for Fig 5-2.
Figure 5-2  Changes in surface K$^b$, D$^b$ and PR-K$^b$ expression on Ad5- and Ad12-infected cells. B6 ConA blasts infected by Ad5 (closed diamonds) or Ad12 (open diamonds) for the indicated time (x-axis) were examined for changes in surface K$^b$, D$^b$, and PR-K$^b$ expression. Cells that received the same treatments as Ad5- or Ad12-infected cells, except for the addition of virus, were also examined (closed circles). Surface K$^b$ (A) and D$^b$ (B) expression was examined using PE-labeled AF6-88.5 mAb and FITC-labeled KH95 mAb respectively. PR-K$^b$ expression (C) was monitored using OVAp and FITC-labeled 25D1.16 mAb, which recognizes specifically the OVAp-K$^b$ complex, as described previously (313). Data shown are from one of two independent identical experiments. Three other experiments examined changes of surface MHC-I on Ad5- or Ad12-infected cells during the first 18 or 24h after virus infection and showed similar results.
5.4.2 Cytotoxicity and IFN-γ production by NK cells co-cultured with Ad5- and Ad12-infected splenocytes.

We next tested whether ConA blasts would become sensitive to NK lysis during the early phase of infection by Ad5 or Ad12. Con A blasts infected with Ad5 or Ad12 remained resistant to NK mediated lysis for the first 6h post-infection (Fig 5-3A). By 9h, Ad5-infected cells became highly sensitive to NK lysis and remained so throughout the rest of the time course. Ad12-infected cells remained resistant and only became sensitive to NK lysis 42h post-infection (Fig 5-3A).

IFN-γ is a major cytokine synthesized by NK cells during viral infection (260, 261, 275). We tested Ad5- and Ad12-infected cells to see whether they would activate NK cells to produce IFN-γ. Fig 5-3B shows the intracellular staining for IFN-γ production by NK cells at t=24h post virus-infection (Ad5, Ad12, or mock). Only Ad5-infected cells induced IFN-γ synthesis. Consistent with their sensitivity to NK lysis, Ad5-infected ConA blasts could induce significant IFN-γ synthesis from NK cells at as early as 9h post-infection (Fig 5-3C). The percentage of IFN-γ producing NK cells increased between 9h and 24h post-infection and reached a maximum value (of ~45% in this particular experiment), suggesting that only a subset of NK cells could be activated by Ad5-infected Con A blasts to produce IFN-γ. Ad12-infected ConA blasts could not stimulate NK cells to synthesize IFN-γ and even suppressed IFN-γ synthesis below that observed in cultures containing mock-infected cells, even though Ad12-infected cells became sensitive to NK lysis by 42h post-infection. These observations suggest that activation of NK cells to produce IFN-γ correlates with the disappearance of surface PR-Kb expression on the Ad5-infected cells. The time that the surface PR-Kb on Ad5-infected cells was down-regulated correlated well with the time the cells became sensitive to NK lysis.

To confirm that Ad12-infection occurred, Ad12-specific CTLs were generated in vitro and Ad12-infected cells were tested for sensitivity to CTL lysis in a 4h ⁵¹Cr-release assay. Lysis of Ad12-infected cells could be detected at 24h post-infection and was further increased by 42h post-infection (Fig 5-3D), suggesting Ad12 viral antigens were presented on target cells by 24h post-infection. Furthermore, some of the CTLs might be recognizing viral antigens that are expressed by both Ad5 and Ad12 because Ad5-infected cells also
Figure 5-3. Activation of NK- and CTL-mediated cytotoxicity and of IFN-γ synthesis.

B6 ConA blasts infected by Ad5 (solid diamonds), Ad12 (open diamonds), or mock (open triangles) for the indicated time (x-axis) were tested by (A) 51Cr release assays for sensitivity to IL-2 activated NK (day 4). Experiments were done with three E/T ratios; shown here is E/T=30. (B) Infected cells were tested to see whether they could activate NK cells to synthesize IFN-γ (see material and methods). BFA was added to the culture 2h after the addition of NK cells to block IFN-γ secretion. Cells were further cultured for 4h to allow the accumulation of IFN-γ in the cytosol of NK cells that were activated by virally infected cells. The time indicated on the x-axis is the time when BFA was added. At the end of 4h culture, NK1.1+ cells were examined for IFN-γ staining. Shown in (B) are dot-plots of IFN-γ staining of NK1.1+ cells co-cultured with ConA blasts infected by Ad5, Ad12, or mock at t=24h post-infection. (C) The results are presented as % NK1.1+ cells that are IFN-γ+ over time. (D) Ad5- and Ad12-infected cells were examined for their susceptibility to lysis mediated by Ad12-specific CTLs in a 4h 51Cr-release assay. Experiments were done with three E/T ratios; shown here is E/T=30. This figure (A-D) is representative of two independent experiments. Similar experiments examining NK sensitivity of Ad5- or Ad12-infected cells during the first 9h, 18h, 24h, or 36h after virus infection all showed similar results. Two other independent experiments examining IFN-γ production during the first 18h after Ad5- or Ad12-infection also showed similar results.
became slightly susceptible to CTL lysis at 24h post-infection (Fig 5-3D). Taken together, although Ad12-infected cells could not activate IFN-γ synthesis by NK cells, they could activate cytotoxicity mediated by both activated NK cells and CTLs.

