Modulation of Ly49A+ Natural Killer Cell Licensing by Major Histocompatibility Complex Class I Alleles

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Modulation of Ly49A\(^+\) Natural Killer Cell Licensing by Major Histocompatibility Complex Class I Alleles

by

Anna Helena Jonsson

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2012

St. Louis, Missouri
ABSTRACT OF THE DISSERTATION

Modulation of Ly49A\(^+\) Natural Killer Cell Licensing by
Major Histocompatibility Complex Class I Alleles

by

Anna Helena Jonsson

Doctor of Philosophy in Biology and Biomedical Sciences
(Immunology)

Washington University in St. Louis, 2012

Professor Wayne M. Yokoyama, Chairperson

Natural killer (NK) cells are innate immune lymphocytes that react to cells lacking self-MHC (major histocompatibility complex) class I molecules, such as transformed or virally infected host cells and allogeneic bone marrow. This reactivity is mediated by inhibitory receptors for MHC class I that block the ability of activation receptors to stimulate NK cells. Since many NK cells lack receptors that recognize self-MHC, the inhibitory receptors also mediate a second function, termed NK cell licensing, to protect against autoreactivity. To become licensed, i.e. functionally competent to be triggered through its activation receptors, an NK cell must engage host MHC class I via at least one of its MHC class I-specific inhibitory receptors, which in mice belong to the Ly49 family of receptors. However, many properties of this process remain unclear.

To explore potential determinants of NK cell licensing on a single Ly49 receptor, we have investigated the relative licensing impacts of the b, d, k, q, r, and s H2 haplotypes on Ly49A\(^+\) NK cells. In ex \textit{vivo} stimulation assays, some Ly49A-MHC class I haplotype combinations produced an intermediate licensing phenotype, indicating that
licensing is not a binary phenomenon. Comparisons of these data with soluble Ly49A
tetramer binding assays indicate that licensing is essentially analog but is saturated by
moderate-binding MHC class I ligands. Interestingly, licensing exhibited a strong inverse
correlation with Ly49A surface accessibility, a measure of cis engagement of Ly49A
with MHC class I expressed on the same cell. Finally, Ly49A-mediated effector
inhibition was found to be more sensitive to MHC class I engagement than licensing of
Ly49A⁺ NK cells, suggesting that licensing establishes a margin of safety against NK cell
autoreactivity.

We have also developed a transgenic mouse model with inducible MHC class I
expression for use in future studies of NK cell licensing. Upon administration of
doxycycline, these mice express a H2Kᵇ-β₂m-ova peptide single-chain MHC class I
trimer, which will license Ly49C⁺ NK cells. Preliminary data suggest that this model of
inducible NK cell licensing will be useful for studies of the kinetics of NK cell licensing
both in vivo and in vitro.
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What follows is an abbreviated statement of acknowledgments for some of the many people who have supported me in so many ways during my PhD research years. A complete description of everyone who has helped and how would be longer than the rest of the dissertation! This in itself is something for which I am very thankful.

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Last but not least, I want to thank my parents, siblings, and grandparents. They may not always understand the science and experiments that make up the roller coaster of graduate school, but their steady love has been a constant source of comfort and support without which my graduate studies would not have been possible.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>B10</td>
<td>C57BL/10</td>
</tr>
<tr>
<td>β2m</td>
<td>β-2-microglobulin</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>cM</td>
<td>centimorgan</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>K^b-ova</td>
<td>H2K^b molecule presenting SIINFEKL peptide from ovalbumin protein</td>
</tr>
<tr>
<td>KIR</td>
<td>killer immunoglobulin-like receptor</td>
</tr>
<tr>
<td>LAK</td>
<td>lymphokine-activated killer</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
</tr>
<tr>
<td>LRC</td>
<td>leukocyte receptor complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MCMV</td>
<td>murine cytomegalovirus</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NKC</td>
<td>natural killer gene complex</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI-3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>Poly-I:C</td>
<td>polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>SCT</td>
<td>single chain trimer</td>
</tr>
<tr>
<td>SCT I, III</td>
<td>first-generation, third-generation single chain trimer</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain-containing inositol 5-phosphatase</td>
</tr>
<tr>
<td>SHP</td>
<td>src homology region 2 domain-containing phosphatase</td>
</tr>
<tr>
<td>SNP</td>
<td>single-nucleotide polymorphism</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>tg</td>
<td>transgene</td>
</tr>
<tr>
<td>TRE</td>
<td>tetracycline response element</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
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CHAPTER 1

INTRODUCTION
Natural killer (NK) cells are innate immune lymphocytes that produce pro-inflammatory cytokines and kill transformed and virally infected cells. Unlike B and T lymphocytes, which use unique somatically rearranged antigen-specific receptors, e.g., the B or T cell receptor, NK cells rely on germline-encoded receptors belonging to several families that often include both activating and inhibitory members. During effector responses, NK cells detect alterations in endogenous protein expression on target cells by integrating signals from a number of these germ line-encoded activation and inhibitory receptors (1, 2). The balance of signals mediated by these receptors determines the NK cell response to the target. In certain disease states, such as cancers and viral infections, the inhibitory signal is decreased or absent, allowing the activation signal to dominate and trigger cytokine production and/or cytotoxic effector mechanisms. Pathological conditions can also induce expression of activating ligands on a target cell that lead to NK cell activation by overpowering inhibitory signals.

Activating and inhibitory NK cell receptors

Activating and inhibitory NK cell receptors belong to the same family of proteins. In humans, the predominant NK cell receptors are the killer-cell immunoglobulin (Ig)-like receptors (KIRs), type I integral membrane proteins that form a polymorphic family within the immunoglobulin superfamily (3, 4). In mice, the major NK cell receptors are type II integral membrane, C-type lectin-like molecules belonging to the Ly49 family (5, 6). Both human and mouse NK cells also express a conserved lectin-like heterodimeric receptor, CD94 coupled with members of the NKG2 family. The lectin-like homodimer
NKG2D is an activation receptor expressed by both human and murine NK cells that binds stress-induced ligands with MHC class I-like structures (7, 8).

Most MHC-specific inhibitory NK cell receptors recognize MHC class Ia molecules or, in the case of NKG2/CD94, the MHC class Ib molecule Qa-1 (in humans, HLA-E), which presents signal peptides from MHC class Ia molecules and thereby provides an indirect measure of MHC class Ia expression (5, 9). Ligands of MHC-specific inhibitory receptors tend to be constitutively expressed on healthy cells but are often downregulated in instances of viral infection or cellular transformation (10-12). Notably, several viruses have developed decoy MHC class I-like receptors in an attempt to avoid NK cell activation, an evolutionary indication of the importance of the antiviral activities of NK cells (13). The inhibitory receptors of the KIR and Ly49 families have an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tails that predominantly associates with the cytoplasmic tyrosine phosphatase, SHP-1, though the ITIM may potentially recruit other signaling molecules (5, 14, 15).

The activating KIRs and Ly49 receptors are structurally related to their inhibitory counterparts but lack cytoplasmic ITIMs. Instead, they have short cytoplasmic tails and associate via charged transmembrane residues with DAP12 or other immunoreceptor tyrosine-based activation motif (ITAM)-containing signaling chains for normal expression and signal transduction (5, 16). Activating receptors outside the KIR and Ly49 families also use separate signaling chains. Ligands of activation receptors include both MHC class I-like molecules and unrelated proteins, though the ligands of many activation receptors remain undetermined. Some activating receptors, like Ly49H, bind to virally encoded ligands (17, 18). Others, like NKG2D, bind host ligands that are constitutively
expressed at low levels on healthy cells and are upregulated in response to cellular stress and other stimuli (8, 19). The upregulation of such ligands is termed “induced-self” and enhances activation of NK cells by diseased cells.

*The “missing self” theory of NK cell function*

NK cells were first identified by their ability to kill tumor cells “naturally,” i.e., without prior exposure (20). This ability distinguished them from T cells, which require previous exposure to the tumor cells in order to efficiently kill them. Since NK cells do not have unique antigen-specific receptors like T cells, it was long a mystery how they identified the tumor as an appropriate target cell. The answer was provided by Klas Kärre and colleagues, who discovered that NK cells preferentially kill target cells that lack MHC class I expression (21). This led to the proposal of the “missing self” theory of NK cell function, which states that NK cells scan host cells for proper expression of MHC class I. Host cells that have downregulated MHC class I, for example as a result of viral infection are thus identified and eliminated by NK cells. In this way, NK cells have a role that is complementary to CD8 T cells, which respond to MHC class I bound to foreign peptides. As virally infected or transformed cells try to evade CD8 T cell attack by down-regulating MHC class I, they become susceptible to NK cell attack instead. The subsequent identification of MHC class I-specific inhibitory Ly49 receptors and KIRs provided a molecular basis for missing-self reactivity and tolerance to normal MHC-expressing cells (22-24).

However, there were two problems with the missing-self theory of NK cell function. The first problem was the lack of NK cell auto-aggression in MHC class I-
deficient mice. According to the missing-self hypothesis, NK cells in MHC class I-deficient hosts should attack self cells because they are unable to engage MHC class I via any inhibitory receptor. However, data from both $\beta_2m^{-}$ mice and TAP1$^{-}$ mice demonstrate that NK cells in these mice are actually hyporesponsive (25, 26). In these mice, there is little or no engagement of any self-specific inhibitory receptor, yet the $\beta_2m^{-}$ NK cells remain inert to healthy self cells. Furthermore, NK cells from MHC class I-deficient mice are poor responders to plate-bound anti-activation receptor antibody cross-linking, which is entirely independent of engagement of the inhibitory Ly49 receptors or other as-yet undefined receptors that may be involved in target recognition (27). These findings suggested that a different mechanism of NK cell tolerance governs these NK cells.

The second problem with the missing-self hypothesis concerns the necessity of pairing inhibitory NK receptors with their cognate MHC class I ligands. Ly49 receptors and KIRs, as well as their MHC ligands, are highly polymorphic. In addition, each inhibitory receptor binds only a subset of MHC class I alleles, and the Ly49 and KIR loci are not genetically linked to the MHC locus. The Ly49s are encoded in the NK gene complex (NKC) on distal mouse chromosome 6 whereas the KIRs are encoded in the leukocyte receptor complex (LRC) on human chromosome 19q13.4 (16, 28). In contrast, the MHC region is on mouse chromosome 17 and human chromosome 6p21, respectively. In other words, the genes for the NK cell receptors and MHC ligands segregate independently.

5
Early models of NK cell self-tolerance

Several models were created to address the gaps in the missing-self theory of NK cell function. One early model proposed that all NK cells express at least one receptor specific for self-MHC, thus ensuring self-tolerance of all NK cells in MHC class I-sufficient hosts. This model was initially based on the finding that NK cell clones established from two normal human donors all appeared to express at least one self-specific inhibitory receptor (29). Several studies in mice also appeared to support this hypothesis. First, expression of Ly49 and KIR alleles during development is sequential and, once established, appears to be fixed for each NK cell (2, 30). Second, the host MHC haplotype affects the frequency of cells expressing a given Ly49. For example, mice that express an MHC class I ligand for Ly49A (e.g. H2D\(^d\)) have a lower frequency of Ly49A\(^+\) NK cells than MHC-congenic mice that lack a ligand for Ly49A (e.g. H2\(^b\)) or MHC-deficient mice (31, 32). Together, these data suggested that NK cells accumulate additional inhibitory receptors until they are able to engage self-MHC and are consistent with the “at least one” hypothesis.

More recent work, however, has shown that a significant fraction of the NK cell population of both mice and humans lacks any known receptor for self-MHC (27, 33, 34). Like NK cells from MHC class I-deficient mice, these “self-blind” cells are hyporesponsive in that they do not kill MHC class I-deficient targets as well as NK cells expressing self-MHC-specific inhibitory receptors. It cannot be formally excluded that these cells express as-yet unidentified inhibitory receptor(s) for self, and the cause of their deficiency in target killing could be related to expression (or lack thereof) of undefined target cell ligands for either inhibitory or activation receptors. However, these
findings suggested that a tolerance mechanism separate from self-MHC engagement by inhibitory NK receptors might be at work.

Another model for tolerance, the receptor calibration model, focused on differences in Ly49 expression levels on individual NK cells from mice with different MHC haplotypes (35-37). Ly49 surface staining is significantly lower on NK cells from hosts with self-MHC that can bind the given Ly49 than on NK cells from mice that lack self-MHC. For example, Ly49A⁺ NK cells from mice expressing self-MHC (e.g. H2D^d) have lower levels of surface Ly49A than Ly49A⁺ NK cells from mice lacking self-MHC (e.g. H2^b or β2m⁻) (31, 35, 38). Recent studies in humans indicate a similar trend of decreased surface inhibitory KIR levels in individuals who express the cognate human leukocyte antigen (HLA) ligand (39). Thus, expression levels of NK cell receptors for MHC are altered in hosts with the cognate MHC ligand.

According to the postulated kinetics of receptor engagement and signaling in the receptor calibration model, an NK cell with a lower level of expression of an inhibitory receptor would require a higher level of ligand expression in order to achieve engagement sufficient to reach the threshold for inhibitory signaling (37). This was the proposed explanation for the finding that Ly49A^low NK cells had a higher threshold for inhibition by H2D^d than Ly49A^hi NK cells (40). In mice, the Ly49A^low NK cells were better able to kill tumor targets expressing reduced H2D^d than Ly49A^high NK cells isolated from a mouse lacking self-MHC, which were completely inhibited (41). Interestingly, in an H2^b MHC class I mosaic mouse, where MHC class I is expressed on some cells but not others, Ly49C levels were reduced on both MHC-deficient and MHC-sufficient NK cells (42). Culture in interleukin (IL)-2 for four days restored normal (i.e. elevated) Ly49C
surface expression on MHC-deficient cells, indicating that the surface expression level is reversible depending on environmental conditions.

Recent findings propose a new interpretation for the data used to support the receptor calibration model: the MHC-dependent reduction in apparent surface expression of Ly49 receptors on NK cells can be explained by cis interactions between Ly49 and MHC class I molecules expressed on the same NK cell (discussed further below) (43, 44). The amount of accessible surface Ly49 is lower on NK cells that co-express self-MHC, giving the appearance of reduced protein levels. In light of the new data on cis engagements, the “receptor calibration” model might thus be better termed the “receptor sequestration” model, where cis binding, instead of an actual decrease in expression, modulates sensitivity to inhibition by self-MHC (44). Regardless, the receptor calibration model still fails to explain why MHC class I-deficient NK cells respond so poorly to MHC class I-deficient target cells and to plate-bound antibody stimulation, both of which elicit robust responses by wild-type NK cells (26, 27). Thus, these older models of NK cell tolerance do not account for observed NK cell functions, particularly in MHC-deficient hosts.

NK cell licensing

In 2005, the Yokoyama lab proposed a new theory, called NK cell licensing, for NK cell tolerance in MHC class I-deficient hosts. NK cell licensing dictates that an NK cell must to engage self-MHC class I in order to be responsive to subsequent stimuli received via its activation receptors, a state termed “licensed” (Figure 1) (27, 45, 46). NK cells that fail to engage self-MHC are unlicensed. Licensing occurs via the MHC class I-
specific Ly49 receptors that were first identified as inhibitory receptors in effector responses, and it requires the ITIM signaling motif (27). NK cell licensing ultimately produces two types of self-tolerant NK cells with regard to self-MHC. Licensed NK cells maintain self-tolerance by direct inhibition via self-MHC class I engagement through the same receptor that conferred licensing. Unlicensed NK cells, which cannot engage self-MHC, are tolerant because they are highly resistant to stimulation received through their activation receptors. Missing-self stimuli (e.g. Concanavalin (ConA)-treated β2m−/− blasts) are not enough to activate unlicensed cells, whereas licensed cells respond robustly to missing-self stimuli.

The licensing model arose from studies examining the responses of freshly isolated NK cells upon target cell-free antibody cross-linking. Prior studies of NK cell tolerance predominantly examined cultured NK cell killing of target cells, a strategy that was useful in dissecting NK cell receptor specificity (22), but that could be problematic if culture conditions affected functional attributes of otherwise naïve NK cells. Furthermore, despite major advances, the entire repertoire of NK cell receptors and ligands involved in target recognition is incompletely understood. To minimize these potential confounding effects, Kim et al used freshly explanted, naïve NK cells that were stimulated with plate-bound antibodies against NK cell activation receptors such as NK1.1 (Nkrl1c), which is expressed on all immature and mature NK cells in C57BL/6 (H2b) mice. They then stained for intracellular interferon γ (IFNγ) as an index of NK cell activation, which, in conjunction with surface staining for lineage markers and Ly49 receptors, allowed for the functional characterization of individual NK cells.
These studies revealed that only NK cells expressing an inhibitory receptor specific for self-MHC class I produced IFNγ upon *ex vivo* plate-bound antibody stimulation (27). For example, Ly49A⁺ NK cells from MHC-congenic or transgenic (tg) mice expressing H2D^d, a known ligand for Ly49A, produced abundant IFNγ upon stimulation. Ly49A⁺ NK cells from mice lacking an MHC ligand for Ly49A, such as the H2^b_ haplotype, produced significantly less IFNγ. Importantly, IFNγ production in response to PMA and ionomycin stimulation was the same for both licensed and unlicensed NK cells, demonstrating that unlicensed cells are equipped to produce IFNγ. Moreover, these findings were recapitulated with antibodies to other NK cell activation receptors, including those with different associated ITAM-containing signaling chains. In addition, production of other cytokines showed similar patterns, and the responsiveness of licensed NK cells was also extended to target killing. Interestingly, CD94/NKG2A expression did not clearly correlate with the licensed phenotype. Taken together, these findings strongly suggested that a receptor specific for self-MHC must be engaged in order for the NK cell to become functionally competent for triggering through an activation receptor, i.e., NK cells are licensed by engagement of self-MHC-specific receptors.

The requirement of the Ly49-MHC interaction in producing functionally competent cells was conclusively demonstrated using a mouse (produced by the Hansen Lab, Washington University, St. Louis, MO) transgenic for a H2K^b_ -ovalbumin (ova) peptide single chain MHC class I trimer (SCT) on an otherwise MHC class I-deficient background (K^b^d_ D^b^d_ β2m^−^) (27). The H2K^b_ -ova SCT is the only expressed MHC class I molecule in these mice, and it is exclusively recognized by Ly49C, as indicated by
SCT-tetramer staining of primary NK cells from β2m-deficient mice (27). SCT-tetramer staining was completely blocked by pre-incubation with an antibody monospecific for Ly49C, establishing that the SCT is only recognized by Ly49C. As predicted by the licensing model, Ly49C⁺ NK cells from these mice produced IFNγ upon plate-bound anti-NK1.1 stimulation, but NK cells lacking Ly49C expression did not. Thus, a self-MHC-specific receptor is required for licensing, and individual NK cells are separately licensed, depending on their expressed receptors.

That individual NK cells are licensed separately based upon their expressed inhibitory receptors provides an explanation for hybrid resistance, a phenomenon that contradicts the classic laws of tissue transplantation. It has long been observed that while F₁ hybrid mice can accept skin grafts from either inbred parental strain, a T cell-dependent process, they reject parental bone marrow (BM) transplants (47, 48). Recipient NK cells are responsible for this BM rejection, and it is dependent on the MHC environment of the recipient (49, 50). Due to the stochastic expression pattern of inhibitory NK receptors, licensing predicts that some NK cells will be licensed only by MHC class I molecules inherited from the mother, while MHC class I molecules inherited from the father will license a different set of NK cells. Because of the co-dominant expression of MHC class I molecules, all cells of the F₁ progeny express both the maternal and paternal MHC molecules, thereby maintaining self-tolerance of the licensed NK cells. However, if BM from one parent is infused into the F₁ progeny, NK cell licensed to the other parent will mediate rejection of those BM cells as “missing self.” Licensing provides a satisfying explanation for how NK cells can determine if a cell expresses the full complement of self-MHC molecules, as in hybrid resistance.
Proposed molecular mechanisms of licensing

The mechanism by which engagement of the self-MHC-specific receptor leads to licensing has not been identified. The question of how an “inhibitory” receptor can have an apparently positive effect on NK cell function is especially intriguing. Experiments have demonstrated that the ITIM of the inhibitory receptor is required, but SHP-1, the phosphatase that mediates effector inhibition, does not appear to be involved (27, 51). Two general models dominate current thought on the molecular mechanism of NK cell licensing: 1) the “arming” or “stimulatory receptor” hypothesis; and 2) the “disarming” or “inhibitory receptor” hypothesis (45, 52). While there are not yet any definitive data to prove or disprove either mechanism, it is useful to review the models and analyze the existing data.

The “arming” mechanism postulates that NK cell licensing is directly induced by the interaction of an inhibitory NK receptor with MHC class I. In other words, signaling from the inhibitory receptor itself induces licensing. The arming mechanism of licensing has been quite controversial because it ostensibly requires an “inhibitory” receptor to produce a “positive” effect. However, with recent discoveries of novel signaling events downstream of ITIMs, including phosphorylation events typically associated with activation receptors (53), this possibility may not be as far-fetched as originally thought. Alternatively, a different signaling milieu may exist during NK cell education as opposed to during effector responses, resulting in different outcomes after the same or similar receptor engagement.
The “disarming” model proposes that self-MHC-specific NK cell receptors oppose constitutive activation signals to induce licensing. In other words, they act akin to their role in inhibiting effector responses by counteracting the signal of a postulated second receptor that presumably recognizes self and activates the NK cell. In the absence of an inhibitory signal from the self-MHC-specific receptor, the unmitigated activation signals would cause the NK cell to become (or remain) unlicensed. Implicit in the disarming model is the engagement of a self-specific activation receptor. Interestingly, no single activation receptor or signaling chain is required for licensing as mice deficient in each of the signaling molecules, DAP10, DAP12, FcRεγ, and CD3ζ, had intact licensing (27). A redundant role for these activation receptors cannot be excluded, however, as the studies only included single knockout mice for each of these proteins.

A recent study from our group tested the arming and disarming models of NK cell function (54). This study used a transgenic C57BL/6 mouse that ubiquitously expresses m157, the murine cytomegalovirus (MCMV)-encoded ligand for the Ly49H NK cell activation receptor (17, 18). The m157 transgenic mice were more susceptible to MCMV infection and were unable to reject m157-transgenic BM, suggesting defects in Ly49H+ NK cells (54). These defects could not be attributed to decreased Ly49H expression or fraction of Ly49H+ NK cells. Interestingly, Ly49H+ NK cells from these m157 transgenic (tg) mice were hyporesponsive to both Ly49H-dependent and Ly49H-independent stimuli in vitro, indicating a generalized hyporesponsiveness to stimulation. Continuous Ly49H-m157 interaction was necessary for these functional defects, which were also observed by the Lanier group using retroviral gene transduction of m157 into hematopoietic stem cells for BM reconstitution (55). Notably, the functional defects also occurred when mature
wild-type NK cells were adoptively transferred to m157tg mice, suggesting mature NK cells can acquire hyporesponsiveness (54). Thus, continuous engagement of an activation receptor results in hyporesponsiveness.

Importantly, NK cell tolerance due to Ly49H-m157 interaction was similar in Ly49H⁺ NK cells regardless of expression of Ly49C, an inhibitory receptor specific for a self-MHC allele in C57BL/6 mice (27, 54). Thus, in this mouse model, NK cell licensing could not override the hyporesponsiveness caused by the constitutive m157-Ly49H activation signal. In other words, engagement of self-specific activation receptors in vivo induces an NK cell tolerance effect that is not affected by self-MHC-specific inhibitory receptors. These findings suggest that self-tolerance to constitutive expression of activating receptors is independent from NK cell licensing.

When and where does NK cell licensing occur?

NK cell licensing dictates that there must be a physical interaction between inhibitory NK receptors and MHC class I to produce a functional NK cell. The details of when, where, and with the assistance of what (if any) accessory cell this occurs remain a mystery. Several pieces of circumstantial evidence suggest that NK cell licensing might take place during maturation in the BM, where complete NK cell development is assumed to occur. First, inhibitory receptors are expressed relatively early in NK cell development (56, 57), and their expression coincides with the acquisition of functional capabilities (30, 58). Second, in ontogeny, these inhibitory receptors appear to be acquired sequentially in an MHC-dependent manner, meaning that an NK cell that can engage self-MHC is less likely to express additional inhibitory receptors than an NK cell
that cannot (yet) engage self-MHC (2, 30-32, 59). Finally, in vivo studies demonstrate that BM NK cells undergo proliferation before reaching full developmental maturity (56). NK cells that have self-MHC-specific receptors proliferate at a higher rate than NK cells that lacking such receptors (27). Nonetheless, there is no direct evidence that NK cell licensing must occur during development or in the environment of the bone marrow.

Is there a particular cell that must display MHC class I to NK cells for the purpose of licensing? Current data would suggest that there is no single cell type responsible for licensing of NK cells. Fetal liver and BM chimera experiments suggest that both hematopoietic and non-hematopoietic compartments play a role (60, 61). Transgenic expression of H2Dd in liver, testis, and intestine, with very low expression levels in thymus, spleen, and kidney, did not induce NK cell licensing (62). However, all of these studies are complicated by the fact that mosaic expression of MHC class I induces tolerance of NK cells to MHC class I-deficient cells (63). In vitro developmental studies suggest that a stromal cell is required for expression of the Ly49 receptors, apparently in an MHC-dependent manner (59, 64). However, whether stromal cells are actually necessary for NK cell licensing is not known. In sum, the minimum requirements for licensing of NK cells have not yet been identified.

Cis and trans engagement of Ly49 receptors with MHC class I

Ly49 receptors have the curious ability to engage their MHC ligand in cis, where Ly49 and MHC class I are expressed on the same cell, as well as in trans, where Ly49 and MHC class I are expressed on different cells (43). Importantly, as the cis and trans binding sites of Ly49 receptors are the same (“site 2”, (65-67)), cis engagement by Ly49
prevents the receptor from interacting with MHC class I in \textit{trans} (43). Indeed, a recent study has shown that \textit{cis} interactions of Ly49 with MHC class I are stable and not displaced by MHC class I presented in \textit{trans} (68). Interestingly, \textit{cis} interactions of Ly49 with MHC class I molecules reduce the ability of NK cells to receive inhibitory signals from target cells. NK cells that expressed both Ly49A and its cognate ligand H2D\textsuperscript{d} were able to kill H2D\textsuperscript{d}-expressing tumor cells whereas Ly49A\textsuperscript{+} NK cells lacking H2D\textsuperscript{d}, and therefore lacking \textit{cis} interactions, were inhibited from killing (43).

Structural studies by the Mariuzza and Margulies groups suggest that the homodimeric Ly49 receptors have two conformations, termed open and closed (69, 70). The open conformation, seen in the Ly49C crystal structure (71), is symmetric and can bind two MHC class I molecules. The closed conformation, seen in the Ly49A crystal structure (65), is asymmetric and can bind only one MHC class I molecule. NMR studies have shown that Ly49 molecules can shift from one conformation to the other, leading to the hypothesis that closed and open conformations may correspond with \textit{cis} and \textit{trans} interactions of Ly49 receptors (69). Indeed, recent work indicates that this is the case. Ly49A can bind two MHC class I molecules in \textit{trans} but only one MHC class I molecule in \textit{cis} (72). The drastically different structural conformations required for those two types of MHC class I engagement may influence the signals mediated by inhibitory Ly49 receptors.

More recently, Held and Mariuzza have proposed that \textit{cis} interactions of Ly49 receptors with MHC class I prevents the recruitment of the Ly49 receptor to the immunological synapse (44). By sequestering inhibitory Ly49 receptors away from the synapse, \textit{cis} interactions make the NK cell more sensitive to activation stimuli. In
essence, this hypothesis is very similar to the receptor calibration model described above and shares the same shortcomings, namely that this model fails to explain the hyporesponsive phenotype of MHC class I-deficient NK cells.

Subsequent research by the Held group indicates that cis engagements of Ly49 with MHC class I are indeed required for NK cell licensing (73). Specifically, NK cells that expressed an engineered Ly49A molecule whose rigid stalk allowed trans but not cis engagements with MHC class I were not licensed. In addition, Chalifour et al suggest that Ly49 receptors that are not bound to MHC class I in cis produce a tonic inhibitory signal that terminates upon cis engagement of Ly49. While NK cells expressing a wild-type Ly49A transgene in the absence of a MHC class I ligand exhibited a reduced response to MHC class I-deficient target cells, NK cells expressing a Ly49A molecule with a mutated ITIM responded as well as non-transgenic NK cells. This finding indicates that unengaged Ly49 receptors produce an ITIM-dependent inhibitory signal.

There has been some debate regarding the roles of cis engagement and true decrease in Ly49 receptor surface expression in producing the observed downregulation of receptors detectable by flow cytometry. Andersson et al reported that acid treatment of NK cells restores Ly49A expression to 43% of levels on NK cells lacking cis ligand, and they ascribe the remaining 57% decrease to true down-regulation of surface expression (74). This is in contrast to data presented by Doucey et al, which showed almost complete recovery of Ly49 levels with acid treatment (43). Notably, in similar experiments, we have found that cis engagement between Ly49 and MHC class I may protect the MHC class I molecule from denaturation (AHJ and WMY, unpublished observations). The extent of cis binding may therefore be underestimated in calculations based on the acid
stripping technique. Nonetheless, it is possible that mechanisms in addition to cis engagements may induce the downregulation of surface Ly49 receptors.

**NK cell licensing: analog or digital?**

While NK cell licensing is well established for strong ligands of inhibitory Ly49 receptor, the effect on NK cell licensing of moderate or weak Ly49-MHC class I interactions remains unclear. The relationship between Ly49-MHC class I affinity and strength of licensing is particularly interesting given the vast polymorphism of both MHC class I genes and inhibitory NK receptors. Does a Ly49-MHC class I interaction above a given threshold lead to full licensing whereas interactions below the threshold have no effect on licensing (a digital model), or is the strength of NK cell licensing proportional to the strength of the Ly49-MHC class I interaction (an analog or quantitative model)? Since Ly49 receptors also mediate effector inhibition, are the Ly49-MHC class I engagement sensitivities of NK cell licensing and effector inhibition the same or different? Answers to these questions would improve our understanding of the behavior of NK cell populations in the diverse MHC contexts of outbred populations.

Recent studies have attempted to elucidate the analog or digital nature of NK cell licensing by investigating the potency of NK cell subsets in mice expressing one, two, or three MHC class I alleles (75, 76). Joncker et al reported that NK cell potency increases with the number of self-specific inhibitory receptors: NK cells expressing two different self-specific inhibitory receptors respond more robustly to stimulation than NK cell subsets expressing only one self-specific inhibitory receptor (76). In addition, Brodin et al used flow cytometry to assess the NK cell potency of Ly49-monopositive populations in
mice expressing selected MHC class I alleles (75). They found that the Ly49A-
monopositive NK cell subset of mice expressing only H2D^d, a known licensing ligand for
Ly49A (27), exhibited a more robust response to NKG2D stimulation than the
corresponding cells from mice expressing only H2D^b, which in turn responded more
strongly than cells from mice lacking MHC class I. Based upon these and other data,
Brodin et al and Joncker et al propose an analog (“rheostat”) model of licensing, in which
the potency of a given NK cell subpopulation is modulated by the nature and number of
Ly49-MHC class I interactions. Data from studies of human NK cells also support a
quantitative model of licensing. For example, Yu et al and Yawata et al both report that
NK cell potency was higher among NK cells that expressed two self-specific KIRs than
among NK cells expressing only one self-specific KIR (2, 77). Thus, the available data
support quantitative modulation of potency of an individual NK cell based upon the
number of self-MHC-specific inhibitory receptors that it expresses.

On the other hand, evidence of the effect of engagement of a single inhibitory
receptor by different MHC alleles is limited, although the simplest prediction of the
rheostat model is that stronger interactions should produce more potent NK cells.
However, most studies of murine NK cells have been confined to just two H2 haplotypes,
H2^b and H2^d. The most detailed study, by Brodin et al, used H2K^b, D^b, and D^d as model
MHC class I molecules. Whereas Ly49A does not bind H2K^b, and has occasionally but
not consistently been reported to bind weakly to H2D^b in older binding studies (e.g., (78,
79)), Ly49A binds strongly to H2D^d. Thus, only a very limited selection of MHC class I
alleles has been used to study the putative quantitative nature of licensing through a
single self-MHC-specific receptor.
More detailed knowledge of the contribution of individual NK cell inhibitory receptors to licensing in different MHC contexts may have clinical implications. Human NK cells display properties consistent with licensing in that NK cells expressing a killer immunoglobulin-like receptor (KIR) with specificity for self-HLA molecules exhibit more robust responsiveness than NK cells without self-HLA specific receptors in the same individual (2, 33, 80). With increasing numbers of studies reporting a connection of NK cell inhibitory receptors and licensing with human anti-viral and anti-cancer responses (e.g., (81-83)), a better understanding of licensing in mice should provide insight into human NK cell biology and its effects on human health and disease.
Figure 1. Licensing of NK cells. An NK cell must engage self-MHC class I in order to become licensed. NK cell licensing requires the ITIM signaling motif (indicated by an asterisk) of the inhibitory Ly49 receptor. Ly49 receptors can engage MHC class I either in *cis* or in *trans*, but the roles of these two types of Ly49A engagement in NK cell licensing are not yet clear. An NK cell that cannot engage self-MHC, either because it does not express any inhibitory NK receptors capable of binding any self-MHC class I molecule or because the host lacks MHC class I (e.g., β2m<sup>−/−</sup> mice), remain unlicensed. There is no known molecular marker of licensing, hence licensing of NK cells is assessed in an *ex vivo* stimulation assay using plate-bound antibodies against NK1.1, an activation receptor expressed by all NK cells on the B6 and B10 backgrounds. In these assays, licensed NK cell populations make IFNγ at a high frequency whereas unlicensed NK cells make IFNγ at a lower frequency.
Ly49A

NK cell

Unlicensed

Non-ligand MHC Class I

Licensed

Ligand MHC Class I, e.g. H-2D^d

cis

trans

NK cell

NK cell

Ly49A

NK cell

NK cell
CHAPTER 2

MATERIALS AND METHODS
Mice. MHC-congenic mice, including C57BL/10 (H2^b), B10.D2 (H2^d), B10.BR (H2^b), B10.D1 (H2^d), B10.RIII (H2^b), and B10.S (H2^b), as well as TAP1−/− mice and Rosa-rTA mice (B6.Cg-Gt(Rosa)26Sor^{m1(rtTA*M2)lac/J}) were purchased from The Jackson Laboratory (Bar Harbor, ME). H2K^{b−/−} H2D^{b−/−} (K^{b−/−} D^{b−/−}) mice were purchased from Taconic Farms (Germantown, NY). D^{d−}-transgenic K^{b−/−} D^{b−/−} mice were obtained by crossing D^{d−}-transgenic mice (D8, expressing a D^{d} genomic construct with transgenic D^{d} expression comparable to H2^{d} mice) provided by D. Margulies (NIAID, Bethesda, MD) to K^{b−/−} D^{b−/−} mice. To generate offspring homozygous, hemizygous, or nullizygous for the D^{d} transgene, K^{b−/−} D^{b−/−} mice homozygous for the D^{d} transgene were bred to K^{b−/−} D^{b−/−} mice, and F1 mice were then bred together. Similarly, B10.BR mice were bred to K^{b−/−} D^{b−/−} mice to produce mice hemizygous for the H2^{k} class I locus. These mice were then compared to commercially obtained parental strains. For the B10.RIII F_{2} hybrid mouse experiments, B10.RIII mice were mated to C57BL/6 mice. The subsequent (B10.RIII x C57BL/6)F_{1} hybrid mice were sibling mated to produce F2 hybrid mice which were then screened using microsatellite markers for the H2 locus and chromosome 10 from B10.RIII mice. Microsatellite screening was performed by the Rheumatic Disease Core Center’s Speed Congenics Lab at Washington University School of Medicine. All mice were used in accordance with institutional guidelines for animal experimentation.

Antibodies and flow cytometry. The following antibodies and reagents were purchased from BD Biosciences (San Jose, CA): Pacific Blue and PerCP Cy5.5 anti-CD3 (145-2C11); PerCP Cy5.5 anti-CD19 (1D3); APC and PE Cy7 anti-NK1.1 (PK136); Alexa 488, PE, or PE Cy7 anti-IFNγ (XMG1.2); FITC anti-H2D^{k} (15-5-5); PE anti-H2D^{d}
(34-5-8S); PE anti-H2Kb (AF6-88.5); and PE- and APC-conjugated streptavidin. PE- and APC-conjugated anti-H2Kb-ova peptide-in-groove mAb were purchased from eBioscience (San Diego, CA). Anti-Ly49A clones A1 and JR9 were produced from hybridomas as previously detailed (22, 84), purified, and labeled with biotin or FITC using standard protocols. For gating on Ly49A-monopositive NK cells, the following antibody clones were used to exclude other inhibitory NK cell receptors: 4LO33 (anti-Ly49C; produced from a hybridoma kindly provided by Suzanne Lemieux, Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Quebec, Canada); 5E6 (anti-Ly49C/I; BD); HBF-719 (anti-Ly49F; BD); 4D11 (anti-Ly49G2; BD or hybridoma from ATCC); YLI-90 (anti-Ly49I; BD); 16a11 (anti-NKG2A; eBioscience). The commercially available antibodies were purchased in PE-conjugated form, except 4D11 (APC). Homemade 4LO33 and 4D11 were used in biotinylated form with streptavidin-PE. Anti-NK1.1 clone PK136 (ATCC) was grown from the hybridoma and purified according to standard protocols. For flow cytometric analysis, RBC-lysed single-cell suspensions of splenocytes were stained in the presence of 2.4g2 supernatants. Samples were analyzed on a FACSCanto (BD Biosciences), and the data were analyzed using FlowJo (TreeStar, Inc., Ashland, OR)

**NK cell stimulation assays.** Splenocytes were harvested and stimulated with 2 μg or 5 μg PK136 (anti-NK1.1) monoclonal antibody (or, in some cases, PK136 ascites) essentially as previously described (27). Briefly, 6-well tissue culture-treated plates were coated with purified mAb in 1 mL PBS. 10^7 naïve splenocytes were added to the washed plates and incubated at 37 C and 5% CO2 for 1 hour, and then further incubated in the presence of brefeldin A (GolgiPlug, BD Biosciences) for an additional 7 hours. IFNγ was
detected by intracellular cytokine staining and flow cytometry as described previously (56). For co-culture experiments, 5 x 10^6 splenocytes from each of two mice were pooled into the same well. In some experiments, alternative stimuli were used, including anti-Ly49H mAbs (3D10), anti-Ly49D mAb (4e5), and IL-12 and IL-18. For anti-Ly49H and –Ly49D stimulation, plates were coated with 5μg of mAb. IL-12 and IL-18 were added to a final concentration of 10 ng/mL and 50 ng/mL, respectively. Stimulation with PMA and ionomycin at final concentrations of 0.5μg/mL and 4 μg/mL, respectively, was used as a positive control.