5.4.3 The involvement of NK1.1 and 5E6 antigens (Ly49C & Ly49I) and PR-K^b in anti-viral cytotoxicity.

NK1.1 antigen has been shown to be an activating receptor in NK recognition (155, 357). The 5E6 antigen, Ly49C, has been shown to be an NK inhibitory receptor recognizing surface PR-K^b (338). To examine the importance of NK1.1 and 5E6 antigens in anti-viral cytotoxicity, soluble NK1.1 mAb and Dynabead bound 5E6 mAb were used to block activating receptor (NK1.1 antigen) and to cross-link the Ly49C inhibitory receptors respectively in ^51^Cr-release assays. In the presence of soluble NK1.1 mAb, lysis of Ad5-infected cells (24h post-infection) dropped from high to near background levels while the lysis of Ad12- or mock-infected cells was little affected (Table 5-1: #1,2), suggesting that the NK1.1 antigen plays an important role in activating NK cytotoxicity during anti-viral immunity. In this experiment, soluble Protein A and Protein G were used to coat the Fc portion of NK1.1 mAb to avoid undesirable ADCC. Dynabeads coated with 5E6 mAb were used to cross-link the 5E6 antigens (Ly49C^B6 and Ly49I^B6) on NK cells. This resulted in a moderate inhibition of lysis of Ad5-infected cells (Table 5-1: #1,3).

As a further control, we examined whether binding of a K^b-specific peptide (OVAp) to surface PR-K^b would result in lysis of Ad12-infected cells and whether the blockage of PR-K^b and Ad5-infected (which resulted in the loss of surface PR-K^b) would have comparable effects on activating NK cytotoxicity. Note that binding of OVAp converts most if not all surface PR-K^b to peptide-bound K^b, which can no longer be recognized by the NK inhibitory receptor, Ly49C^B6 (338). Ad12- or mock-infected cells, when pre-pulsed with OVAp, became sensitive to NK lysis (Table 5-1: #1,4). Ad5-infected cells, already sensitive to NK lysis, showed little change in sensitivity on being pre-pulsed with OVAp. These observations suggest that the loss of PR-K^b on Ad5-infected cells can serve as a trigger of NK cytotoxicity and further suggest that the presence of PR-K^b on Ad12- and mock-infected cells protects them from lysis.
Table 5-1. The involvement of NK receptor in the lysis of virus-infected cells

<table>
<thead>
<tr>
<th>T=24h</th>
<th>% specific lysis (± s.e.m.)†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ad5</td>
</tr>
<tr>
<td>1. -</td>
<td>54.9 (±2.9)</td>
</tr>
<tr>
<td>2. + NK1.1 mAb</td>
<td>7.7 (±0.8)</td>
</tr>
<tr>
<td>3. +5E6 mAb on dynabeads</td>
<td>19.4 (±0.9)</td>
</tr>
<tr>
<td>4. + OVAp</td>
<td>52.6 (±2.5)</td>
</tr>
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Note: † experiments were done with three E/T ratio (3, 10, 30); shown here is E/T=30. This is a representative of four independent experiments.
5.4.4 PR-K\textsuperscript{b} can partly but not completely protect RMA-S cells from NK lysis.

The TAP-deficient cell line, RMA-S, has served as a prototype for defining and studying PR-MHC-I molecules and should express PR-K\textsuperscript{b} molecules. We therefore examined what role these PR-K\textsuperscript{b} molecules might play in protecting RMA-S cells from NK cytotoxicity. RMA-S cells have been shown to express low levels of PR-K\textsuperscript{b} molecules, whose surface expression can be increased by culturing the cells at room temperature (32, 74). Here, the susceptibility of RMA-S cells to NK lysis was examined in a \textsuperscript{51}Cr release assay performed at 26\textdegree C. Figure 5-4 shows that RMA-S cells cultured overnight at 26\textdegree C (hence, RMA-S\textsubscript{26}) were less sensitive to NK lysis (Fig 5-4A, dashed line) than RMA-S cells cultured overnight at 37\textdegree C (Fig 5-4B, dashed line), possibly because the increased surface expression of PR-K\textsuperscript{b} conferred partial NK resistance. On pre-pulsing with OVAp (solid lines), both RMA-S\textsubscript{26} and RMA-S\textsubscript{37} cells became more sensitive to NK lysis, with the increase being much greater for RMA-S\textsubscript{26} cells.
Figure 5-4  PR-K<sup>b</sup> molecules in protecting RMA-S from NK cytotoxicity. RMA-S cells were cultured at (A) 26°C or (B) 37°C overnight, pre-pulsed with either OVA<sub>p</sub> (solid lines) or nothing (dashed lines), and then tested for lysis by B6 NK cells (H-2<sup>b</sup>) in a 5h <sup>51</sup>Cr-release assay at 26°C. Data are representative of 3 independent experiments.
5.5 DISCUSSION:

NK cells can contribute to the first line of defence during a viral infection (261, 270, 271). According to the "missing-self" hypothesis, NK cytotoxicity is inhibited by the recognition of self-MHC-I on normal autologous cells (11). Therefore, viruses that down-regulate surface MHC-I expression during the early phase of infection will activate NK cytotoxicity and cytokine synthesis and thus, be eliminated by NK cells. In this study, Ad5-infected cells that lost surface PR-K$^b$ expression by 9h post-infection (but with no detectable decrease in total surface K$^b$ or D$^b$ expression) became sensitive to NK lysis and could trigger NK IFN-γ synthesis during the first 24h of infection. In contrast, Ad12-infected cells had increased surface K$^b$, D$^b$ and PR-K$^b$ expression, were protected from NK lysis, and could not trigger NK IFN-γ synthesis. NK-mediated immune responses involve both activating and inhibitory receptors (24, 126, 168). Both blocking an NK activating receptor (NK1.1), and cross-linking NK inhibitory receptors (Ly49C and Ly49I) to induce an inhibitory signal resulted in reduced NK cytotoxicity against Ad5-infected cells (Table 5-1). In particular, Ly49C$^{B6}$ has been implicated in recognizing PR-K$^b$ as an inhibitory ligand (338). Blocking the recognition of PR-K$^b$ by adding OVAp, which binds to PR-K$^b$ with high affinity, did not further increase the lysis of Ad5-infected cells but rendered previously resistant mock- and Ad12-infected cells highly sensitive to NK-lysis (Table 5-1). These observations provide strong evidence that PR-K$^b$ is a "self-marker" for NK recognition whose absence is sufficient to trigger lysis of Ad5-infected cells. One cannot rule out the possibility that there are other changes taking place with the same kinetics in Ad5-infected cells which increase their susceptibility to NK lysis and/or their ability to induce IFN-γ synthesis.

This is the first study examining the relationship between MHC-I expression on virally infected primary cells and activation of autologous NK cells. Several viruses have evolved to develop a variety of strategies in global or selective down-regulation of surface MHC-I to prevent their antigens from being recognized by CTLs (48, 49, 66, 67, 352) but cells infected by these viruses may still be detected by NK cells. Surface PR-K$^b$ molecules, which constitute only ~10% of total surface K$^b$, are very unstable with a $t_{1/2}$ of less than 1h (74) (Su & Miller, in preparation). Consequently, changes that affect the continuous supply of PR-K$^b$ from the ER may be detected within 1h by NK cells expressing inhibitory receptors for PR-K$^b$. In contrast, the loss of pM-K$^b$ or pM-D$^b$ ($t_{1/2}$ >20h) induced by viral infection may
not be detected until much later than 20h. In this study, the loss of PR-K^b was detected within 9h post Ad5-infection (Fig 5-2C) and correlated with sensitivity to NK lysis (Fig 5-3A) and IFN-γ production (Fig 5-3C). This time is less than the completion time of one viral replication cycle (352, 358). Loss of PR-K^b on Ad5-infected cells has not been reported previously; mechanisms involved in the down-regulation of host PR-K^b expression will require further investigation. It may be a direct effect of viral infection on MHC-I biogenesis or an indirect effect of early viral gene products on other cellular processes. The E1A region (an early transcriptional unit) of Ad5 (267, 359) has been implicated in increasing surface MHC-I expression (264, 348, 360) and confers NK susceptibility to Ad5-transformed cells (350). It is possible that viral products encoded by the Ad5 E1A region may be responsible for the decreased surface PR-K^b. Note that BFA, which blocks export of newly synthesized protein from the ER to the cell surface, leads to loss of PR-K^b within 1h of addition (298, 309).