**Ly49A tetramer synthesis and binding assays.** Recombinant soluble BirA-tagged Ly49A monomers were synthesized, purified, and refolded as previously described from a construct generously provided by N. Matsumoto (University of Tokyo, Tokyo, Japan) (78). The monomers were tetramerized with APC-conjugated streptavidin (BD Biosciences) immediately prior to cell staining.

**Ly49A+ LAK cell preparation.** Splenocytes were harvested from H2D^d-transgenic K^b/- D^b/- mice, and nylon wool column non-adherent cells were isolated and cultured in R10 media supplemented with 800 U/mL IL-2. On day 6, Ly49A+ and Ly49A- LAK cells were isolated as previously described (22). Cells were used on Day 9. Ly49A+ LAK cell purity (Ly49A+ NK1.1+ CD3+) ranged from 79.1 to 93.4% (mean ± SD: 87.3 ± 5.5%), and Ly49A- LAK cell purity (Ly49A- NK1.1+ CD3+) ranged from 88.7 to 95.5% (mean ± SD: 92.6 ± 2.3%).

**Cytotoxicity assays.** Four-hour ^51^Cr-release assays were performed according to standard protocols using Day 9 Ly49A+ or Ly49A- LAK cells as effectors. In some experiments, splenocytes from mice treated with poly-I:C (InvivoGen, San Diego, CA)
were used as effector cells. Con A blast target cells were produced by culturing splenocytes in R10 plus 6 μg/mL concanavalin A for two days. JR9 F(ab’)$_2$ fragments for blocking experiments were produced from purified antibody using Pierce Mouse IgG$_1$ Fab and F(ab’)$_2$ Preparation Kit (Thermo Scientific). MAR F(ab’)$_2$ fragments were produced by conventional pepsin digestion and protein A purification.

*In vivo peptide administration.* The VSV-8 peptide RGYVYQGL was purchased from Sigma (St. Louis, MO), and the NUbo68–75 peptide VNVDYSKL and its variant VNVDASKA were purchased from EZBiolab (Westfield, IN). The peptides were reconstituted in a minimal volume of PBS under sterile conditions. For ip peptide administration, peptide concentrations were adjusted with sterile PBS to the appropriate volume. For peptide administration by osmotic pump (ALZET, Cupertino, CA), peptides were used at the stock concentration in PBS, 40 mM for VNVDYSKL and 10 mM for VNVDASKA. This produced delivery rates of 480 nmol/day and 120 nmol/day for VNVDYSKL and VNVDASKA, respectively. Osmotic pumps were primed, loaded, and surgically implanted following manufacturers instructions. Surgical procedures followed institutional guidelines and recommendations for sedation and anesthesia. The mice were monitored daily during the entire course of the experiment.

*K$_b$-ova SCT III and H2D$^d$ tetramer synthesis.* A K$_b$-ova SCT III tetramer cDNA construct was produced by cloning a BirA tag onto the K$_b$-ova SCT III cDNA sequence of a plasmid with a pET21a backbone, kindly provided by the Hansen Lab (Washington University School of Medicine). This construct was transformed into BL21 (DE3) RIL competent cells. The H2D$^d$ heavy chain and $\beta_2$m constructs were kinds gifts of D. Margulies (NIAID, Bethesda, MD). For protein production, transformed BL21 (DE3)
RIL cells were grown overnight in 100 mL Luria broth (LB) supplemented with 100 
µg/mL ampicillin and 50 µg/mL chloramphenicol. The overnight culture was then used 
to inoculate 3L of LB without antibiotics. After the culture had grown to an OD of 
approximately 0.8, protein production was induced by the addition of IPTG to a final 
concentration of 1 mM. The bacteria were harvested by centrifugation after an additional 
four hours of culture. Inclusion bodies were produced and the protein refolded following 
established protocols (85). The H2D₄ tetramer was refolded with RGPGRAFVTI peptide 
(Sigma, St. Louis, MO) from the gp160 protein of HIV. The concentrated refolding 
reaction was applied to a S75 size exclusion column and eluted with sizing column buffer 
(150 mM NaCl, 20 mM HEPES pH 7.4, 0.05% sodium azide). Biotinylation was 
performed with BirA biotin ligase kit (Avidity, Aurora, CO) following the 
manufacturer’s protocol, except for the incubation, which was carried out at 30°C for 90 
minutes. The biotinylated protein was purified on an anion exchange column as described 
(85). The relevant fractions were pooled, concentrated, and equilibrated into PBS 
supplemented with protease inhibitors (1mM EDTA, 1 µg/mL leupeptin, 1 µg/mL 
peptatin, 200 µM PMSF). Protein was quantitated by UV absorbance.

*Kb*-ova SCT III tetramer binding and competition assays. For simple binding 
assays, 4.4 x 10⁵ CHO cells stably transfected with Ly49C were stained on ice for 30 
minutes in a reaction volume of 50 µL. The indicated final concentration of labeled 
conventional H2Kᵇ-ova tetramers, first-generation Kᵇ-ova SCT tetramers, or third-
generation Kᵇ-ova SCT tetramers were produced from 2.2 µM stocks for all tetramers. 
H2D₄ tetramers were used as a negative control for Ly49C binding. In the competition 
assay, unlabelled third-generation Kᵇ-ova SCT tetramers were mixed with labeled
tetramers and then added to 5x10^5 CHO cells stably transfected with Ly49C in a total staining volume of 50 μL. A final concentration of 8.8 nM of labeled tetramer was used with the indicated amount of unlabeled tetramer.

Construction of rtTA transgene. The rtTA transgene was produced by excising the P_CMV promoter from pTetOn Advanced (Clontech, Mountain View, CA) with AccI and EcoRI and inserting a short adaptor segment containing DraIII and SalI sites. (All restriction enzymes were purchased from New England Biolabs, Ipswich, MA.) The H2K^b promoter was obtained from the vector pHSE3’S3-C, a kind gift from James Darnell (Rockefeller University, New York, NY), as a Bgl II SalI fragment and cloned into the DraIII- and SalI-digested pTet-On adaptor-rtTA plasmid. The IgH enhancer element was excised from pHSE3’S3-C with EcoRI and then Klenow-treated to produce blunt ends. This fragment was then ligated into pTet-On K^b promoter-rtTA plasmid that had been digested with HindIII and treated with Klenow. The completed construct was sequenced for verification.

Construction of K^b-ova single-chain trimer (SCT) transgene. The third-generation K^b-ova SCT construct was produced from three separate plasmids kindly provided by Ted Hansen (Washington University School of Medicine, St. Louis, MO). First, H2K^b exons 1 and 2 were amplified from plasmid WU649, which encodes the genomic sequence of the first three exons of H2K^b. This fragment was then used as a reverse megaprimer with a conventional forward primer for amplification of the 5’ region of the third-generation K^b-ova SCT consisting of the peptide, β2m, and early H2K^b exon 1 from the plasmid WU899, which encodes a cDNA form of the third-generation K^b-ova SCT. The resulting 1.2 kb fragment was then digested with BstBI and PmlI and ligated into a
similarly digested WU649 plasmid. The resulting plasmid was then digested with Xbal and ligated into Xbal-digested WU596, which encodes the remainder of the genomic H2K\textsuperscript{b} sequence. To clone the entire K\textsuperscript{b}-ova SCT sequence into pTRE-tight (Clontech), the K\textsuperscript{b}-ova SCT fragment was amplified using primers carrying NotI and HindIII sites, respectively. After digest by these enzymes, this fragment was cloned into a similarly digested pTRE-tight plasmid. The completed construct was sequenced for verification.

**Preparation of transgene DNA for injection into oocytes.** The 4.5 kb K\textsuperscript{b}-ova SCT transgene was isolated by gel purification of XhoI digestion of pTRE-tight-K\textsuperscript{b}-ova SCT III. The SCT transgene DNA was electo-eluted from the gel fragment and recovered from the electrophoresis buffer using an Elutip-d column (Whatman, Kent, United Kingdom). The transgene DNA was ethanol precipitated and resuspended in microinjection buffer under sterile conditions. The rtTA transgene was isolated as a 5.2 kb BstZ171 and BsrBI fragment and processed in a similar manner. Injections into C57BL/6 oocytes were performed by the Transgenic Knock-out Micro-injection Core Facility, Washington University School of Medicine.

**PCR screening of SCT tg, rtTA tg, and Rosa-rtTA mice.** SCT mice were screened by PCR amplification of a 250 bp band by the primers (5’-TAG GCG TGT ACG GTG GGA G-3’) and (5’-TGC CTC CAC CTC CGC TAC-3’). To identify the presence of the rtTA gene in rtTA tg or Rosa-rtTA mice, the primers (5’-ACA AGA GCA AAG TCA TAA ACG G-3’) and (5’-CAG CGG AAT GAC TTG GCG TTG-3’) were used to amplify a 250 bp fragment. Both of these PCR reactions used Rag1 as an internal control band, using primers (5’-GAG GTT CCG CTA CGA CTC TG-3’) and (5’-CCG GAC AAG TTT TTC ATC GT-3’) to produce a 474 bp band. Homozygous or heterozygous
Rosa-rtTA knockin mice were distinguished using a PCR protocol provided by The Jackson Laboratory, which uses the primers (5’-AAA GTC GCT CTG AGT TGT TAT-3’), (5’-GCG AAG AGT TTG TCC TCA ACC-3’), and (5’-GGA GCG GGA GAA ATG GAT ATG-3’) to produce a 340 bp band from the rtTA knockin gene and a 650 bp band from the wild-type Rosa locus.

*SCT and rtTA Southern blots.* To assess copy-numbers of each transgene, conventional Southern blots were performed. Briefly, 4 µg of tail DNA was digested with XbaI (SCT) or Xho I and BamHI (rtTA). The digest fragments were separated by agarose gel electrophoresis. After depurination, denaturation, and neutralization, the DNA was transferred to a Hybond N+ membrane (GE Healthcare, Waukesha, WI). After drying, the membrane was hybridized to 32P-labeled a SCT- or rtTA-specific probe in the presence of 50% formamide hybridization buffer containing salmon sperm DNA. The SCT probe was produced by PCR amplification of 650 bp fragment using primers (5’-CGG GAA TAC AAT GGC GAC AC-3’) and (5’-GTC CCC TCC TTT TCC ACC TG-3’). The rtTA probe was produced by PCR amplification of a 700 bp fragment using primers (5’-ACA AGA GCA AAG TCA TAA ACG’3’) and (5’-GCA GGC AGC ATA TCA AGG TC-3’). The probes were radiolabeled using a Rediprime II kit (GE Healthcare).

*Doxycycline administration.* Doxycycline (Sigma, St. Louis, MO) was administered at 2 mg/mL in drinking water supplemented with 5% w/v sucrose or 4% grape-flavored KoolAid (Kraft Foods, Northfield, IL). The doxycycline drinking water was replaced every 2 to 4 days and was supplied in dark water bottles to protect the doxycycline from light. For ip administration, doxycycline was dissolved in PBS (6 mg/mL) and filter-sterilized. Each mouse received 500 µL (3 mg) per dose.
**In vitro licensing of NK cells.** Nylon wool column non-adherent cells from doxycycline-naïve SCT tg rtTA tg β2m−/− mice were isolated and cultured in R10 media supplemented with 800 U/mL IL-2 and 1 μg/mL doxycycline. On day 6, Kb-ova+ and Kb-ova− LAK cells were isolated using panning as previously described (22). Cells were used in conventional 51Cr-release assays on Day 9.

**T cell stimulation assays.** For CD3+CD28 stimulation, 6-well plates were coated with 25 μg purified anti-CD3 mAb (145-2C11). After addition of 5x10⁶ RBC-lysed splenocytes in 1 mL R10 media, anti-CD28 mAb was added to a final concentration of 2 μg/mL. For ConA stimulation, sterile-filtered ConA (Sigma) in PBS was added to 5x10⁶ RBC-lysed splenocytes in 1 mL R10 media to a final concentration of 6 μg/mL. Large-scale PMA/ionomycin stimulation was performed by adding PMA and ionomycin to 5x10⁶ RBC-lysed splenocytes to a final concentration of 0.5μg/mL and 4 μg/mL, respectively, in a total volume of 2 mL. These large-scale stimulations were incubated at 37C, 5% CO₂ for one hour. BFA was then added, followed by an additional 7 hours of incubation. The cells were then stained for intracellular IFNγ production using conventional methods. For small-scale T cell stimulation assays, 10⁵ cells were stimulated with PMA and ionomycin at concentrations of 0.5μg/mL and 4 μg/mL, respectively, in a final volume of 100 μL. BFA was added after the first 15 minutes of a four-hour incubation. Cells were then stained using conventional methods.

**Statistical analysis.** Statistical calculations were performed using GraphPad Prism software (Treestar, Inc.). Statistical significance of differences between two groups was calculated using the unpaired two-tailed t-test. Correlations between groups of values were analyzed using two-tailed correlation regression.
CHAPTER 3

EFFECT OF MHC CLASS I AFFINITY ON NK CELL LICENSING
**Introduction**

NK cell licensing is a mechanism of self-tolerance that requires an NK cell to engage self-MHC class I in order to become functionally competent. In mice, NK cell licensing is mediated by inhibitory Ly49 receptors such as Ly49A. NK cell licensing has previously only been studied in the presence or absence of a known strong MHC class I ligand of a given inhibitory Ly49 receptor. However, both MHC class I molecules and inhibitory NK receptors are highly polymorphic, resulting in Ly49-MHC class I interactions of varying affinities. How does the strength of a given Ly49-MHC class I interaction affect licensing?

Other groups have recently proposed a “rheostat” model of NK cell licensing (75, 76). In this model, the strength of NK cell licensing is proportional to the strength of the inhibitory input received by the cell. However, most of the work supporting this rheostat model compared NK cells that expressed different numbers of self-specific inhibitory receptors in a given MHC class I environment. Experiments addressing the effect of a single inhibitory NK receptor paired with different MHC class I molecules used a very limited selection of MHC class I alleles and lacked any binding data. Thus, whether licensing through a given Ly49 receptor follows the rheostat model remains unclear. In this chapter, we describe our studies of licensing of Ly49A⁺ NK cells in more diverse MHC contexts. Paired with assays of Ly49A engagement in *cis* and in *trans*, these studies elucidate the associations between strength of binding and licensing.
Results

 Licensing of Ly49A+ NK cells in MHC-congenic mice

The licensing status of NK cells must be determined functionally, as no molecular marker of licensed NK cells has yet been identified. To assess licensing, we used a target cell-free system of ex vivo stimulation of naïve splenocytes with immobilized monoclonal antibodies against NK1.1, an activation receptor expressed on all NK cells of mice with the C57BL/6 and C57BL/10 backgrounds (56). Stimulation through NK1.1 allowed us to compare the activation of individual NK cells through a universally expressed NK cell activation receptor, unlike target cell-based stimulation assays, which activate NK cells through multiple receptors and pathways that are incompletely defined and may differ from NK cell to NK cell. Intracellular cytokine staining and flow cytometry allow simultaneous characterization of surface receptor expression and cytokine response to stimulation. In the ex vivo NK1.1 stimulation assay, a higher frequency of licensed NK cells produce IFNγ, whereas few unlicensed NK cells produce IFNγ (27).

IFNγ production by bulk Ly49A+ NK cells from six MHC-congenic strains and MHC class I-deficient (Kb−/− Db−/−) mice was measured by intracellular cytokine staining and flow cytometry (Figure 2). To rule out possible licensing contributions by other inhibitory receptors in these diverse MHC contexts, we also assessed IFNγ production by Ly49A-monopositive (Ly49A+ C− F− G2− T NKG2A−) NK cells (Figures 3A and B). The patterns of IFNγ production by bulk Ly49A+ and Ly49A-monopositive NK cells were nearly identical, indicating that they are due to the effects of licensing through Ly49A. Furthermore, IFNγ production by NK cell populations lacking Ly49A in addition to other inhibitory Ly49 and NKG2A receptors does not reproduce the pattern, confirming that
the IFNg production frequency by Ly49A-expressing cells is not due to the background activity level of NK cells from a particular MHC-congenic mouse (Figure 3C). The only exception is the H2f haplotype (B10.RIII mice), in which Ly49- and NKG2A-negative NK cells displayed an elevated IFNg production frequency compared with the other strains. However, as Ly49A-monopositive NK cells from H2f mice produce IFNg at a significantly higher frequency than these Ly49- and NKG2A-negative NK cells, Ly49A has a clear licensing effect in this MHC haplotype. (The B10.RIII strain is discussed further in this chapter as well as in Chapter 7.)

Three MHC haplotypes (H2b, c, or) were included in which the function of Ly49A on NK cells had not previously been analyzed. Ly49A+ NK cells from H2f mice produced IFNg at high frequencies, suggesting that this haplotype encodes an as-yet unidentified MHC molecule(s) able to engage Ly49A. In contrast, Ly49A+ NK cells of the H2d and H2e MHC haplotypes produced IFNg at lower frequencies, indicating weak licensing. In H2b mice, which express H2Db, a putative weak ligand of Ly49A, Ly49A+ NK cells produced IFNg at a similarly low frequency, confirming that the H2b haplotype poorly licenses Ly49A+ NK cells (75). As shown previously (27), NK1.1 cross-linking leads to a high frequency of IFNg production by Ly49A+ NK cells from H2d mice, which express the Ly49A ligand H2Dd, but by very few Ly49A+ NK cells from MHC class I-deficient mice. NK cells from H2b mice, which express H2Db, a known ligand for Ly49A, also produced IFNg at a high frequency (70, 86).

The dotted lines in Figures 2B and 3B indicate the mean IFNg production by Ly49A+ NK cells from mice of the H2d haplotype. As H2Dd is known to clearly license Ly49A+ NK cells, MHC haplotypes with IFNg production frequencies above this level
can be considered strongly licensing MHC haplotypes for Ly49A. Three MHC haplotypes (H2\textsuperscript{a}, H2\textsuperscript{b}, and H2\textsuperscript{b}) fall below this line but exhibit stronger IFN\textgamma responses by Ly49A\textsuperscript{+} NK cells than MHC class I-deficient cells. The low level of licensing of Ly49A\textsuperscript{+} cells of the H2\textsuperscript{b} haplotype likely represents the same weak licensing impact observed by Brodin et al in mice expressing H2D\textsuperscript{b} either alone or in combination with H2K\textsuperscript{b} (75). By extension, the H2\textsuperscript{a} and H2\textsuperscript{b} MHC haplotypes also offer weak licensing environments to Ly49A\textsuperscript{+} NK cells.