Unlike Ad5, Ad12 has evolved to circumvent NK detection. Ad12 increased surface K^b, D^b and PR-K^b expression on the host cells (Fig 5-2C); Ad12-infected cells did not activate NK cytotoxicity until 42h post-infection. Mechanisms involved in how Ad12 up-regulate MHC-I are still not clearly defined. The E1A region of Ad12, which was shown to down-regulate the transcription of peptide transporter genes, TAP1 and TAP2 (361), may be responsible for the increased output of PR-K^b. The TAP heterodimeric complex has been shown to facilitate the loading of peptide to newly synthesized MHC-I (74, 324); reduced expression of the TAP complex may result in increased export of PR-MHC-I and decreased export of stable MHC-I (74). The Ad12 E1A region has also been shown to be responsible for increased K^b mRNA expression in the cytosol of Ad12-infected mouse embryonic cells (362), consistent with the up-regulated surface K^b and D^b observed in this study, and the resistance of Ad12-transformed cells to NK cytotoxicity (269, 350). However, the Ad12 gene products responsible for regulating host MHC-I remain to be identified.

It is generally agreed that whether NK recognition leads to lysis results from the balance of activating and inhibitory signals (24, 25, 309). Mouse activating receptors, Ly49D has been shown to recognize surface MHC-I (42, 130, 363) and hence, increased surface MHC-I expression may trigger NK activating receptors. On pre-pulsing with OVA_p, Ad12-infected cells were slightly more sensitive to NK lysis than mock- or Ad5-infected
cells (Table 5-1: #4). It is possible that an up-regulated MHC-I could trigger an activating signal, but that the increased activating signal was not strong enough to overcome the inhibitory signal and therefore, Ad12-infected cells remained resistant to NK lysis during the first 24h of infection (Table 5-1). When the inhibitory signal contributed by PR-K<sup>b</sup> recognition was removed by the OVAp pre-pulse, the activating signal overrode any remaining inhibitory signals and the Ad12-infected cells became sensitive to NK lysis (Table 5-1: #4). Why Ad12-infected cells became susceptible to NK lysis at 42h post-infection cannot be explained with changes in surface MHC-I expression and requires further study. Physiological ligands for most NK activating receptors remain largely unknown and have been speculated to be adhesion molecules, carbohydrate, lipids and/or heat-shock proteins (144). Identification and characterization of NK activating ligands will be extremely valuable in understanding NK recognition.

The presence of PR-K<sup>b</sup> molecules on RMA-S cells was not sufficient to confer resistance to NK lysis (Fig 5-4). We estimate that an RMA-S cell expresses ~2 x 10<sup>4</sup> PR-K<sup>b</sup> when cultured at 37°C and that this increases about 5-fold when cultured at 26°C, whereas only ~4000 PR-K<sup>b</sup> on a B6 ConA blast are sufficient to protect from NK lysis (data not shown). However, removing the PR-K<sup>b</sup> on RMA-S cells by pulsing with OVAp did increase the observed level of lysis, particularly for cells cultured at 26°C (Fig 5-4), consistent with PR-K<sup>b</sup> being the ligand for an inhibitory receptor. We hypothesize that RMA-S, a transformed cell line, carries high levels of ligand(s) for NK activating receptors and that the presence of the inhibitory signal provided by the PR-K<sup>b</sup> alone is not sufficient to counteract these activating signals. Data of Correa and Raulet (1995) can be interpreted to support this hypothesis. They transfected RMA-S cells with D<sup>e</sup> and stabilized D<sup>e</sup> expression by incubating the cells with a high affinity D<sup>e</sup>-binding peptide. They then tested these cells for sensitivity to lysis by Ly49A<sup>e</sup> B6 NK cells. Ly49A is an inhibitory receptor for which D<sup>e</sup> associated with high affinity peptide is a ligand (41, 282). The cells were only partially protected.

Recently, Franksson et al. (1999) and Michaëlsson et al. (2000) have addressed the peptide-dependency of K<sup>b</sup> to Ly49C. In one study, RMA-S cells were pulsed with OVAp or other K<sup>b</sup>-binding peptides overnight, washed to remove excess peptide before analyzing their susceptibility to 5E6<sup>+</sup> LAK cells in a cytotoxicity assay. In contrast to our study, it is found
that RMA-S cells pre-pulsed with OVAp were protected from lysis by 5E6+ LAK cells (364). It remained unclear why other K\textsuperscript{b}-binding peptides, which bind and stabilize surface K\textsuperscript{b} molecules equally well, show different protective capacity to 5E6+ LAK cells in their study. Furthermore, since OVAp (but not other peptide tested) has been shown to stabilize both K\textsuperscript{b} and D\textsuperscript{b} on the RMA-S cell surface (74), it is possible that PR-K\textsuperscript{b} and PR-D\textsuperscript{b} molecules were regenerated before the assay. In our previous study, we have shown that PR-K\textsuperscript{b} molecules could be regenerated within 1½ hr and render the cells resistant to NK lysis (309, 338). Michaëlsson et al. (2000) demonstrated specific interaction between Ly49C and tetramers of H-2K\textsuperscript{b} in flow cytometry. This finding cannot be easily reconciled with our finding that in the presence of exogenous OVAp, OVAp-pre-pulsed B6 ConA blasts of D\textsuperscript{b}-deficient mice (which express K\textsuperscript{b} only) did not bind to the COS-7 cells that were transfected with Ly49C cDNA in cell-adhesion assay (338). It is possible that Ly49C-COS-7 transfectants are different from other transfectants or naïve NK cells used in Michaëlsson’s study (365). Alternatively, tetramers of bacterially expressed, non-glycosylated MHC class I molecules refolded with different peptides might be different from the native surface K\textsuperscript{b} molecules on ConA blasts for NK recognition.

In conclusion, this study provides evidence for the physiological significance of surface PR-K\textsuperscript{b} and its receptor, Ly49C\textsuperscript{B6}, in NK mediated anti-viral responses. This study also provides evidence for an extension of the “missing-self” hypothesis: PR-MHC-I should be included in the hypothesis as one of the “self-markers”. That is, NK cells survey tissues for normal expression of both pH-associated MHC-I (as suggested by study of Ly49A, (41, 282, 309)) and PR-MHC-I (as suggested by the study of Ly49C\textsuperscript{B6}, (338)) and lyse targets when either of them is absent. Since NK cells play a critical role in the early anti-viral immune response, it will be advantageous for the host immune system if NK cells can recognize virally infected cells during the early stage of infection before the completion of viral replication. Hence, NK recognition of PR-MHC-I (because of its short t½ and low surface expression) might be of significant importance in controlling virus load before the acquired immune responses mediated by CTLs and/or antibodies are in action.
CHAPTER 6

DISCUSSION
6.1 Significance of Findings:

The current model of NK recognition suggests that there are multiple receptors on a single NK cell surface. The outcome and specificity of a NK-target cell interaction (also specificity) depend on the interplay between signals derived from activating and inhibitory receptors (Fig 6-1) (25, 168). In 1992, Karlhofer and Yokoyama identified Ly49A molecule as the NK inhibitory receptor for mouse H-2D$^d$ molecule (20). This seminal finding provided the first direct evidence to support the molecular mechanism of NK recognition, the "Missing-self" hypothesis, as first proposed by Kärre et al. in 1985 (47). Specifically, it explains in part how quantitative changes in MHC-I level can trigger NK recognition. For example, when dysreglated cells lose all or most of the MHC-I expression during tumorigenesis or infections (47), the inhibitory effect on NK cells, exerted through the inhibitory receptors, is lost, conferring susceptibility to NK lysis. However, there are exceptions to the "missing-self" hypothesis, suggesting its incompleteness in depicting the mechanisms of NK recognition (29-31). For example, cells infected by HSV or transformed by adenovirus type V (Ad5) became susceptible to NK lysis but had no reduced total surface MHC-I expression (262, 264). On the contrary, cells transformed by adenovirus type XII (Ad12) had reduced expression of total surface MHC-I but remained resistant to NK lysis (263). It is conceivable that the original "missing-self" hypothesis examined only changes of total stable surface MHC-I on the target cells, and might have overlooked changes of sub-populations of surface MHC-I.