*Genetic contamination of B10.RIII strain does not affect licensing phenotype*

While the MHC-congenic strains used herein have a long history as tools for the study of lymphocyte biology, the potential for interference from incidental genetic contamination remains. Although we do not know of genetic contamination in the other MHC-congenic strains, the B10.RIII (H2\textsuperscript{f}) strain contains a large MHC-donor RIII-derived region on chromosome 10 that correlates with increased susceptibility to models of arthritis, independent of the MHC locus (87, 88). To test whether this genetic contamination was responsible for the observed high frequency of IFN\textgamma production by B10.RIII NK cells, we crossed B10.RIII mice with C57BL/6 mice to produce F\textsubscript{2} hybrid mice that were homozygous for RIII-derived genetic segments on either chromosome 10 or at the MHC locus. Such mice were identified by genotyping of microsatellite markers. In the PK136 stimulation assay, Ly49A\textsuperscript{+} NK cells from mice of the H2\textsuperscript{f} MHC haplotype but lacking the RIII-derived chromosome 10 region produced IFN\textgamma at a frequency similar to mice encoding both RIII-derived regions (Figure 4). In contrast, Ly49A\textsuperscript{+} NK cells from mice of the H2\textsuperscript{b} MHC haplotype that contained the RIII-derived chromosome 10
segment did not produce IFNγ beyond the level of H2b mice lacking the RIII-derived region on chromosome 10. Thus, the licensing phenotype of Ly49A+ NK cells in B10.RIII mice is not affected by the genetic contamination on chromosome 10 of this strain.

**Measurement of Ly49A-MHC class I interactions in trans**

Using the panel of MHC-congenic mice that differentially license Ly49A+ NK cells, we next investigated whether the affinity of Ly49A engagement by these MHC haplotypes correlated with NK cell licensing. The most quantitative method to measure the strength of interactions between proteins is surface plasmon resonance. However, to apply this technique to our system would require cloning, expression, and purification of every MHC class I molecules in the MHC-congenic strains, about 20 molecules in all. Moreover, the peptide specificities of many of the MHC molecules are not known, precluding efficient refolding of these molecules. Instead, we selected flow cytometry-based methods that used soluble Ly49A tetramers and MHC-congeneric cells to provide relative measures of affinity.

To make Ly49A tetramers, we expressed, refolded, purified, and biotinylated monomeric, soluble, BirA-tagged Ly49A molecules using a construct and protocol kindly provided by Naoki Matsumoto (University of Tokyo, Japan). Initially, we hoped to measure relative off-rates of binding using the techniques of tetramer decay: the off-rate of binding is proportional to the decay in tetramer fluorescence bound to MHC-congeneric cells. However, the off-rate of Ly49A engagement with even a known high-affinity MHC ligand was too fast to allow for meaningful comparisons with lower-affinity MHC class I
ligands (data not shown). Attempts to compare on-rates of Ly49A-tetramer binding using similar methods also failed as the on-rates were too fast for useful comparisons (data not shown). We therefore opted to use steady-state soluble Ly49A tetramer binding as our measure of the strength of Ly49A-MHC class I engagement.

We measured relative Ly49A affinities by binding of soluble Ly49A tetramers to naïve splenocytes from MHC-congenic and K\(^{b/c}\) D\(^{b/c}\) mice. As expected, MHC haplotypes that exhibited strong licensing of Ly49A\(^{+}\) NK cells bound more Ly49A tetramer than cells of the haplotypes with weak licensing (Figure 5A). Surprisingly, cells of the H2\(^{d}\) haplotype bound much more Ly49A tetramer than any of the other MHC haplotypes, yet its level of licensing of Ly49A\(^{+}\) NK cells was not higher than H2\(^{f}\) and H2\(^{k}\) (Figures 2B and 3B). These results suggest that licensing is saturated by a certain threshold of Ly49A engagement with self-MHC.

Measurement of Ly49A-MHC class I interactions in cis

Ly49A and MHC class I molecules can also interact in cis, i.e. on the surface of the same cell (43, 74). The gold standard for measuring Ly49A-MHC class I interactions in cis is to denature the MHC class I molecules with a brief incubation in acidic buffer, thereby releasing bound Ly49A. However, we were unable to use these techniques on our MHC congenic cells. First, we did not have any way to verify that all MHC class I molecules present in the six MHC-congenic strains were equally susceptible to acid treatment: antibodies are available only to a few of the MHC class I molecules studied in this paper. Also, the MHC-congenic mice are not Ly49A transgenic, unlike the cells used in acid stripping studies published by the Held group (43, 89), and assays focused solely
on endogenous Ly49A⁺ NK cell populations are not adequately robust to detect differences between our seven groups.

The extent of cis engagement can also be measured indirectly by the MFI of anti-Ly49A monoclonal antibodies such as A1: cis engagements block binding of the antibody and result in a lower MFI (43). The MFI of anti-Ly49A staining of Ly49A⁺ NK cells from MHC-congenic mice produces a spectrum of values, with H2d, H2f, and H2k approaching saturation (Figure 5B). Similar results were obtained with a second anti-Ly49A monoclonal antibody, JR9, that is partially blocked by cis engagement of Ly49A and MHC class I (data not shown) (43). With either antibody, the results suggest that cis interactions of Ly49A with various MHC haplotypes are analog in character because, like tetramer binding, nearly all pair-wise comparisons of MHC haplotypes produce a statistically significant difference. However, the differences between the MHC haplotypes with the lowest Ly49A surface accessibility were very small in magnitude, suggesting that these haplotypes had reached saturation of cis binding.

Correlations of strength of licensing with strength of Ly49A-MHC class I engagements

Interestingly, putative cis engagements of Ly49A with MHC class I correlate better with strength of licensing than does Ly49A tetramer binding, a measure of trans interactions (R² = 0.83 and 0.31, respectively) (Figures 5C and D). In particular, NK cell licensing and cis engagement of Ly49A have similar saturation thresholds, suggesting that cis engagement of Ly49A with MHC class I is a strong determinant of licensing, as recently proposed by Chalifour et al for Ly49A and H2Dd and H2Dk (73). Curiously, H2q, which only weakly licenses Ly49A⁺ NK cells, exhibits statistically significant levels
of Ly49A tetramer binding and cis engagement of Ly49A as compared to the other weakly licensing MHC haplotypes, H2\(^a\) and H2\(^b\). This finding suggests that a threshold of Ly49A binding beyond that of H2\(^a\) must be achieved for strong licensing to occur, such as that observed with H2\(^k\).

**Discussion**

Inhibitory NK receptors have two functions. As the name suggests, one function is to inhibit activation of NK cell effector responses during encounters with healthy self cells. The second, more recently discovered role relates to self-tolerance. In a process termed NK cell licensing, NK cells must engage self-MHC class I with an “inhibitory” receptor in order to attain full functional competence (27). NK cell licensing explains how NK cells are able to detect missing self while maintaining self-tolerance despite the complex expression patterns and MHC class I specificities of inhibitory NK receptors of the Ly49 and KIR families.

The initial studies of licensing used mice that either expressed or lacked a known MHC class I ligand of a given MHC class I receptor (27). However, given that MHC molecules and inhibitory NK receptors are both highly polymorphic and that inhibitory NK receptors bind some but not all MHC class I alleles, the natural state of both human and mouse populations is likely to be much more complicated. The multitude of possible pairings of MHC class I alleles and inhibitory NK receptor alleles raises a number of questions with regard to NK cell licensing. Do only strong Ly49-MHC class I interactions lead to licensing? Is licensing digital (on-off) or analog (gradual, quantitative)? Is there a minimum affinity threshold for licensing? If licensing is analog,
is there a saturation threshold for licensing, beyond which stronger interactions with inhibitory NK receptor interactions do not translate into stronger licensing?

During the course of our studies, two other groups proposed a theory of quantitative (“rheostat”) licensing to govern the licensing state of a given NK cell (75, 76). This theory states that the strength of NK cell licensing varies with the strength of the licensing stimuli received by a given individual NK cell. This rheostat model of licensing was based on data collected from NK cells that express different numbers of self-MHC-specific inhibitory receptors. These studies indicated that NK cells that express two self-MHC-specific inhibitory receptors respond more strongly to stimulation than NK cells possessing just one self-MHC-specific inhibitory receptor (75, 76). While this is an interesting and important finding, it does not address whether a single inhibitory NK receptor, such as Ly49A, modulates the licensing status of an NK cell based upon the strength of its interaction with MHC class I. Does a given inhibitory NK cell receptor provide a digital on-off signal favoring licensing of the NK cell in the presence of self-MHC, or does the strength of the engagement of the inhibitory NK receptor by self-MHC modulate the strength of the consequent licensing? A small number of experiments presented in one report did investigate NK cells expressing a single inhibitory receptor in the context of one, two, or three MHC class I alleles (75). However, these assays were not very informative, because the range of MHC contexts was too narrow. In addition, no binding assays were done, so there was no quantitation of interactions between the given inhibitory NK receptor and its putative MHC class I ligands.

Studies to thoroughly and directly address whether the rheostat model applies to licensing by a single inhibitory NK receptor require a larger and more diverse group of
MHC class I molecules than offered by these previous studies of licensing. MHC-congenic mice on a C57BL/10 background represent an ideal model system in which to study NK cells in a variety of MHC class I contexts. These mice possess the same genetic background (except for a small region in B10.RIII mice, as discussed above), including the same haplotype of the Ly49 gene family in the NK gene complex, which displays profound genetic differences between inbred strains of mice (90). Moreover, these mice allow study of MHC class I alleles that have generally not been studied in detail in NK cell biology. Paired with studies of binding between Ly49A and the various MHC haplotypes, this MHC-congenic system is an excellent model in which to determine the relationship between strength of NK cell licensing and strength of binding of an inhibitory NK receptor to MHC class I.

Our findings are largely consistent with a quantitative (rheostat) model of NK cell licensing, with several new and important principles. First, NK cell licensing is saturated by a relatively low apparent “affinity” of Ly49A engagement, as determined by Ly49A tetramer binding. This is best exemplified by the H2d haplotype, whose uniquely high level of Ly49A engagement did not translate into stronger licensing compared to MHC haplotypes with more moderate binding to Ly49A. One possibility is that the H2d mice (B10.D2) have some unidentified genetic defect that dampens NK cell potency independent of NK cell licensing. However, H2Dd transgenic mice (D\textsuperscript{d+/-}) on a K\textsuperscript{b/-} D\textsuperscript{b/-} C57BL/6 background bind Ly49A tetramers nearly as well as mice expressing the endogenous H2\textsuperscript{d/d} locus and yet did not exhibit levels of licensing beyond that of B10.D2 mice (data not shown). Differences in avidity are also a possible explanation. However, as described in Chapter 4, changes in MHC gene dosage, even of the moderate-binding
MHC haplotype H2k, did not affect strength of licensing, indicating that the saturation threshold of Ly49A-mediated licensing encompasses low-avidity interactions.

Previous data regarding a saturation threshold for NK cell licensing were inconclusive in studies of NK cells expressing different numbers of self-MHC-specific Ly49 receptors. Joncker et al reported increased NK cell potency with up to three simultaneously expressed self-specific inhibitory receptors (76). However, Brodin et al observed saturation of NK cell licensing by NK cells from H2b mice that expressed three self-specific receptors: these NK cells were not more potent than NK cells expressing two of the three receptors (75). On the other hand, the inclusion of two additional receptors, i.e. the very small population of NK cells that expresses five inhibitory receptors, produced a boosted response to stimulation. In contrast, our data with additional MHC alleles demonstrate that licensing through Ly49A has an “affinity”-based saturation threshold when examined in different MHC contexts. Additional studies are needed to determine if this phenomenon extends to other MHC class I-specific inhibitory receptors, which have different MHC specificities from Ly49A.

Interestingly, Ly49A surface accessibility by anti-Ly49A monoclonal antibodies, a surrogate measure of cis interactions of Ly49A and MHC class I (43), exhibited a much stronger correlation with NK cell licensing than did measures of trans interactions. Importantly, the saturation pattern of putative Ly49A cis engagements coincided with that of licensing of Ly49A+ NK cells. We cannot formally rule out that the observed pattern of anti-Ly49A antibody binding is due to actual differences in Ly49A surface expression. However, when interpreted as measures of cis engagements, as previously described, these findings are consistent with a recent study by Chalifour et al, which
reported that *cis* interactions of Ly49A and H2D<sup>d</sup> or H2D<sup>k</sup> are required for licensing (73). Indeed, our data provide additional evidence for an important role for *cis* interactions in licensing, and extend previous observations to additional MHC haplotypes.

The degree of *cis* binding also correlates with a phenotype of weak licensing of Ly49A<sup>+</sup> NK cells of H2<sup>b</sup>, H2<sup>s</sup>, and H2<sup>q</sup> haplotypes. Ly49A<sup>+</sup> and Ly49A-monopositive NK cells from MHC-congenic strains of the H2<sup>b</sup>, H2<sup>s</sup>, and H2<sup>q</sup> haplotypes make IFN<sub>γ</sub> at a significantly higher frequency than cells from MHC class I-deficient mice. On the other hand, this frequency of IFN<sub>γ</sub> production is significantly lower than the corresponding cells from MHC haplotypes that strongly license NK cells (H2<sup>d</sup>, H2<sup>e</sup>, H2<sup>s</sup>). Interestingly, the Ly49A<sup>+</sup> NK cell populations of the H2<sup>d</sup>, H2<sup>e</sup>, and H2<sup>b</sup> MHC haplotypes do not produce IFN<sub>γ</sub> at a higher frequency than the corresponding Ly49A<sup>−</sup> populations. Conventionally, this would argue against a licensing impact of Ly49A in these MHC haplotypes. However, recent work indicates that unengaged Ly49A receptors actually suppress NK cell responsiveness to stimulation (73). Our observations support this view, as Ly49A-monopositive NK cells from MHC class I-deficient mice produced IFN<sub>γ</sub> at a lower frequency than the corresponding Ly49- and NKG2A-negative population. Thus, our findings of similar IFN<sub>γ</sub> responses by Ly49A<sup>+</sup> and Ly49A<sup>−</sup> populations of the H2<sup>q</sup>, H2<sup>s</sup>, and H2<sup>b</sup> haplotypes are consistent with the presence of a weak licensing interaction for Ly49A.

In sum, the strength of NK cell licensing mediated by Ly49A is proportional to the strength of the Ly49A-MHC class I interaction, but only up to a relatively modest saturation threshold. In other words, NK cell licensing through a given inhibitory NK receptor is only consistent with the rheostat model at low MHC class I affinities. In
addition, *cis* engagements of Ly49A and MHC class I exhibited the same saturation threshold as Ly49A-mediated licensing, supporting a role for *cis* engagements in NK cell licensing.
Figure 2. IFNγ production by Ly49A+ NK cells varies with MHC haplotype. (A) Representative flow cytometry dot plots of IFNγ production by NK cells (NK1.1+ CD3− CD19−) stimulated with plate-bound anti-NK1.1 antibody (5 µg PK136). (B) Average frequency ± SD of IFNγ production by bulk Ly49A+ NK cells (NK1.1+ CD3− CD19−) stimulated with plate-bound anti-NK1.1 antibody (5 µg PK136). N=9 or 10 per group except Kb−/− Db−/− (N=7). Pooled results from four independent experiments. The dotted line indicates the mean IFNγ production frequency of Ly49A+ NK cells of the H2d haplotype, which is known to strongly license Ly49A+ NK cells. For each MHC haplotype, the data are shown in the same rank order in Figures 2, 3, 5, and 8, according to the MFI of anti-Ly49A staining depicted in Figure 5B. *: p<0.05; **: p<0.01; ***: p<0.001.
Figure 3. The MHC-dependent pattern of IFNγ production by Ly49A⁺ NK cells is not due to other inhibitory NK receptors or background NK cell reactivity. (A) Representative gating scheme for flow cytometric analysis of intracellular IFNγ production by Ly49A-monopositive NK cells (Ly49A⁺ NK1.1⁺ CD3⁻ CD19⁻ NKG2A⁻ Ly49C⁻ Ly49F⁻ Ly49G2⁻ Ly49I). The number in the final dot plot represents the percent of IFNγ⁺ cells among the Ly49A⁺ population. (B) Average frequency ± SD of IFNγ production by naïve Ly49A-monopositive NK cells (Ly49A⁺ NK1.1⁺ CD3⁻ CD19⁻ NKG2A⁻ Ly49C⁻ Ly49F⁻ Ly49I; Ly49G2⁺ NK cells were also gated out in two of three experiments) stimulated with plate-bound anti-NK1.1 antibody (5 µg PK136). The dotted line indicates the mean IFNγ production frequency of Ly49A⁺ NK cells of the H2d haplotype, which is known to strongly license Ly49A⁺ NK cells. N=6 or 7 per group, pooled results from three independent experiments. See Materials and Methods for antibody clones used. (C) Average frequency ± SD of IFNγ production by Ly49A⁻ Ly49C⁻ Ly49F⁻ Ly49I⁻ NKG2A⁻ NK cells (Ly49G2⁺ NK cells were also gated out in two of three experiments) incubated with plate-bound anti-NK1.1 antibody (5 µg PK136). N=6 or 7 per group, pooled results from the same three independent experiments displayed in (B). *: p<0.05; **: p<0.01.
Figure 4. IFNγ production by NK cells from B10.RIII mice is not influenced by a known region of genetic contamination. IFNγ production by Ly49A⁺ NK cells from mice expressing RIII- or C57BL/6 (B6)-derived regions on chromosome 10 or at the MHC locus. B10.RIII mice were crossed with B6 mice, and the F2 pups were typed by microsatellite analysis to select for mice homozygous for the RIII- or B6-derived segments on chromosome 10 and chromosome 17, as indicated. Bars represent average frequency ± SD of IFNγ production by naïve Ly49A⁺ NK cells (NK1.1⁺ CD3⁻ CD19⁻) incubated with plate-bound anti-NK1.1 antibody (2 µg PK136). N=9 per group. Pooled results from three independent experiments. *: p<0.05; **: p<0.001
Figure 5. Correlations between licensing, soluble Ly49A tetramer binding, and putative cis binding of Ly49A. (A) Naïve splenocytes from MHC-congenic and \( K^{b/-} \) D\(^{b/-} \) mice were stained with soluble Ly49A tetramers. The histogram on the left displays representative Ly49A tetramer staining data for one mouse in each group. The bar graph on the right shows mean fluorescence intensity (MFI) ± SEM with N=3 for each group. Representative of three independent experiments. (B) Naïve splenocytes from MHC-congenic mice were stained for Ly49A (mAb A1), NK1.1, CD3, and CD19. The histogram on the left displays representative anti-Ly49A staining data for one mouse in each group. The bar graph on the right shows MFI ± SEM of Ly49A staining of Ly49A\(^+\) NK cells (NK1.1\(^+\) CD3\(^-\) CD19\(^-\)) with N=3 for each group. Representative of two independent experiments. (C) Correlation analysis of frequency of IFN\( \gamma \) production by Ly49A-monopositive NK cells from and Ly49A tetramer staining of MHC-congenic and MHC class I-deficient mice. For details on data, see Figures 3B and 5B. (D) Correlation analysis of frequency of IFN\( \gamma \) production by and anti-Ly49A staining MFI of Ly49A-monopositive NK cells from MHC-congenic mice. For details on data, see Figures 3B and 5B. *: \( p<0.05 \); **: \( p<0.01 \); ***: \( p<0.001 \).
CHAPTER 4

EFFECT OF MHC CLASS I HAPLOINSUFFICIENCY
ON NK CELL LICENSING
**Introduction**

MHC class I genes are co-dominantly expressed, so homozygous cells express twice as much of a given MHC class I molecules as a heterozygous (or hemizygous) cell. Licensing of NK cells requires MHC class I expression, but the effects of MHC class I haploinsufficiency on licensing is unclear. Given the great diversity of MHC class I alleles in outbred populations, MHC class I heterozygosity is very common. Since inhibitory NK receptors bind some but not all MHC class I alleles, functional haploinsufficiency of a MHC class I ligand of a given KIR is also common. Does this haploinsufficiency of the MHC class ligand affect the strength of NK cell licensing? In this chapter, we describe licensing studies in several genetic models of MHC class I haploinsufficiency that indicate that a single copy of a MHC class I gene is sufficient for licensing of Ly49A⁺ NK cells.