We have addressed the issue of whether the NK receptors have the ability to recognize different forms of MHC-I molecules. There are two major sub-populations of surface MHC-I: peptide-receptive MHC-I or "PR-MHC-I", and MHC-I associated with high affinity peptides (pH) (74, 328). The latter constitutes the majority of cell surface MHC-I molecules and has a long half-life (~20h). PR-MHC-I is unstable and was shown for K$^b$ and D$^b$ to constitute ~10% of total surface MHC-I. Loss of PR-MHC-I cannot be easily detected by mAb against total surface MHC-I, and may have been overlooked while examining the relation between loss of MHC-I and susceptibility of the target cells to NK lysis. In this study, we have shown that NK cells are inhibited by the presence of PR-MHC-I. Blocking PR-MHC-I with pH or with MHC-I specific mAb rendered the cells susceptible to lysis by
Figure 6-1  A two-receptor model for NK recognition. This model predicts that NK cells express two types of receptors, one for activation, presumably upon interaction with a target cell ligand. Another receptor interacts with target cell MHC-I (pH-MHC-I and/or PR-MHC-I) then delivers an inhibitory signal that disrupts signalling through the activation receptor.
autologous NK cells. Such effect is reversible, because MHC-I are continuously synthesized and exported to the cell surface from the ER. When cells pre-pulsed with pH were re-cultured in the absence of exogenous pH, PR-MHC-I accumulated on the cell surface and again restored the resistance to NK lysis. Our results suggest that there are NK inhibitory receptors recognizing PR-D^b, PR-K^b, PR-K^d, and/or PR-D^d. In searching for NK inhibitory receptors recognizing PR-MHC-I, we found that an NK inhibitory receptor, Ly49C^b6 recognized PR-K^b. There may be many more NK inhibitory receptors recognizing PR-MHC-I that have yet to be identified.

Since both pH-MHC-I and PR-MHC-I are implicated in NK recognition (41, 282, 338), we examined the generation, stability, and peptide binding capacity of MHC-I on the surface of normal spleen cells. It was found that there are two sub-populations of MHC-I with very different half-lives. Approximate 50% of surface K^b are associated with relatively high affinity peptide and are quasi-stable with a t^(1/2) of ~20h; the remaining 50% of surface K^b are unstable with a t^(1/2) of ~0.5-0.6h. The NK inhibitory ligand, PR-MHC-I falls within the unstable MHC-I sub-populations and the half-life of PR-K^b was measured to be 21-48min. We argue that surveillance of different forms of MHC-I molecules of different half-lives is well suited for the innate defense against some and perhaps all virus infections where NK-response is involved. When the export of MHC-I to the cell surface of the infected cell is disrupted during a viral infection, the level of PR-MHC-I with a shorter half-life, surface expression will be changed within 1-2 hr and be recognized by NK cells that express the inhibitory receptors for PR-MHC-I. Further changes in the level of pH-MHC-I (t^(1/2) of ~20 hr) will then be detected by the NK cells bearing their cognate inhibitory receptors (eg. Ly49A).

We therefore tested whether there is qualitative changes in MHC-I level during viral infections that may account for the NK susceptibility. In the study of virus infection, we showed that Ad5-infected cells had no reduction in the expression of total surface K^b or D^b but yet became susceptible to lysis by autologous NK cells 9h after the infection. This observation is consistent with the reported studies of Ad5-transformed cells (264, 269, 270), but seems to contradict with the proposed "missing-self" hypothesis. We then examined PR-K^b expression on Ad5-infected cells and found that surface PR-K^b expression was greatly reduced suggesting that PR-K^b may be one of the self-markers described in the "missing-self" hypothesis. Indeed, when NK inhibitory receptors, Ly49C, which recognize PR-K^b
were cross-linked with mAb to induce a negative signal, lysis of Ad5-infected cells was blocked. This study showed that PR-K\(^b\) played a role in NK-mediated response to Ad5-infection and suggests that NK cells survey tissues for normal expression of both p\(_H\)-MHC-I and PR-MHC-I and lyse targets when either of them is aberrant or absent. NK cells have been shown to be crucial in the control of MCMV infection in mice (259, 366). Interestingly, there is also evidence to suggest mechanisms of viral evasion of NK cell recognition. Murine cytomegalovirus (MCMV) encodes a MHC-I homologue (m144), which has been shown to associate with \(\beta_2\)m but not peptide, resembling an empty murine MHC-I (221, 223, 226, 227). Functional studies suggest that the MCMV encoded MHC-I homologue contributes to immune evasion through interference with NK-mediated clearance (229). Human cytomegalovirus (HCMV) also encodes a MHC-I homologue UL18, which unlike m144, associates with both \(\beta_2\)m and endogenous peptide and acts as ligand for an NK inhibitory receptor, LIR-1 (52, 367). NK receptor(s) recognizing m144 have not yet been identified and may be the same NK inhibitory receptor(s) that recognize PR-MHC-I (52, 224, 225). Taken together, this study supports a role of NK cells in the control of viral infections, and reveals a novel mechanism of NK surveillance of viral infected cells.