**Results**

*NK cell licensing is insensitive to gene dosage of H2D<sup>d</sup>*

To address the role of avidity of the Ly49A-MHC class I interaction in NK cell licensing, we used mice homozygous, hemizygous, or nullizygous for an H2D<sup>d</sup> transgene (genomic D<sup>d</sup> construct with D<sup>d</sup> expression comparable to H2<sup>d</sup> mice) on an otherwise MHC class I-deficient background. As expected from the co-dominant expression pattern of MHC class I genes, cells from mice homozygous for H2D<sup>d</sup> (D<sup>d tg/tg</sup>) express twice as much H2D<sup>d</sup> as cells from hemizygous mice (D<sup>d tg/--;</sup>) (Figure 6A). Accordingly, D<sup>d tg/tg</sup> cells also bind more soluble Ly49A tetramer than D<sup>d tg/--;</sup> cells (Figure 6B). However, the licensing of Ly49A⁺ NK cells from D<sup>d tg/tg</sup> and D<sup>d tg/--;</sup> mice was not significantly different
at either of two anti-NK1.1 stimulation doses tested (Figures 6C and D). These results indicate that saturation of NK cell licensing is maintained for high-affinity Ly49A-MHC class I interactions even at lower avidities. Importantly, Ly49A exhibited similar cis engagement in D\textsuperscript{d}tg/tg and D\textsuperscript{d}tg/c mice (Figure 6E), demonstrating the same correlation of cis engagement with strength of licensing as observed in MHC-congenic mice (see Chapter 3).

*A single gene copy of H2\textsuperscript{k} class I molecules, moderate-affinity ligands of Ly49A, is sufficient for strong NK cell licensing*

Since cells of the H2\textsuperscript{d} haplotype have a uniquely high affinity and/or avidity for Ly49A among the MHC haplotypes, we also developed a system based on H2\textsuperscript{k}, a MHC class I haplotype with a lower reactivity for Ly49A in MHC-congenic mice (Figure 5A). We crossed B10.BR (H2\textsuperscript{k/k}) mice with K\textsuperscript{b/c} D\textsuperscript{b/c} (H2\textsuperscript{k/c}) mice to create F\textsubscript{1} hybrid mice hemizygous for H2D\textsuperscript{k} (H2\textsuperscript{k/c}). As expected, H2\textsuperscript{k/k} cells express twice the level of H2D\textsuperscript{k} as H2\textsuperscript{k/c} cells and also bind twice as much soluble Ly49A tetramer (Figures 7A and B). However, there was no consistent statistically significant difference in licensing among Ly49A\textsuperscript{+} NK cells at two different anti-NK1.1 stimulation doses (Figures 7C and D). Furthermore, cis engagement of Ly49A was similar on H2\textsuperscript{k/k} and H2\textsuperscript{k/c} Ly49A\textsuperscript{+} NK cells (Figure 7E), providing yet more data that cis engagement of Ly49A has the same saturation threshold as NK cell licensing. In sum, these data indicate that haploinsufficiency of even a moderate-affinity self-MHC molecule has little or no effect on NK cell licensing.
**Discussion**

The studies described herein indicate that licensing of Ly49A⁺ NK cells occurs just as well with one gene dose of an MHC class I ligand as it does with two. Previous studies addressing gene dosage have focused on human systems and provide contradictory results. In several epidemiological studies, homozygosity for a cognate HLA allele ligand for a KIR was required for protection from infectious diseases, whereas in other studies, one HLA allele was sufficient for protection (81, 91-93). For example, individuals homozygous for the genes encoding KIR2DL3 and its cognate ligand, HLA-C1, have improved resolution of hepatitis C (91). In contrast, even a single gene dose of HLA-Bw4, when expressed with KIR3DL1, slows progression to AIDS (93). Similarly inconsistent results were reported in studies of human NK cell stimulation *ex vivo*. For example, licensing effects on KIR3DL1⁺ NK cells were only seen in donors homozygous for the HLA ligand for KIR3DL1 (80). This contrasts with data presented by others groups, in which a single copy of the HLA ligand for KIR2DL1 or KIR2DL2 (i.e. HLA-C2 and HLA-C1, respectively) was enough to induce robust licensing of the corresponding NK cell populations (2, 33).

There are several possible explanations for the discrepancies in these human studies. First, different KIRs may have different avidity thresholds, such that some KIRs require homozygous expression of a cognate HLA ligand for robust licensing. However, a single KIR, KIR3DL1, was found to require homozygous expression of its ligand HLA-Bw4 in *ex vivo* stimulation studies, yet conferred a protective effect against progression to AIDS even in individuals that were heterozygous for HLA-Bw4 (93).
Second, in the epidemiological studies, individuals that are homozygous and heterozygous for HLA ligand of a given KIR are often pooled together into the same experimental group (“individuals with at least one ligand”), making it impossible to distinguish differential effects of one versus two gene doses of cognate HLA ligands of a given KIR. Moreover, human NK cell studies generally divide the HLA alleles into two broad categories representing HLA molecules that generally do or do not bind the KIR of interest. The HLA typing is often based on binding by a group-specific monoclonal antibody, not sequencing for identification of exact alleles. With this type of categorization, the strength of the interaction between the KIR of interest and the various alleles of the cognate HLA ligand group may vary widely. As described for murine NK cells in Chapter 3, these differences in KIR affinity may result in different strengths of NK cell licensing and thus introduce variability in the groups that reduces the sensitivity of the assay to detect licensing effects. Furthermore, individuals who are homozygous for HLA genes belonging to a particular group are more likely to express an HLA allele with high affinity for a given KIR. This in turn increases the likelihood that the individual will have strongly licensed NK cells compared with individuals who have only one potential HLA ligand allele. In short, the finding that homozygosity of HLA expression is necessary for licensing in some studies may be a result of imprecise categorization of HLA alleles.

Caution must also be exercised in extrapolating the details of studies of licensing of Ly49-expressing murine NK cells to KIR-expressing human NK cells and vice versa. For example, KIRs and Ly49 receptors may use different biophysical mechanisms for licensing. Accumulating evidence indicates that \textit{cis} interactions determine licensing of
murine NK cells (73). Since there is no evidence that KIRs can engage HLA molecules in cis, this mechanism is less likely for human NK cell licensing (44). Different mechanisms of MHC class I engagement may explain why some KIRs require cognate HLA ligand homozygosity to produce a detectible licensing phenotype whereas Ly49A mediates licensing even in MHC class I haploinsufficient settings.

In fact, the ability of Ly49A to mediate strong licensing even with just one gene dose of an MHC class I ligand may be explained by differences in relative surface expression levels of MHC class I and Ly49A. In studies of MHC class I (H2Dd) transfer from donor cells to Ly49A+ NK cells, H2Dd levels reached a maximum of 16 to 30% of endogenous levels (94, 95). These data suggest that endogenous MHC class I molecules outnumber Ly49A receptors by several-fold on the surface of NK cells. In this way, high avidity may compensate for the moderate affinity of some MHC class I molecules for Ly49A such that even moderate Ly49A ligands induce robust licensing. This effect may be especially strong for cis binding of Ly49A and MHC class I, since cis binding occurs in an essentially two-dimensional environment. Furthermore, if cis binding determines licensing and cis binding is overwhelmed by the number of MHC class I molecules, then decreasing the number of MHC class I molecules while maintaining the same affinity should not affect the degree of licensing. Indeed, these are the results observed in Ly49A+ NK cells from mice expressing one versus two copies of an H2Dd transgene or endogenous H2k class I locus. Future quantitative studies are needed to confirm this hypothesis.

Mice with different gene doses of an MHC class I ligand provide an interesting contrast to studies of mice with chimeric expression of an MHC class I molecule. While
haploinsufficiency of H2D\textsuperscript{d} produced fully functional Ly49A\textsuperscript{+} NK cells, chimeric expression of H2D\textsuperscript{d} on even as many as 80% of cells failed to license Ly49A\textsuperscript{+} NK cells, as NK cells from these mice were incapable of rejecting H2D\textsuperscript{d}-deficient cells (63). Interestingly, the unlicensed state NK cells from H2D\textsuperscript{d}-chimeric mice was reversed by separating H2D\textsuperscript{d\textsuperscript{+}} and H2D\textsuperscript{d\textsuperscript{-}} NK cells for 24 hours after incubation together (or apart) in IL-2 for four days (63). Thus, while NK cells are licensed by half-maximal levels of MHC class I on all cells, as shown herein, the lack of a given MHC class I molecule on even a fraction of cells can greatly impact the licensing status of NK cells. The molecular basis of this discrimination remains unknown.

Since half-maximal MHC class I expression is sufficient for NK cell licensing, the minimum MHC class I avidity threshold of Ly49A-mediated NK cell licensing has still not been determined. Is this avidity threshold the same for high- and moderate-affinity MHC class I ligands of Ly49A? Is strength of licensing proportional to MHC class I expression below this MHC avidity threshold, or are NK cells simply unlicensed? Investigating these issues will require a system of fine control of MHC class I expression. We hope that the inducible MHC class I expression system described in Chapter 6 may fulfill these requirements and provide answers to our remaining questions about MHC class I avidity and NK cell licensing.
Figure 6. Licensing of Ly49A⁺ NK cells is not affected by haploinsufficiency of an H2D⁰ transgene. (A) H2D⁰ expression on splenocytes from mice homozygous (red), hemizygous (blue), or nullizygous (green) for a D⁰ transgene. Each histogram represents one mouse. Representative of four independent experiments. (B) Ly49A tetramer staining of splenocytes, histograms colored as in A, from a single experiment. (C) Frequency of IFNγ production by anti-NK1.1-stimulated (5 µg PK136) Ly49A⁺ NK cells from mice of the indicated D⁰ transgene genotype. Similar results were obtained when Ly49C, I, and G2⁺ cells were gated out of the analysis. Frequency was normalized to the average frequency of IFNγ production by D⁰/²g/²g Ly49A⁺ NK cells in each experiment. Each symbol represents one mouse, pooled from two independent experiments. (D) As in (C), except at a stimulation dose of 2 µg PK136. (E) MFI ± SEM of anti-Ly49A staining (mAb JR9) on Ly49A⁺ NK cells from littermates of the indicated D⁰ transgene genotype. N=4 or 6 per group. Representative of three independent experiments.
Figure 7. Licensing of Ly49A⁺ NK cells is not affected by haploinsufficiency of H2k MHC class I genes, moderate-affinity ligands of Ly49A. (A) H2Dk expression on splenocytes from mice homozygous (red), hemizygous (blue), or nullizygous (green) for endogenous H2k class I genes. Each histogram represents one mouse. Representative of three independent experiments. (B) Ly49A tetramer staining of splenocytes, histograms colored as in E, from a single experiment. (C) Frequency of IFNγ production by anti-NK1.1-stimulated (5 μg PK136) Ly49A⁺ NK cells from mice of the indicated MHC class I genotype. Frequency was normalized to the average frequency of IFNγ production by H2k/k Ly49A⁺ NK cells in each experiment. Each symbol represents one mouse, pooled from three independent experiments. (D) As in (C), except at a stimulation dose of 2 μg PK136. (E) MFI ± SEM of anti-Ly49A staining (mAb JR9) on Ly49A⁺ NK cells (NK1.1⁺ CD3⁺ CD19⁺) from mice of the indicated MHC class I genotype. N=5 and 6 for the H2k/k and H2k⁻/⁻ groups, respectively. Results from a Kb⁻/⁻ D⁻/⁻ mouse is shown for reference. Representative of three independent experiments.
CHAPTER 5

MHC SPECIFICITY AND SENSITIVITY OF
LY49A-MEDIATED EFFECTOR INHIBITION
Introduction

Ly49A has two functions on NK cells: (1) licensing, and (2) inhibition of effector function, i.e., the inhibition of target cell cytotoxicity in the presence of a target cell ligand for Ly49A. However, whether licensing and effector inhibition use the same signaling cascades is not known. This issue is further complicated by the fact that Ly49 receptors can engage MHC class I both in trans and in cis. The binding site for Ly49A on MHC class I are the same for both of these binding orientations, i.e. “site 2” (65, 67, 96). While effector inhibition by definition requires trans engagement of Ly49 receptors, recent studies suggest that NK cell licensing is mediated, at least in part, by cis interactions of Ly49 receptors with MHC class I (73). These potential differences in mechanism raise the possibility that the MHC specificities and sensitivities of NK cell licensing and effector function may differ. In this chapter, we describe experiments to assess whether these two functions of Ly49A share the same MHC specificities and avidity thresholds. We found that while the MHC specificities were largely the same, effector inhibition was more sensitive to MHC class I engagement than NK cell licensing.

Results

Design and development of the effector inhibition assay

To determine whether effector inhibition correlates with MHC haplotype in the same way as licensing, we assessed inhibition of Ly49A+ effector cells by Con A-activated blasts from MHC-congenic mice in traditional §1Cr-release cytotoxicity assays. As effector cells, we used Ly49A+ lymphokine-activated killer (LAK) cells cultured from splenocytes of H2Dd-transgenic Kb/c Db/c mice. Because these effector cells developed in
a licensing MHC background for Ly49A NK cells, they maximize the potency of the resulting Ly49A⁺ LAK cells. Importantly, using the same pool of effector cells in all wells in each experiment eliminated licensing as a cause of differences in cytotoxicity.

Since only a small percentage of NK cells express Ly49A, we needed to separate the Ly49A⁺ and Ly49A⁻ LAK cells. The current preferred approach for separating cell populations is cell sorting by FACS. However, LAK cells do not handle this type of cell sorting well. Despite many attempts at optimization of sorting conditions, the LAK cells exhibited poor viability and proliferation. We therefore used a technique called panning, which was previously used to isolate Ly49A⁺ LAK cells in early studies of Ly49A function (22). Panning uses antibody-coated tissue culture flasks to capture cells that express the molecule of interest. Following panning, adherent cells are lifted, washed thoroughly, and cultured for an additional two days to allow clearance of any remaining anti-Ly49A antibody, which might otherwise interfere with engagement to MHC class I on the target cells. Ly49A⁺ LAK cell purities following panning ranged from 79.1 to 93.4% (mean ± SD: 87.3 ± 5.5%). To make the target cells, splenocytes were cultured in media containing ConA for two days. ConA is a T cell mitogen that makes otherwise healthy splenocytes susceptible to NK cell attack.

*Specific inhibition of Ly49A⁺ LAK cell activity by MHC-congenic target cells*

The chromium release assays revealed differences in the susceptibility of MHC-congenic target cells to lysis by Ly49A⁺ LAK cells. Some MHC haplotypes, such as H2d, were better at inhibiting attack by Ly49A⁺ LAK cells than other MHC haplotypes, like H2b, or MHC class I-deficient cells (Figure 8A). To more easily visualize the data, we
converted the percent specific lysis data to percent MHC-specific inhibition, normalizing
to MHC class I-deficient cells (Figure 8B). The relative levels of Ly49A-mediated
effector inhibition by different MHC haplotypes produced the same hierarchy as NK cell
licensing (Figures 2 and 3). These observed levels of lysis were Ly49A dependent,
because Ly49A− LAKs failed to produce a similar pattern of inhibition and addition of a
blocking antibody against Ly49A restored Ly49A+ LAK killing of normally resistant
MHC-congenic target cells (Figure 9).

While the general hierarchy for effector inhibition was similar to that of NK cell
licensing, we also observed some differences from NK cell licensing. For example, the
H2q haplotype robustly inhibited LAK killing yet had only a weak licensing effect.
Similarly, the H2s haplotype, which showed minimal licensing of Ly49A+ NK cells, still
inhibited Ly49A+ LAK killing significantly more than the H2b MHC haplotype. These
apparently different thresholds suggest that NK cell licensing and effector inhibition
utilize separate molecular signaling pathways or, if mediated by the same signaling
cascades, are controlled by differential threshold mechanisms.

A single gene dose of an MHC class I ligand of Ly49A is sufficient for maximal inhibition

To determine whether MHC class I haploinsufficiency affects the ability of a
target cell to inhibit killing by Ly49A+ LAK cells, we studied killing of target cells
expressing either one or two gene doses of H2Dd or the H2k class I alleles. Target cells
hemizygous for a transgene encoding H2Dd, a strong MHC class I ligand of Ly49A,
inhibited killing by Ly49A+ LAKs as well as their homozygous counterparts (Figure
10A). Similarly, target cells hemizygous for the H2k class I genes were as efficient at
inhibiting Ly49A⁺ LAK cell attack as cells homozygous for these genes (Figure 10B). Taken together, these results indicate that effector inhibition, like licensing, is saturated by relatively low MHC class I avidity.

**Discussion**

Ly49A-mediated effector inhibition is more sensitive to MHC class I engagement than is Ly49A-mediated licensing. This finding reveals a fundamental principle in our understanding of the role of NK cell responses to normal cells as well as in pathological contexts such as viral infection and tumor development. Some caution should be exercised in comparing assays that use different output measures (i.e., cytokine production versus cytotoxicity), as they may be regulated in different ways. In addition, it is possible that IL-2 activation affects the stimulation threshold of the NK cells. For example, previous studies have indicated that IL-2 treatment can overcome the hyporesponsive phenotype of unlicensed NK cells (27). However, as cytokine activation of NK cells would be most likely to raise the threshold necessary for inhibition by MHC class I, naïve NK cells may be even more sensitive to MHC class I engagement than the LAK cells studied herein. Furthermore, similar cytokine-rich environments are likely present during the pro-inflammatory phase of initial immune responses. Regardless, these findings reveal an interesting and important relationship between licensing and NK cell effector function.

These results are also consistent with the findings of a recent study investigating the relative contributions of licensed and unlicensed NK cells in the response to MCMV infection (51). In this study, unlicensed cells were found to respond more robustly to
MHC class I-expressing target cells transfected with a ligand for an activating receptor. The licensed cells were prevented from responding because of the inhibitory signals received from MHC class I engagement. In other words, while licensed cells respond potently to MHC class I-deficient stimuli, MHC class I expression by target cells efficiently prevents activation of licensed NK cells. These findings are consistent with our results that even cytokine-stimulated NK cells (i.e., LAK cells) are extremely sensitive to inhibition via MHC class I engagement.

The importance of this dominance of effector inhibition is illustrated by another recent study, in which the inflammatory environment of a viral infection led to rejection of otherwise healthy MHC class I-deficient bone marrow in mixed bone marrow chimeric mice (97). In lethally irradiated mice reconstituted with equal numbers of wild-type and $\beta_2m^{-/-}$ bone marrow, the $\beta_2m^{-/-}$ cells were eradicated within 10 days of MCMV infection. The wild-type bone marrow cells did not decrease in proportion with the $\beta_2m^{-/-}$ bone marrow cells, indicating that inhibitory signals produced by MHC class I engagement of inhibitory NK receptors prevented NK cells activated by the viral infection from killing wild-type bone marrow cells. Thus, in inflammatory contexts, the ability to shut down aberrant NK cell activation is important for maintaining self-tolerance. (The mechanisms by which unlicensed cells, such as cells that do not express self-MHC-specific inhibitory NK receptors, maintain tolerance to self-cells during and after viral infections remain unclear.)

The difference in threshold of NK cell licensing and effector inhibition also has important implications for our understanding of the signaling cascades that mediate these events. While Ly49A-mediated effector inhibition relies mainly upon signaling initiated
by SHP-1, the precise signaling pathway required for licensing has not been identified (14, 98, 99). However, the differences in threshold support the view that different signaling cascades may underlie these two functions of Ly49A. While the ITIM is known to be required for both NK cell licensing and effector inhibition, it is increasingly evident that ITIMs can initiate a number of diverse signaling cascades. For example, recent work has implicated a signaling pathway involving phosphorylation of Crk in signaling by the ITIMs of inhibitory KIRs in human NK cells (53). Clearly, ITIM signaling in NK cells is more complex than simple recruitment of SHP-1.

The hypothesis that NK cell licensing uses a signaling cascade separate from effector function leads to a host of new questions. First, in teleological terms, how does an NK cell know the difference between licensing and effector inhibition? What molecular mechanisms regulate whether a particular Ly49A-MHC class I interaction leads to effector inhibition or licensing? One possibility is that cis versus trans engagement of Ly49A by MHC class I may distinguish licensing interactions from inhibitory stimuli. Cis interactions of Ly49A appear to be required for licensing of Ly49A+ NK cells, as NK cells expressing a Ly49A molecule that could not engage MHC class I in cis remained unlicensed (73). Further studies using a variety of Ly49A mutants indicate that cis engagement and trans engagement induce different structural conformations of the Ly49A molecule, including differences in the stoichiometry of the Ly49A-MHC class I interaction (72). These structural differences may be associated with differences in intracellular signaling cascades. In fact, one group has proposed an entirely new way in which to view licensing by suggesting that unengaged Ly49 receptors mediate a tonic inhibitory signal that is terminated upon cis engagement by MHC class I
The molecular components of this putative ITIM-dependent tonic signal have not been identified.

However, data from a number of studies clearly indicate that cis interactions of Ly49 with MHC class I are not sufficient for NK cell licensing and thus may not be the distinguishing feature of licensing versus effector inhibition mediated by Ly49 receptors. For example, H2D\textsuperscript{d}-expressing NK cells from H2D\textsuperscript{d}-mosaic mice are tolerant to H2D\textsuperscript{d}-negative cells (63). Only when cultured separately for a given amount of time, i.e., in an environment with more abundant trans interactions of Ly49A and MHC class I, do the cells acquire the ability to respond to target cells lacking H2D\textsuperscript{d}. Thus, the question remains of how interactions of Ly49A and MHC class I can mediate both licensing and effector inhibition, which not only have different downstream effects on the NK cell but also feature different thresholds for strength of MHC class I engagement.
Figure 8. Ly49A-mediated inhibition of effector function is more sensitive than NK cell licensing. (A) Aggregate results of three independent $^{51}$Cr-release assays of Ly49A$^+$ LAK cells from H2D$^d$-transgenic K$^{b/-}$ D$^{b/-}$ mice killing Con A-activated MHC-congenic splenocytes. Each condition was tested in triplicate in each experiment. (B) Specific inhibition $\pm$ SEM of killing of MHC-congenic Con A blasts by Ly49A$^+$ LAK cells at an E:T of 8:1. Specific inhibition = 100 x (Specific lysis of K$^{b/-}$ D$^{b/-}$ Con A blasts – Specific lysis of MHC-congenic Con A blasts) / Specific lysis of K$^{b/-}$ D$^{b/-}$ Con A blasts. Aggregate results from three independent experiments in which each condition was assayed in triplicate. *: $p<0.05$; ***: $p<0.001$. 
Figure 9. Ly49A-dependence of inhibition of cytotoxicity by MHC-congenic target cells. (A) Cytotoxicity by Ly49A⁻ LAK cells. Aggregate results of three individual 4-hour $^{51}$Cr-release assays of Ly49A⁻ LAK cells from H2D$^d$-transgenic K$^{b/-}$ D$^{b/-}$ mice killing Con A-activated MHC-congenic splenocytes. Each condition was tested in triplicate in each experiment. (B) JR9 blockade of Ly49A restores killing of otherwise resistant MHC-congenic target cells. Specific lysis of MHC-congenic ConA blasts by Ly49A⁺ LAK cells either untreated or pre-incubated with 20 μg/mL (Experiment 1) or 10 μg/mL (Experiment 2) of anti-Ly49A (mAb JR9) or control (MAR) F(ab')$_2$ fragments, as previously described.(84, 101)Specific lysis of ConA blasts from H2$^b$ mice and K$^{b/-}$ D$^{b/-}$ mice decreased in the presence of anti-Ly49A F(ab')$_2$ fragments, presumably because of inhibitory signals produced by cross-linking Ly49A receptors. The data in each column represent the results of triplicate wells from one experiment.
Cytotoxicity by Ly49A⁺ LAK cells

A

% Specific Lysis

0 10 20 30 40 50 60 70

E:T

2:1 8:1

B

% Specific Lysis

0 10 20 30 40 50 60 70

none F(ab')2

control JR9

H2d - Expt 1

H2d - Expt 2

H2k - Expt 1

H2k - Expt 2

H2r - Expt 1

H2r - Expt 2

H2q - Expt 1

H2q - Expt 2

H2s - Expt 1

H2s - Expt 2

Kb⁻/− Db⁻/− - Expt 1

Kb⁻/− Db⁻/− - Expt 2

Cytotoxicity by Ly49A⁺ LAK cells

2:1 8:1

% Specific Lysis

0 10 20 30 40 50 60 70
Figure 10. Ly49A-mediated inhibition of effector function is not affected by haploinsufficiency of MHC class I expression. (A) Specific lysis of Con A blasts homozygous, hemizygous, or nullizygous for a D^d transgene by Ly49A^+ LAK cells. Representative of two independent experiments. (B) Specific lysis of Con A blasts homozygous, hemizygous, or nullizygous for endogenous H2K^k and H2D^k genes by Ly49A^+ LAK cells. Representative of two independent experiments.
CHAPTER 6

DEVELOPMENT OF MODELS OF
INDUCIBLE NK CELL LICENSING
Introduction

Many questions remain about the kinetics and other determinants of NK cell licensing. How long does it take for an NK cells to become licensed? Can NK cells be licensed as mature cells? What other changes occur in conjunction with licensing, e.g., changes in surface receptor expression, sizes of NK cell subpopulations, or activation states of signal transduction molecules and transcription factors? These questions are difficult to address in wild-type mice for a number of reasons. First, licensing of NK cell populations in wild-type mice is unsynchronized. Second, since there are no known molecular markers of NK cells actively undergoing licensing, we cannot selectively focus on the relevant NK cell populations, even using methods such as flow cytometry and microscopy. In mice with static NK cell populations, we can at best compare NK cells from mice with normal MHC class I expression (e.g. B6 mice) and mice lacking MHC class I expression (e.g. β2m−/− mice). However, such comparisons are complicated by the potential of other effects caused by the genetic deficiency of the MHC class I-deficient model. For example, β2m-deficiency also affects expression and function of a number of MHC class Ib molecules, which have important functions both within and beyond the immune system (102). All MHC class I-deficient mice will also differ from wild-type mice in that they have defective CD8 T cell development, which may indirectly affect NK cells.

By creating a mouse model with inducible expression of MHC class I, we hope to overcome many of these problems. Because the initiation of MHC class I expression is exogenously controlled, licensing of NK cells will be synchronized. This provides easy access to bulk NK cell populations that are in the midst of the molecular events that
comprise NK cell licensing. A system of synchronized licensing will also allow us to study whether mature NK cells are as capable of being licensed as developing NK cells. Lastly, because all NK cells develop in the same MHC class I-deficient environment prior to MHC class I expression, a system of inducible licensing provides well-matched controls, including both NK cells that lack inhibitory NK receptors for the expressed MHC molecule and NK cells that express a cognate inhibitory NK receptor but exist in mice in which MHC class I expression was not induced.

Results

Administering peptide to TAP1-deficient mice to induce NK cell licensing

Our first design for a system of inducible licensing was based on models used to study T cell biology. In their studies of T cell selection and activation, Martin and Bevan used three consecutive daily intraperitoneal (ip) injections of 100 nmol of peptide into TAP-deficient, TCR-transgenic mice to induce stabilization of a specific peptide-MHC class I molecule (103). Since TAP1-deficient mice have unlicensed NK cells due to their very low surface expression of MHC class I (25), we hypothesized that peptide-stabilization of MHC class I could induce licensing of NK cells (Figure 11). To test this hypothesis, we gave TAP1<sup>−/−</sup> mice daily ip injections of 100 nmol of one of two H-2K<sup>b</sup>-specific peptides or an irrelevant peptide, respectively, for three days. However, no NK cell licensing was observed (data not shown).

One concern in these experiments was that the peptide bolus was filtered out of the bloodstream too quickly to have more than a very transient effect on class I surface expression. A longer duration of peptide treatment might also be helpful in case NK cell
licensing takes longer than three days. To address these issues, subcutaneous osmotic pumps were surgically implanted to deliver peptide at a constant rate of 20 nmol per hour (480 nmol per day) for 14 days. Again, the NK cells remained unlicensed (Figure 12A). However, there was also no detectible increase in H2Kb in the peptide-treated mice difference compared with mice that received PBS vehicle alone, raising the possibility that the peptide was simply not stabilizing MHC class I (Figure 12B). Alterations in T cell populations did not provide internal controls, as T cells in TAP1−/− mice were not affected by peptide treatment, presumably because specific peptide-MHC-reactive T cells are too rare in a non-TCR tg setting (data not shown).

In order to use thymocyte deletion and peripheral T cell expansion as internal controls for effective peptide delivery and functional stabilization of MHC class I, we crossed TAP1−/− mice to the N15 TCR transgenic line. Eight consecutive daily ip injections of 400 nmol of the N15 TCR agonist peptide VSV-8 (RGYVYQGL) into N15 TCR tg TAP1−/− mice produced the expected changes to the T cell compartments (Figure 13). Specifically, there was a reduction in the size of the double-positive thymocyte population, indicating deletion of peptide-reactive developing T cells, and an expansion of the splenic CD8 T cell compartment. Consistent with our earlier findings, these changes did not take place in TAP1−/− littermates that lacked the N15 TCR tg but were treated in parallel with the same dose and batch of peptide.

Surprisingly, splenocytes from N15 TCR tg TAP1+/+ mice, but not non-TCR tg TAP1−/− littermates, exhibited a low but detectible level of H2Kb, perhaps as a result of cytokine release secondary to CD8 T cell activation (Figure 14A). However, the NK cells remained unlicensed, even in the presence of mildly elevated H2Kb, because NK cells
from mice treated with VSV peptide did not respond more robustly to stimulation through NK1.1 than did NK cells from TAP1−/− mice that did not receive peptide (Figure 14B).

It is plausible that the extent of class I stabilization in this system is not adequate to provide a licensing stimulus to NK cells. Only in some experiments were we even able to detect the increase by flow cytometry, and the greatest change, in N15 TCR tg TAP1−/− mice, only produced detectible expression on less than 10% of cells (Figure 14A). While this was enough for changes in T cell populations, it did not affect NK cell licensing. NK cells clearly require a higher level of MHC class I than TCR-tg T cells do to produce detectible changes. Supporting this hypothesis, studies of mice with mosaic expression of MHC class I transgenes indicate that NK cells remain tolerant to MHC class I-deficient cells even when such cells represent only 20% of cells (63). NK cell licensing is thus likely to require MHC class I surface expression on the vast majority of cells. Peptide administration to TAP1−/− mice was not able to reach this threshold level of surface class I, even at high doses.

*Design of a Tet-On system of MHC class I expression*

To achieve more robust inducible MHC class I expression, we designed a “Tet-on” transgenic mouse model for pharmacological induction of expression of a single MHC class I molecule (Figure 15). In the absence of doxycycline, the mice will lack all MHC class I molecules. Upon doxycycline treatment, a single MHC class I molecule is upregulated, leading to licensing of a single NK cell subset. The Tet-On system requires two transgenes (Figure 16). One transgene is composed of the H2Kb promoter and IgH
enhancer driving expression of rtTA, a doxycycline-responsive transactivator protein. The H2K\textsuperscript{b} promoter was selected in order to provide a physiological expression pattern for our inducible MHC class I molecule. A later iteration of this model uses a mouse with rtTA knocked in to the Rosa locus (discussed further below). The other transgene encodes the H-2K\textsuperscript{b} class I molecule as a single chain trimer (SCT) driven by a tetracycline response element (TRE-tight). The rtTA transactivator and TRE-tight both feature enhancements over early Tet-on systems to minimize leakiness of transgene expression in the absence of doxycycline (104, 105).

The single chain trimer selected for this system consists of the class I molecule H-2K\textsuperscript{b} with murine \(\beta_2m\) and the ovalbumin-derived SIINFEKL peptide. Previous studies have shown that a constitutively expressed K\textsuperscript{b}-ova SCT transgene can license Ly49C\textsuperscript{+} NK cells (27). In fact, on an MHC class I-deficient background, the K\textsuperscript{b}-ova SCT – Ly49C interaction is the only licensing stimulus present (27). This low background licensing level will permit detection of subtle changes in the Ly49C\textsuperscript{+} NK cell population. The SCT design chosen for this particular project is the “third-generation” SCT, which features two amino acid substitutions that increase its stability relative to the form used in the earlier transgenic strain (106). One substitution relieves structural constraints of one of the linkers. The other enables a disulfide bond to connect the peptide-\(\beta_2m\) linker to the H-2K\textsuperscript{b} heavy chain, thereby preventing the peptide from slipping out of the peptide-binding groove. Improving the stability of SIINFEKL in the peptide binding groove may enhance the ability of the SCT to interact with Ly49C, which has been shown to be somewhat peptide-selective (107).
Verification of binding of the third-generation SCT to Ly49C

The ability of this third-generation SCT (SCT III) to bind Ly49C has not been previously verified. To do this, we produced a soluble form of SCT III and multimerized it with APC-conjugated or unlabeled streptavidin. We then assessed binding of the SCT III tetramers to CHO cells stably expressing Ly49C. For comparison, we used conventional K<sup>b</sup>-ova tetramers and first-generation K<sup>b</sup>-ova SCT tetramers (SCT I), both kindly provided by Steven Truscott of the Hansen lab. The third-generation SCT displayed reactivity similar to the first-generation SCT, though neither of these tetramers bound with the high reactivity of the conventional K<sup>b</sup>-ova tetramers (Figure 17A). In blocking experiments in which unlabeled and labeled tetramers were added simultaneously, SCT III blocked all three types of tetramer in a dose-dependent fashion (Figure 17B). Together, these results indicate that while the third-generation SCT does not bind Ly49C as well as the conventional K<sup>b</sup>-ova class I molecule, it binds approximately as well as the first-generation SCT, which has been shown to induce licensing of Ly49C<sup>+</sup> NK cells in vivo (27).

Production and screening of founders of SCT and rtTA transgenic mouse lines

The construct for the SCT transgene was produced from a cDNA construct of SCT III and a genomic construct of wild-type H2K<sup>b</sup> and cloned into the pTRE-Tight plasmid containing a modified tetracycline response element. The rtTA transgene was produced by replacing the P<sub>CMV</sub> promoter of the pTet-On-Advanced plasmid encoding the next-generation Tet-On transactivator rtTAs-M2 with a H2K<sup>b</sup> promoter. An IgH enhancer element was inserted at the 3’ end of the rtTA sequence. Linearized rtTA and SCT
constructs were submitted to the Transgenic Core facility for injection into fertilized C57BL/6 mouse embryos. The resulting mice were screened for the presence of the transgene by PCR. Number of insertion sites and copy number were assessed by Southern blots. The studies described herein use rtTA rounder line #2 and SCT founder line #26, which each carry fewer than five repeats of their respective transgene (Figure 18).

Characterization of SCT expression in SCT tg rtTA tg β2m+/– mice

After separately backcrossing the SCT tg and rtTA tg lines to a β2m+/– background, we bred the transgenic mice together to produce rtTA tg SCT tg β2m+/– offspring. Kb-ova expression was detected as early as 48 hours after administration of 2 mg/mL doxycycline in the drinking water (Figure 19A). However, Kb-ova was only expressed on a portion of the cells in the blood and spleen, even after several weeks of doxycycline treatment (Figure 19B). There was some variability between individual mice, but over a series of experiments, Kb-ova was expressed on approximately 10-15% of splenocytes, with similar expression levels on T cells and B cells. Surprisingly, expression on NK cells was consistently higher than on the other cell types, usually in the range of 20-30%. Among NK cells, Kb-ova surface expression was higher on Ly49C+ NK cells than on Ly49C– NK cells, although even Ly49C– NK cells had a higher frequency of Kb-ova+ cells than non-NK cell types (Figure 19C). The preference for Kb-ova expression on Ly49C+ NK cells is likely due to MHC class I transfer via trogocytosis mediated by specific interactions of Ly49C and Kb-ova. Trogocytosis-mediated transfer of MHC class I molecules has been reported for inhibitory Ly49 receptors and their wild-type cognate MHC class I receptors (94, 95).
We would prefer to have a system with more robust K\textsuperscript{b}-ova SCT expression. We have therefore tested different methods to improve SCT expression in the rtTA tg SCT tg mice, such as switching from 5% sucrose in the drinking water to 4% grape KoolAid, to better mask the bitter taste of doxycycline. We have also tested the efficacy of intraperitoneal injections of doxycycline, which produce modestly higher frequencies of K\textsuperscript{b}-ova SCT expression. Unfortunately, none of these approaches have produced frequencies of K\textsuperscript{b}-ova expression above 50% among NK cells and 20% among bulk splenocytes in the rtTA tg SCT tg β\textsubscript{2}m\textsuperscript{-} mice.

\textit{NK cells in doxycycline-treated rtTA tg SCT tg mice are unlicensed}

Since no molecular marker of licensing has been defined, we assess licensing through \textit{ex vivo} activation receptor (NK1.1) cross-linking of NK cells. Subsequent intracellular cytokine staining and flow cytometry allow calculations of IFN\textsubscript{γ} production frequencies by specific NK cell subpopulations, such as Ly49C\textsuperscript{+} NK cells. There are two ways to measure licensing using these data: (1) the absolute frequency of IFN\textsubscript{γ} production by Ly49C\textsuperscript{+} NK cells in mice that do or do not express an MHC ligand, and (2) the “licensing ratio” of IFN\textsubscript{γ} production frequencies by Ly49C\textsuperscript{+} NK cells versus Ly49C\textsuperscript{-} NK cells in the same animal. Previous studies demonstrate that mice that express a constitutive K\textsuperscript{b}-ova SCT transgene on an otherwise MHC class I-deficient background have Ly49C\textsuperscript{+} NK cells that are licensed according to both measures (27).