6.2 Concluding Remark- more questions than answer

This study has identified a new mechanism in NK recognition but at the same time, raised many questions. Every Ly49 receptor seems to have more than one ligand. Ly49C\(^{B6}\) binds to PR-K\(^b\), H-2\(^d\), and H-2\(^s\), whereas Ly49A binds to D\(^d\), D\(^k\) and K\(^b\). It is not known whether Ly49C\(^{B6}\) also binds to the PR form of H-2\(^d\) and H-2\(^s\). If yes, does the conformation of PR-K\(^b\), required for Ly49C\(^{B6}\) recognition, resemble the conformation of PR-H-2\(^s\) or PR-H-2\(^d\)? Although, it is interesting, it is technically difficult to address this question. The major obstacle is that PR-MHC-I are not stable and therefore, their conformations could not be accurately examined using crystallography and compared. In contrast, we could easily examine whether Ly49A recognizing p\(_H\)-D\(^k\). If yes, the Ly49A recognition sites on p\(_H\)-D\(^d\) and p\(_H\)-D\(^k\) may be identified by comparing the crystal structures of p\(_H\)-D\(^d\) and p\(_H\)-D\(^k\).

Furthermore, m144 is an MHC-I homolog encoded by MCMV and has been found to
be expressed as an empty MHC-I on the host cell surface (223, 226). Functional study of m144 suggests that it functions as a decoy to inhibit NK cytotoxicity, and thus further supports the physiological relevance of PR-MHC-I in anti-viral responses (227, 229). NK receptor for m144 has not yet been identified. Soluble m144 (if retain the correct conformation) will be a valuable reagent to be used in searching for receptors recognizing PR-MHC-I. Identification of receptors specific for PR-MHC-I (other than H-2b) will facilitate the evaluation of this newly identified NK recognition mechanism in other strains of mice.

With the inclusion of PR-MHC-I as a self-marker, the “missing-self” hypothesis still does not seem to be complete. In this study, Ad12-infected cells remained resistant to NK lysis up to 42h after the infection. After 42 hr, these cells became susceptible to NK lysis, despite no changes in the surface Kb, Db or PR-Kb expression. It is possible that Ad12-infection has up-regulated a triggering molecule, or down-regulated some yet-to-be identified “self-marker” which may or may not be MHC-I. PR-Db may be a “self-marker” and its expression may be selectively down-regulated. To test this hypothesis, PR-Db expression can be examined using a radiolabelled peptide, specific for Db. Alternatively, Ad12 infection could be studied using splenocytes and autologous NK cells from a Db-deficient B6 mouse which expresses only Kb. Furthermore, PR-Kb constitutes only ~10% of total surface Kb and we have shown that ~50% surface Kb are unstable; perhaps, the other 40% of unstable surface Kb is also a ligand for NK inhibitory receptors and therefore, serves as a “self-marker”. Identification and characterization of this 40% of unstable surface Kb might yield some insight into NK recognition and/or the stability of surface MHC-I. It should be noted that the putative “self-marker” does not have to be MHC-I although most NK inhibitory receptors seem to recognize MHC-I. The newly identified inhibitory receptor, NKR-P1B does not seem to recognize MHC-I and may be recognizing some carbohydrate moieties. It is equally possible that ligands for NK activating receptor(s) were up regulated around 42h after the Ad12-infection.

The large and ever increasing number of putative NK cell receptors indicates that the entire field of NK cell receptors is advancing rapidly. Many receptors have been described and several are well characterized with respect to their ligand specificity and functional activities. These recent advances in our understanding of NK receptors, as well as
availability of specific reagents, have presented a testable framework for further elucidation of the role of NK cells in immune surveillance and the molecular mechanism of NK recognition. The real challenge now is to understand the interplay of these multiple receptors in various physiological settings such as viral infections (272, 368, 369), pregnancy (370-372) or tumor surveillance (373, 374). What is the signaling pathway involved in NK activation and/or inhibition? Does the affinity/avidity of individual receptor-ligand interaction influence the intensity of signaling relative to the inter-dependent critical thresholds—ultimately determining either the repression or activation of NK functions? Many in vitro findings about NK recognition should be validated in vivo. The implementation of cellular experimental systems to reliably identify and characterize NK inhibitory and activating ligands involved in NK recognition in vivo might provide some answers to these questions and help to explain some differences in observations made in different in vitro systems.
### APPENDIX A. SUMMARY OF NK RECEPTORS