In our inducible K\textsuperscript{b}-ova SCT mice, Ly49C\textsuperscript{+} NK cells from rtTA tg\textsuperscript{+} SCT tg\textsuperscript{+} β\textsubscript{2}m\textsuperscript{-} mice treated with doxycycline do not exhibit an increased absolute frequency of IFN\textsubscript{γ} production by Ly49C\textsuperscript{+} NK cells compared to single-tg littermate controls, but they
do have an elevated licensing ratio (Figure 20). These results appear incongruous, as different measures of licensing seem to lead to opposing conclusions. However, we believe they may be a consequence of the mosaic K⁰-ova SCT expression pattern. Since only a minority of cells expresses K⁰-ova SCT, licensing of Ly49C⁺ NK cells is generally suppressed, consistent with previous reports of mice with constitutive mosaic expression of an MHC class I molecule (63). However, the few cells that do respond to stimulation show evidence of a productive interaction through Ly49C, as Ly49C⁺ NK cells are more likely to respond than Ly49C⁻ NK cells. Interestingly, neither frequency of IFNγ production nor the licensing ratio differed significantly between the K⁰-ova⁺ and K⁰-ova⁻ Ly49C⁺ NK cell subsets (Figure 21). This result argues against a role for cis binding in NK cell licensing. However, since no NK cell subset was robustly licensed (i.e., had a high frequency of IFNγ production in addition to an elevated licensing ratio), it is difficult to make any firm conclusions based on this system.

License of NK cells treated with doxycycline in vitro

We have recently begun investigating whether NK cells can become licensed during culture in vitro. To do this, naïve rtTA tg⁺ SCTtg⁺ β₂m⁻/- nylon wool non-adherent splenocytes were cultured in media containing doxycycline as well as high doses of IL-2. A fraction of the LAK cells produced by these culture conditions expressed K⁰-ova SCT, mimicking the mosaic expression pattern observed in vivo. These cells were isolated by panning with anti-K⁰-ova antibodies and cultured for an additional two days before being used as effector cells in ⁵¹Cr-release cytotoxicity assays against MHC class I-deficient target cells.
Unfortunately, the panning technique does not work as well for K\(^b\)-ova\(^+\) cell enrichment as it does for Ly49A\(^+\) cells. In two experiments, the frequencies of K\(^b\)-ova SCT expression in the positive fractions were 20% and 54%, respectively (Figure 22A and data not shown), considerably lower than the 80-90% purity obtained for Ly49A\(^+\) NK cells (data not shown). However, despite these low purities, LAK cells enriched for K\(^b\)-ova SCT expression were more potent at killing β\(_2\)m\(^{−/−}\) ConA blast target cells than the corresponding K\(^b\)-ova-depleted LAK cell fraction (Figure 22B). In the experiment with better K\(^b\)-ova purity, the specific lysis of β\(_2\)m\(^{−/−}\) ConA blasts was nearly as high as for B6 LAKs. The observed killing was due to missing-self detection because the K\(^b\)-ova\(^+\) cells did not kill B6 ConA blasts (Figure 22C). The K\(^b\)-ova-depleted LAK cell fraction killed β\(_2\)m\(^{−/−}\) ConA blasts at the same low level as the control single-transgenic β\(_2\)m\(^{−/−}\) LAKs, indicating that only NK cells that express K\(^b\)-ova SCT or are in environments rich in K\(^b\)-ova expression become licensed \textit{in vitro}.

\textit{Expression of K\(^b\)-ova SCT in SCT tg Rosa-rtTA knock-in mice}

The Rosa-rtTA strain is a knock-in mouse on a C57BL/6 background that carries a next-generation rtTA gene in the Rosa locus. This knock-in mouse has been shown to drive expression of target genes in many tissues, including spleen, bone marrow, and thymus (108). To see if this rtTA gene could rescue the mosaic cellular expression profiles of our rtTA mice, we crossed the Rosa-rtTA mice with our SCT tg β\(_2\)m\(^{−/−}\) mice. The resulting Rosa-rtTA\(^+\) SCT tg β\(_2\)m\(^{−/−}\) mice are not useful for studying licensing since they already express endogenous MHC class I. However, a peptide-in-groove antibody
highly specific for H2K\textsuperscript{b} presenting the SIINFEKL (ova) peptide allowed us to detect K\textsuperscript{b}-ova expression in these mice in spite of the presence of background H2K\textsuperscript{b}.

When Rosa-rtTA\textsuperscript{+} SCT tg β2m\textsuperscript{+/−} mice were treated with ip injections of 3 mg doxycycline every other day for seven days (four injections), nearly all splenocytes expressed K\textsuperscript{b}-ova (Figure 23A). This high frequency of expression was observed in two independent experiments, and followed a unimodal pattern. Expression was robust on bulk splenocytes as well as on individual cell populations of NK cells and T cells, though NK cells expressed K\textsuperscript{b}-ova at a higher level than T cells (Figure 23A). Interestingly, the Rosa-rtTA\textsuperscript{+} SCT tg β2m\textsuperscript{+/−} mice, but not single-transgenic control mice, exhibited increased expression of CD69, an activation marker (Figure 23B). The cells expressing CD69 were mainly CD8-negative T cells. The cause of this activation is not yet clear. However, this issue will be investigated in future Rosa-rtTA\textsuperscript{+} SCT tg β2m\textsuperscript{+/−} mice, as general immune activation and inflammation suggested by CD69 expression on T cells could affect NK cell function and thus interfere with our licensing studies.

**Discussion**

A functional system of inducible NK cell licensing would allow us to ask a number of questions that are difficult to address in wild-type systems. Beyond the basic question of kinetics – how long does licensing of an NK cell take? – a system of inducible licensing can also be used to investigate the molecular events surrounding NK cell licensing. For example, what changes in gene expression occur in conjunction with NK cell licensing? Are certain signaling molecules or transcription factors upregulated or activated in licensed (or unlicensed) cells? Through the use of bone marrow chimeras,
we can also assess the relative contributions of hematopoietic and non-hematopoietic cell types to NK cell licensing in a cleaner context than previous studies. Experiments can even be designed to test whether NK cells can be licensed ex vivo, in the absence of any stromal environment.

The benefits and opportunities of a system of inducible NK cell licensing are substantial, but so are the challenges in designing such a system. Because NK cell licensing requires MHC class I expression, our designs focused on regulating MHC class I surface expression. However, our first two attempts at producing MHC class I-dependent systems of inducible NK cell licensing failed. The first, which used peptide administration to stabilize H2Kb in TAP1-deficient mice, produced H2Kb levels that were, at best, barely detectible. These levels were sufficient for changes in TCR tg CD8 T cell populations but were inadequate for NK cell licensing. The second, a Tet-On transgenic system of expression of a single chain trimer form of MHC class I, produced mice with mosaic expression of the Kb-ova peptide-MHC. While as many as 40% of NK cells expressed Kb-ova upon treatment of the mice with doxycycline, the licensing state of these NK cells was too ambiguous to be informative.

Fortunately, the replacement of one of the two transgenes of the Tet-On system yielded mice with reproducible expression of the Kb-ova molecule on virtually all splenocytes. Upon ip administration of doxycycline every other day for a period of seven days, Rosa-rtTA+ SCT tg β2m+/- splenocytes showed nearly uniform expression of Kb-ova. Curiously, an earlier experiment in which Rosa-rtTA+ SCT tg β2m+/- mice were given 2 mg/mL doxycycline in 4% KoolAid for eight days did not produce this level of expression. In that experiment, the two Rosa-rtTA+ SCT tg β2m+/- mice exhibited Kb-ova
expression on 10 and 35% of splenocytes, respectively (data not shown). Thus, it seems that oral doxycycline does not achieve the doses necessary for full expression of the SCT transgene. It should also be noted that ip injections of 3 mg of doxycycline either every day for three days or every other day for eight days failed to produce universal Kb-ova expression in mice carrying our in-house rtTA tg with the SCT tg, suggesting that the rtTA tg has some inherent defect that affects expression of Kb-ova. In sum, while more breeding is necessary to generate Rosa-rtTA+ SCT tg mice on a β2m−/− background, expression experiments using Rosa-rtTA+ SCT tg β2m+/− mice appear extremely promising for future studies of NK cell licensing.

In the meantime, it is useful to consider the reasons why the two initial systems failed to induce full NK cell licensing. Only a minority of TAP1-deficient mice that received injections of peptide exhibited detectible stabilization of H2Kb, and even then, the levels of expression were very low. This small increase in H2Kb was enough to induce significant changes in the T cell compartments, as peptide-MHC-specific TCR tg T cells were deleted in the thymus and expanded in the periphery. These changes reiterate how exquisitely sensitive T cells are to their cognate peptide-MHC ligand. Notably, non-TCR tg T cells were not affected by peptide administration, presumably because T cells specific for the particular Kb-peptide molecule were too rare to produce detectible changes in thymic or splenic T cell compartments within the short time frame of the experiment. A recent study by the Hansen lab has demonstrated that mice that constitutively express a single type of Kb-peptide molecule can select a diverse CD8 T cell population (109). However, the CD8 T cell compartment in these mice was smaller than in B6 wild-type mice, indicating that the thymic selection process is less efficient in
animals with severely restricted peptide-MHC expression. This reduced rate of development of T cells by a single K\textsuperscript{b}-peptide molecule may explain why peptide injections into TAP1\textsuperscript{-/-} mice did not produce detectible changes in the T cell compartments in the course of less than two weeks.

Unlike T cells, NK cells are not polyclonal, so expression of H2K\textsuperscript{b} above the threshold necessary for licensing should produce significant changes in the Ly49C\textsuperscript{+} NK cell subset that binds K\textsuperscript{b}-ova. However, licensing of Ly49C\textsuperscript{+} NK cells was not observed in any of the peptide administration experiments, indicating that the barely detectable levels of K\textsuperscript{b} stabilization produced by these models is below the level required for NK cell licensing. Further studies are needed to verify this conclusion, as it is possible that H2K\textsuperscript{b} was stabilized in a mosaic fashion, which is known to impair NK cell licensing (63).

Our second design used rtTA and SCT double-transgenic mice for doxycycline-inducible expression of K\textsuperscript{b}-ova single-chain trimer. However, mice carrying the SCT with our MHC promoter-driven rtTA tg exhibited mosaic K\textsuperscript{b}-ova expression, not the intended universal expression. Only approximately 15% of splenocytes and 30% of NK cells expressed K\textsuperscript{b}-ova in these mice despite attempts to improve these levels. Interestingly, K\textsuperscript{b}-ova expression was not classically bimodal, as K\textsuperscript{b}-ova\textsuperscript{+} cells exhibited a range of K\textsuperscript{b}-ova expression levels. Regardless, NK cells in these mice were not fully licensed: While there was evidence of a productive Ly49C-K\textsuperscript{b}-ova interaction in the elevated licensing ratio, Ly49C\textsuperscript{+} NK cells failed to make IFN\gamma at a higher frequency than Ly49C\textsuperscript{+} NK cells from MHC class I-deficient control mice.
The finding that mice with induced mosaic expression of K\textsuperscript{b}-ova do not have licensed NK cells is not surprising, as previous studies of mice with constitutive mosaic expression of MHC class I did not exhibit a phenotype consistent with licensing. The DL6-transgenic mouse line expresses a fusion protein composed of the α1 and α2 domains of H2D\textsuperscript{d} and the α3 domain of H2L\textsuperscript{d} in a mosaic fashion on 10-80% of splenocytes, including the NK cell compartment (63). DL6 mice, which have normal expression of H2K\textsuperscript{b} and H2D\textsuperscript{b}, are tolerant to both B6 BM (i.e. lacking H2D\textsuperscript{d}) and H2D\textsuperscript{d}-transgenic B6 BM, although they can reject β2m\textsuperscript{−/−} BM with normal kinetics. Similar results were obtained in two H2D\textsuperscript{d}-mosaic mouse models created using Cre-lox systems (60). However, administration of type I interferon inducers or estrogen receptor antagonists were required to induce mosaicism, and these agents may affect licensing. Notably, tolerance to H2D\textsuperscript{d}-deficient cells was also observed in vitro, but this tolerance of DL6 NK cells to B6 lymphoblasts was reversed by separating H2D\textsuperscript{d+} and H2D\textsuperscript{d−} NK cells for 24 hours after incubation together (or apart) in IL-2 for four days (63). This result suggests that continued exposure to tolerizing cells is needed to maintain tolerance.

Encouragingly, Rosa-rtTA\textsuperscript{+} SCT tg mice exhibit a unimodal elevation of K\textsuperscript{b}-ova expression upon treatment with doxycycline. Breeding is underway to obtain Rosa-rtTA\textsuperscript{+} SCT tg mice on a β2m\textsuperscript{−/−} background to assess licensing of Ly49C\textsuperscript{+} NK cells by induction of K\textsuperscript{b}-ova expression in vivo. After the principle of inducible NK cell licensing is established in these mice, the parameters of K\textsuperscript{b}-ova expression (e.g., surface levels and time span) can be manipulated through changes in the doses and duration of doxycycline treatment. These mice are also promising tools for studies of NK cell licensing in vitro, as the problematic step of separating K\textsuperscript{b}-ova\textsuperscript{+} and K\textsuperscript{b}-ova\textsuperscript{−} NK cells would be unnecessary.
Such studies may help us identify the minimal cellular and molecular components necessary for NK cell licensing. We look forward with great anticipation to the many studies planned for this new mouse model.
**Figure 11. Schematic of peptide-induced licensing of NK cells in TAPI⁻/⁻ mice.**

TAPI⁻/⁻ have low surface expression of MHC class I due to defective peptide loading. Consequently, NK cells in TAPI⁻/⁻ mice are unlicensed. Exogenous peptide can increase MHC class I surface expression by binding to and stabilizing MHC class I molecules found on the cell surface. Stabilization of H2Kᵇ by specific peptides may lead to licensing of Ly49C⁺ NK cells.
Peptide

TAP1/–

Increased surface H-2Kb

Licensed?

Cis

Trans

NK cell

NK cell

Unlicensed

Low surface MHC class I

Ly49C

Peptide
Figure 12. Administration of an H2K<sup>b</sup>-specific peptide does not lead to licensing of Ly49C<sup>c</sup> NK cells or to detectible H2K<sup>b</sup> surface expression. (A) Osmotic pumps containing the K<sup>b</sup>-specific peptide VNVDYSKL, an irrelevant peptide VNVDASKA, or PBS were implanted into TAP1<sup>−/−</sup> mice. VNVDYSKL peptide was administered at a rate of 20 nmol per hour (480 nmol per day) for 14 days. At the end of this period, splenocytes were isolated and stimulated with a 1:3000 dilution of PK136 (anti-NK1.1) ascites, and IFN<sub>γ</sub> production was assessed by intracellular flow cytometry. Dot plots are gated on NK cells (NK1.1<sup>+</sup> CD3<sup>−</sup> CD19<sup>−</sup>). Percentages indicate the frequency of IFN<sub>γ</sub> production by Ly49C<sup>+</sup> NK cells. Representative results from one of three mice in each group are shown. Results from peptide-naïve B6 and TAP1<sup>−/−</sup> mice from the same NK1.1 stimulation experiment are shown for reference. (B) Naïve splenocytes were stained for H2K<sup>b</sup> to assess peptide stabilization of MHC class I. Each histogram represents one mouse. N=3 for VNVDYSKL and PBS. N=1 for peptide-naïve B6 and TAP1<sup>−/−</sup> controls.
A

<table>
<thead>
<tr>
<th></th>
<th>VNVDYSKL</th>
<th>VNVDASKA</th>
<th>PBS</th>
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<tbody>
<tr>
<td>Ly49C</td>
<td>1.2%</td>
<td>1.1%</td>
<td>0.7%</td>
</tr>
<tr>
<td>IFNγ</td>
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Naïve B6 control  Naïve TAP1−/− control

B

- **VNVDYSKL** (n=3)
- **PBS** (n=3)
- **Naïve B6**
- **Naïve TAP1−/−**

![Graph showing H2Kb distribution](image)
Figure 13. VSV peptide produces detectible changes in the T cell populations of N15 TCR tg TAP1+/− mice. N15 TCR tg TAP1+/− and non-tg TAP1−/− mice were given daily intraperitoneal injections of 400 nmol (382 µg) VSV-8 peptide (RGVYQGL) or PBS for eight days. On day nine, (A) thymocytes and (B) splenocytes were harvested and stained for CD4 and CD8. Dot plots are representative of two mice per group.
Figure 14. VSV-8 peptide treatment produces low levels of H2K<sup>b</sup> stabilization but does not induce licensing of NK cells. (A) N15 TCR tg TAP1<sup>+</sup> and non-tg TAP1<sup>−/−</sup> mice were given daily intraperitoneal injections of 400 nmol (382 µg) VSV-8 peptide (RGYVYQGL) or PBS for eight days. On day nine, splenocytes were harvested and stained for H2K<sup>b</sup>. Each histogram represents one mouse. Numbers listed to the right of the histogram indicate the H2K<sup>b</sup> MFI values for each mouse in each group. (B) Splenocytes were stimulated with 5 µg PK136 (anti-NK1.1) and assessed for IFNγ production by intracellular flow cytometry. Cells shown are gated on NK cells (NK1.1<sup>+</sup> CD3<sup>−</sup> CD19<sup>−</sup>). The percentage in each plot indicates the frequency of IFNγ production by Ly49C<sup>+</sup> NK cells.
A

![Graph showing H2Kb expression](image)

- H2b control: 30350
- N15+ peptide: 7265, 5389
- N15- peptide: 3540, 4449
- N15+ PBS: 4469, 4449
- N15- PBS: 3144, 4705
- Unstained: 1170

B

**N15 TCR** tg TAP1−/−

- VSV peptide: 1.6%
- PBS: 1.3%
- H2Kb control: 21.7%

**Non-tg TAP1−/−**

- VSV peptide: 2.9%
- PBS: 2.1%
- H2Kb control: 21.7%

**Fluorescence-Activated Cell Sorting (FACS) plots**

- Ly49C vs. IFNγ
Figure 15. Schematic of doxycycline-induced expression of K\textsuperscript{b}-ova single-chain trimer (SCT) to license Ly49C\textsuperscript{+} NK cells. In the absence of doxycycline, these mice lack all MHC class I molecules due to β\textsubscript{2}m-deficiency. Consequently, NK cells are unlicensed. Upon administration of doxycycline, K\textsuperscript{b}-ova SCT expression is initiated, potentially leading to licensing of Ly49C\textsuperscript{+} NK cells. Manipulating the dose and duration of doxycycline treatment in this inducible system may provide information regarding the kinetics of NK cell licensing.
Doxycycline

rtTA tg
K\(^b\)-ova SCT tg
\(-2\)m\(^{-}\)

Dose? Duration?

Ly49C

NK cell

Unlicensed

No surface MHC class I

Licensed?