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Activating/Inhibitory</th>
<th>Functional structure / motif</th>
<th>Mab</th>
<th>Ligand (Binding assays)</th>
</tr>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKR-P1A</td>
<td>Mouse</td>
<td>A</td>
<td>CxCP</td>
<td>1C10</td>
<td>?</td>
</tr>
<tr>
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<td>I</td>
<td>ITIM</td>
<td>1C10</td>
<td>?</td>
</tr>
<tr>
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<td>CxCP, Associates with Fcγ</td>
<td>PK136, 1C10, 1F10</td>
<td>?</td>
</tr>
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<td>A</td>
<td>CxCP</td>
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<td>?</td>
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<td>D^4, D^6, K^b</td>
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<td>ITIM</td>
<td>14B11</td>
<td>?</td>
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<td>ITIM</td>
<td>4D11</td>
<td>D^4, L^d</td>
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<td>ITIM</td>
<td>5E6, 14B11</td>
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<td>Receptor</td>
<td>Species</td>
<td>Activating/Inhibitory</td>
<td>Functional structure / motif</td>
<td>Mab</td>
<td>Ligand (Binding assays)</td>
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<td>-------------------------</td>
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<td>CD94</td>
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<td>Co-receptor</td>
<td>Associates with NKG2s or form homodimer</td>
<td>HP3B1</td>
<td>?</td>
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<td>Qa-1</td>
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<td>I</td>
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<td>MICA</td>
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<td>Human</td>
<td>?</td>
<td>Associates with CD94</td>
<td>?</td>
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</tbody>
</table>

**Members of Immunoglobulin (Ig) Superfamily**

<p>| 2B4      | Mouse, human     | A                     | (TxYxxI/V), resembling ITIM                    | 2B4 (mouse), 158 (human), c1.7 (human) | CD48                    |
| LAG3     | Mouse            | A                     |                                               | Anti-serum            |                         |
| Gp49B1   | Mouse            | I                     | ITIM                                           | B23.1                | ?                       |
| CD16     | Human, Mouse     | A                     | Associates with CD3ζ                          | 3G8 (human)          | IgG                     |
| CD2      | Human            | A(?)                  |                                               | RM2-1                | CD58                    |</p>
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Activating/Inhibitory</th>
<th>Functional structure / motif</th>
<th>Mo6</th>
<th>Ligand (Binding assays)</th>
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<td>ITIM</td>
<td>XA141, EB6, HP-3E4 y249, GL183</td>
<td>HLA-Cw2, Cw4, Cw5, Cw6</td>
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<tr>
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<td>I</td>
<td>ITIM</td>
<td></td>
<td>HLA-Cw1, Cw3, Cw7, Cw8</td>
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<tr>
<td>KAR (KIR2DS1, p50.1)</td>
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<td>A</td>
<td>Associates with DAP12, CxCP</td>
<td>EB6</td>
<td>HLA-Cw2, Cw4, Cw5, Cw6</td>
</tr>
<tr>
<td>KAR (KIR2DS2, p50.2)</td>
<td>Human</td>
<td>A</td>
<td>Associates with DAP12, CxCP</td>
<td>GL183</td>
<td>HLA-Cw1, Cw3, Cw7, Cw8</td>
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<tr>
<td>KIR3DL1 (p70, NKB1)</td>
<td>Human</td>
<td>I</td>
<td>ITIM</td>
<td>Z27, DX9</td>
<td>HLA-Bw4, B27</td>
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<tr>
<td>KIR3DL2 (p140)</td>
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<td>I (?)</td>
<td>ITIM</td>
<td>Q66, 5,133</td>
<td>HLA-A3, A11</td>
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<td>Associates with FcRγ, CD3ζ</td>
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<td>NKp44</td>
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<td>Associates with DAP12</td>
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<tr>
<td>LIR-1 (ILT2, MIR-7)</td>
<td>Human</td>
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<td>M401</td>
<td>U1,18 (HCMV) B7, -G1, -E</td>
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<tr>
<td>AIRM-1</td>
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<td>I</td>
<td></td>
<td></td>
<td>Sialic acid</td>
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<tr>
<td>LIAR (p40)</td>
<td>Mouse</td>
<td></td>
<td></td>
<td>DX26</td>
<td>?</td>
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<td><strong>Members of other family</strong></td>
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<tr>
<td>CD28</td>
<td>Mouse</td>
<td></td>
<td>disulfide-linked homodimer</td>
<td></td>
<td>B7-1, B7-2</td>
</tr>
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APPENDIX B.  REFERENCES


224. Vitale, M., R. Castriconi, S. Parolini, D. Pende, M. L. Hsu, L. Moretta, D. Cosman, and A. Moretta. 1999. The leukocyte Ig-like receptor (LIR)-1 for the cytomegalovirus UL18 protein displays a broad specificity for different HLA class I alleles: analysis of LIR-1 + NK cell clones. *International Immunology* 11:29.


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