Expression of K\(^b\)-ova SCT

NK cell

Cis

Trans
**Figure 16. Schematic of rtTA and SCT gene functions.** The “Tet-On” system is composed of two genes: rtTA, a doxycycline-activated transcriptional activator, and K\textsuperscript{b}-ova, a third-generation MHC class I single chain trimer (SCT) composed of H2K\textsuperscript{b}, β\textsubscript{2}m, and the ovalbumin peptide SIINFEKL (designed by the Hansen Lab, Washington University in St. Louis). We produced transgenic mice that carry an rtTA gene under control of an MHC class I promoter. We have also obtained mice in which the rtTA gene has been knocked in to the Rosa locus. Both mice should produce wide tissue expression of rtTA. The K\textsuperscript{b}-ova transgene is under control of a tetracycline response element (TRE), to which rtTA binds in the presence of doxycycline. (A) In the absence of doxycycline, rtTA is expressed but is inactive and does not bind to the TRE. Consequently, the K\textsuperscript{b}-ova SCT gene is not expressed. (B) Upon treatment with doxycycline, rtTA is activated, binds to the TRE, and induces expression of the K\textsuperscript{b}-ova SCT.
MHC I promoter

rtTA-Advanced

No doxycycline

rtTA

rtTA

Kb-ova SCT III tg

No MHC Class I

Doxycycline

rtTA

rtTA

rtTA

MHC Class I as SCT
Figure 17. Third-generation K\textsuperscript{b}-ova SCT tetramer binds to Ly49C as well as first-generation K\textsuperscript{b}-ova SCT tetramer but not as well as conventional H2K\textsuperscript{b}-ova tetramers. (A) 4.4 x 10\textsuperscript{5} CHO cells stably transfected with Ly49C were stained with the indicated final concentration of labeled conventional H2K\textsuperscript{b}-ova tetramers, first-generation K\textsuperscript{b}-ova SCT tetramers, or third-generation K\textsuperscript{b}-ova SCT tetramers. H2D\textsuperscript{d} tetramers were used as a negative control. (B) Unlabelled third-generation K\textsuperscript{b}-ova SCT tetramers were added simultaneously with the indicated labeled tetramer to 5x10\textsuperscript{5} CHO cells stably transfected with Ly49C. A final concentration of 8.8 nM of labeled tetramer were used in each condition with variable amounts of unlabeled tetramer. Results were normalized to the MFI of unblocked tetramer staining for each tetramer.
A

![Graph A](image)

B

![Graph B](image)
Figure 18. Southern blots of SCT tg and rtTA tg founders. (A) Conventional Southern blot of genomic tail DNA (4 µg) from each of the indicated SCT tg founders digested with XbaI, transferred to a membrane, and hybridized with a SCT-specific probe. Film was exposed for 6 hours. (B) Conventional Southern blot of genomic tail DNA (4 µg) from each of the indicated rtTA tg founders digested with XhoI and BamHI, transferred to a membrane, and hybridized with a rtTA-specific probe. Film was exposed for two days.
Figure 19. Expression of K\textsuperscript{b}-ova SCT on rtTA tg SCT tg \(\beta_2m^{+/-}\) mice. (A) K\textsuperscript{b}-ova expression was detected as soon as 48 hours after administration of 2 mg/mL of doxycycline in drinking water. Bulk splenocytes, T cells (CD\textsuperscript{3+} NK1.1\textsuperscript{+}), and NK cells (NK1.1\textsuperscript{+} CD\textsuperscript{3-}) are shown. Percentages of cells expressing K\textsuperscript{b}-ova are indicated. Each histogram represents one mouse. Red and blue histograms represent rtTA tg SCT tg \(\beta_2m^{+/-}\) mice. Green and orange histograms represent rtTA tg \(\beta_2m^{+/-}\) littermate controls. (B) K\textsuperscript{b}-ova expression of bulk splenocytes, T cells, NK cells, and B cells after 16 days of treatment with 2 mg/mL doxycycline in the drinking water. Red and blue histograms represent rtTA tg SCT tg \(\beta_2m^{+/-}\) mice. Green and orange histograms represent SCT tg \(\beta_2m^{+/-}\) littermate controls. (C) K\textsuperscript{b}-ova expression on Ly49\textsuperscript{+} and Ly49\textsuperscript{-} cells from a representative rtTA tg SCT tg \(\beta_2m^{+/-}\) mouse (red and pink, respectively) and rtTA tg \(\beta_2m^{+/-}\) mouse (dark green and light green, respectively) after 48 hours of 2 mg/mL doxycycline in drinking water.
Figure 20. Ly49C⁺ NK cells in mice with induced mosaic expression of Kᵇ-ova SCT have a low frequency of IFNγ production but an elevated licensing ratio. Mice were treated for 16 days with 2 mg/mL of doxycycline in the drinking water, except one rtTA tg SCT tg β₂mᵪ mouse, which received just water (“no doxy”). Splenocytes were then harvested and stimulated with plate-bound PK136, and IFNγ production was assessed by flow cytometry. (A) Representative flow cytometry plots of IFNγ production by NK cells (NK1.1⁺ CD3⁻ CD19⁻). (B) Frequency of IFNγ production by Ly49C⁺ NK cells. Each symbol represents one mouse, pooled from two independent experiments. The single-tg group includes both a rtTA tg β₂mᵪ⁻ and SCT tg β₂mᵪ⁻ littermate controls. The non-functional transgene group includes mice from rtTA founder lines that do not produce Kᵇ-ova SCT expression upon doxycycline treatment. (C) The licensing ratio is the frequency of IFNγ production by Ly49C⁺ NK cells divided by the frequency of IFNγ production by Ly49C⁻ NK cells. Data are taken from the same samples shown in (B). *: p<0.05.
A

B6

rTA tg SCT tg

SCT tg only

Ly49C

IFNγ

10^2

10^4

10^6

10^8

10^10

10^12

22.3%

5.27%

5.48%

12.0%

3.61%

5.55%

1.86

1.46

0.99

B

%IFNγ+ among Ly49C+ NK cells

0

5

10

15

20

25

n.s.

B6

SCT+ rTA+

No doxy

Single tg

Non-rTA tgs

C

Licensing ratio

0

1

2

B6

SCT+ rTA+

No doxy

Single tg

Non-rTA tgs
Figure 21. Ly49C$^+$ NK cells that express K$^b$-ova do not have a stronger licensing phenotype than Ly49C$^+$ NK cells that do not exhibit surface expression of K$^b$-ova. Mice were treated for 16 days with 2 mg/mL of doxycycline in the drinking water. Splenocytes were then harvested and stimulated with plate-bound PK136, and IFN$\gamma$ production was assessed by flow cytometry. (A) Representative gating of K$^b$-ova$^+$ and K$^b$-ova$^-$ NK cells in a rtTA tg SCT tg $\beta_{2m}^-$ mouse. (B) Frequency of IFN$\gamma$ production by Ly49C$^+$ NK cells (NK1.1$^+$ CD3$^+$ CD19$^-$). Each symbol represents one mouse, pooled from two independent experiments. (C) The licensing ratio is the frequency of IFN$\gamma$ production by Ly49C$^+$ NK cells divided by the frequency of IFN$\gamma$ production by Ly49C$^-$ NK cells. Data are taken from the same samples shown in (B).
Figure 22. Licensing of NK cells upon K\textsuperscript{b}-ova expression in vitro. (A) Naïve nylon-wool non-adherent splenocytes from rtTA tg SCT tg β\textsubscript{2}m\textsuperscript{−/−} mice, SCT tg β\textsubscript{2}m\textsuperscript{−/−} littermate controls, and B6 controls were cultured in high-dose IL-2 produce LAK cells. Doxycycline (1 μg/mL) was added to the media of all three cultures. On day 6, K\textsuperscript{b}-ova\textsuperscript{+} NK cells were enriched from the rtTA tg SCT tg β\textsubscript{2}m\textsuperscript{−/−} LAK cell culture using panning. The K\textsuperscript{b}-ova-depleted cell fraction was also saved and cultured separately. On day 9, the LAK cells were harvested and stained for K\textsuperscript{b}-ova to assess purity. (B) Day 9 LAK cells shown in (A) were used as effector cells with β\textsubscript{2}m\textsuperscript{−/−} ConA blast target cells in a conventional \textsuperscript{51}Cr-release assay. (C) Day 9 LAK cells shown in (A) were used as effector cells with B6 ConA blast target cells in a conventional \textsuperscript{51}Cr-release assay. Similar results were obtained in a second independent experiment.
Figure 23. K<sub>b</sub>-ova expression in Rosa-rtTA<sup>+</sup> SCT tg β<sub>2</sub>m<sup>+/−</sup> mice treated with doxycycline. (A) Rosa-rtTA<sup>+</sup> SCT tg β<sub>2</sub>m<sup>+/−</sup> mice (red and orange) and SCT tg β<sub>2</sub>m<sup>+/−</sup> littermate controls (dark and light blue) received intraperitoneal injections of 3 mg doxycycline every other day for seven days (four injections). On day 8, splenocytes were harvested and stained for K<sub>b</sub>-ova expression using 25D-1.16, which does not bind to endogenous H2K<sub>b</sub> molecules. Each histogram represents one mouse. Bulk splenocytes, T cells (CD3<sup>+</sup> NK1.1<sup>+</sup>) and NK cells (NK1.1<sup>+</sup> CD3<sup>−</sup>) are shown. (B) CD69 expression of cells shown in (A).
A

Live cells

%Kb-ova+

82.1
70.1
4.6
4.7

71.7
66.9
1.2
1.2

98.2
88.7
5.6
3.9

T cells

NK cells

rtTA+ Kb-ova SCT tg
Kb-ova SCT tg only

B

Live cells

12.1
13.7
4.6
5.7

T cells

18.3
20.1
7.9
9.1

NK cells

CD69
CD69
CD69
CHAPTER 7

CHARACTERIZATION AND ATTEMPTED MAPPING OF HYPERREACTIVITY PHENOTYPE OF NK CELLS OF B10.RIII MICE
**Introduction**

NK cells from the MHC-congenic strain B10.RIII, which carries the RIII-derived H2\(^{e}\) MHC locus on a C57BL/10 (B10) background, produce an unusually robust IFN\(\gamma\) response to stimulation through NK1.1. The phenotype of B10.RIII mice was first noticed in plate-bound PK136 (anti-NK1.1) stimulation assays of a panel of MHC-congenic strains (see Chapter 3). The B10.RIII strain is susceptible to several models of autoimmune disease, including collagen-induced arthritis and experimental autoimmune uveoretinitis (110, 111). The observation of a B10.RIII-specific NK cell hyperreactivity phenotype raised the possibility that the NK cell phenotype and autoimmune susceptibility could be connected. If so, identifying the cause of the NK cell hyperreactivity would not only teach us about NK cell biology but may also provide new insight into the causes of autoimmune diseases. In this chapter, we characterize the hyperreactivity phenotype of B10.RIII mice and attempt to map a genetic source.

**Results**

*Characterization of the NK cell phenotype of B10.RIII mice*

NK cells from B10.RIII mice (H2\(^{e}\) on a C57BL/10 background) exhibited a uniquely robust IFN\(\gamma\) response to stimulation through the activation receptor NK1.1. In B10.RIII mice, 38.9\(\pm\)16.2\% (mean \(\pm\) standard deviation) of B10.RIII Ly49A\(^{+}\) NK cells produced IFN\(\gamma\), whereas B10.D2, an H2\(^{d}\) strain known to license Ly49A\(^{+}\) NK cells, exhibited an IFN\(\gamma\) production frequency of 25.9\(\pm\)4.6\% (Figure 24A). Moreover, the average frequency of IFN\(\gamma\) production by Ly49/NKG2A-negative cells, which lack all MHC class I-specific inhibitory receptors, was 21.9\(\pm\)5.7\% among cells from B10.RIII
mice but only 14.3±7.1% and 10.2±5.5% among B10.D2 or B10 mice, respectively. While the high frequency of IFNγ production by Ly49A⁺ NK cells could, in theory, be the result of extremely strong Ly49A-dependent licensing, this is unlikely. First, data from the other MHC-congenic strains indicates that Ly49A-dependent licensing is saturated by a relatively low threshold of MHC class I engagement. Second, B10.RIII NK cells lacking all known MHC class I-specific inhibitory receptors also exhibited elevated IFNγ responses compared with other MHC-congenic strains. This latter result in particular suggests instead a general hyperreactivity phenotype of B10.RIII NK cells, independent of any superimposed NK cell licensing.

To investigate whether the strong IFNγ response was specific to stimulation through NK1.1, we measured the IFNγ responses of B10.RIII NK cells to a number of different stimuli (Figure 24B). These stimuli included cross-linking of activating Ly49D and Ly49H receptors, IL-12 + IL-18 cytokine stimulation, and the strong non-specific activators PMA and ionomycin, which bypass the membrane-proximal steps of immune cell activation. B10.RIII NK cells responded more robustly than B10 NK cells to the three types of receptor cross-linking, i.e., signaling via NK1.1, Ly49D, and Ly49H. While these three receptors use different signaling chains (FceRIγ for NK1.1, DAP12 for Ly49D and Ly49H), they all rely on ITAM-dependent signaling, suggesting that a signaling component common to these three receptors might mediate the B10.RIII hyperreactivity. Responses to cytokine stimulation and PMA/ionomycin activation were similar among B10.RIII and B10 NK cells, indicating that not all activation pathways are affected by the hyperactivation phenotype of B10.RIII mice.
To test whether this hyperreactivity phenotype affected NK cell cytotoxicity in addition to cytokine production, we performed a $^{51}$Cr-release assay of poly-I:C-activated splenocytes killing YAC-1 and $\beta_2m^{-}$ ConA blast target cells. B10.RIII cells exhibited elevated killing against both target cell types, indicating that cytotoxicity is also enhanced in these mice (Figure 25). That both targets were killed better by B10.RIII cells provides further evidence of a general hyperreactivity phenotype of NK cells in these mice. Interestingly, there was no difference in killing by splenocytes from mice that did not receive poly-I:C (data not shown).

Next, we inquired whether the NK cell phenotype is cell-intrinsic or cell-extrinsic. To do this, splenocytes from B10 and B10.RIII mice were co-cultured during stimulation with anti-NK1.1 monoclonal antibodies or other stimuli. The cells were then separated by H2K$^b$ staining during analysis of the flow cytometry data. In this assay, B10.RIII NK cells and B10 NK cells cultured together during the stimulation produced IFN$\gamma$ at the same frequency as the cells cultured separately (Figure 26). In other words, B10 NK cells did not acquire the hyperreactivity phenotype of the B10.RIII NK cells with which they were cultured. Thus, the hyperreactivity phenotype is cell intrinsic, effectively ruling out a soluble or membrane-bound extracellular factor as the mediator of the phenotype.

**B10.RIII T cells are also hyperreactive**

Because the hyperreactivity phenotype of B10.RIII NK cells seemed to affect several activation pathways and effector functions, we hypothesized that this phenotype may also affect other cell types with similar functions. To test this, we assessed IFN$\gamma$ production by CD8 T cells upon stimulation with antibodies against CD3 and CD28, to
mimic endogenous engagement with TCR and co-stimulatory molecules. We also
stimulated the cells with the T cell mitogen ConA as well as PMA/ionomycin. Compared
with B10 T cells, T cells from B10.RIII mice responded more robustly to all three types
of stimulation (Figure 27). The B10.RIII T cell response to PMA/ionomycin was
especially robust. These results suggested that the phenotype identified in NK cells is also
present in T cells and is likely caused by a factor (or multiple factors) shared by both cell
types.

**B10.RIII mice carry a large region of RIII-derived genetic contamination**

A literature search uncovered two papers that identified a large region of parental
strain RIII-derived genetic contamination on chromosome 10 (Dong et al., Genomics
1996; Nandakumar and Holmdahl, EJI 2005). Using a collagen-induced arthritis model as
a phenotypic assay, Nandakumar and Holmdahl identified this region as being
responsible for the autoimmune susceptibility of B10.RIII mice. However, the molecular
mechanism was not elucidated, nor were the gene(s) responsible identified.

Since this RIII-derived region on chromosome 10 contributed to the autoimmune
susceptibility of the B10.RIII strain, we hypothesized that this region may also be
responsible for the lymphocyte hyperreactivity phenotype. To investigate this possibility,
we used microsatellite analysis to confirm the presence and location of this RIII-derived
region in B10.RIII mice purchased from The Jackson Laboratory (Figure 28A). The
region is very large, at least 34 megabases (32 cM) in size and comprising hundreds of
genes (Figure 28B). We also performed microsatellite analysis of the rest of the B10.RIII
genome to see if other large regions of RIII DNA remained in this strain (Figure 28A).
Only a small number of isolated microsatellite markers diverged from the expected B10 background genotype, indicating that the chromosome 10 region was the only large RIII-derived genetic contamination in these mice.

*Design of chimeric mice to assess the contributions of the chromosome 10 region and the MHC locus to lymphocyte hyperreactivity*

To assess the relative contributions of the MHC locus and the chromosome 10 region on the lymphocyte hyperreactivity phenotype, we produced a set of (B10.RIIIxB6) chimeric mice that carried either the RIII- or B6-derived regions of chromosome 10 and the MHC locus on chromosome 17 (Figure 29A). These four chimeric mouse strains are referred to as 10^RIII H2^γ, 10^RIII H2^b, 10^B6 H2^b, and 10^B6 H2^γ, based upon their genotypes at the region of B10.RIII genetic contamination of chromosome 10 and at the MHC locus, respectively.

*All chimeric mice lack the B10.RIII lymphocyte hyperreactivity phenotype*

Surprisingly, none of the four chimeric mouse lines exhibited strong NK cell hyperreactivity in response to anti-NK1.1 stimulation (Figure 29B). While there were statistically significant differences between most groups, the magnitude of these differences was relatively small. Most importantly, 10^RIII H2^b NK cells had a lower IFNγ production frequency than 10^B6 H2^b NK cells, indicating that the RIII-derived region on chromosome 10 does not confer the NK cell hyperreactivity phenotype observed in B10.RIII mice. The elevated IFNγ production frequency of 10^RIII H2^γ and 10^B6 H2^γ NK
cells compared with $10^{B6} H2^b$ and $10^{RIII} H2^b$ NK cells may be due to differential licensing of NK cell subsets by the H2r and H2b MHC loci.

Similarly, T cells from the four chimeric mice also failed to reproduce the hyperreactivity phenotype upon PMA/ionomycin stimulation (Figure 29C). As observed in NK cells, the RIII-derived region on chromosome 10 did not confer hyperreactivity to the T cells, as $10^{RIII} H2^r$ and $10^{RIII} H2^b$ T cells produced IFNγ at lower frequencies than $10^{B6} H2^b$ and $10^{B6} H2^r$ T cells. T cells from $10^{B6} H2^r$ mice had the highest average IFNγ production frequency, but the magnitude of the difference from the other groups was very small compared with previous observations in B10.RIII T cells (Figure 27). In short, none of the four (B10.RIIIxB6) chimeric strains exhibited the NK or T cell hyperreactivity observed in B10.RIII mice.

This loss of phenotype became especially apparent in direct comparisons of B10.RIII mice purchased from The Jackson Laboratory with $10^{RIII} H2^r$ chimeric mice bred on campus (Figure 30). B10.RIII T and NK cells exhibit consistently elevated, though variable, IFNγ responses compared with cells from $10^{RIII} H2^r$ mice. On average, approximately twice as many B10.RIII NK cells than $10^{RIII} H2^r$ NK cells produced IFNγ in response to anti-NK1.1 stimulation, and PMA/ionomycin-stimulated B10.RIII T cells produced IFNγ at an over three-fold higher frequency than $10^{RIII} H2^r$ T cells. Consistent with our earlier findings, PMA/ionomycin stimulation produced similar responses by NK cells from B10.RIII mice and the four (B10.RIIIxB6) chimeric mice. In sum, even the (B10.RIIIxB6) chimeric mice carrying the RIII-derived regions on both chromosome 10 and at the MHC locus failed to reproduce the hyperreactivity phenotype of B10.RIII mice.

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**Discussion**

NK cells from B10.RIII mice exhibit a more robust response to NK1.1 stimulation than any of the other MHC congenic strains tested. Even NK cells that lacked all MHC-specific inhibitory receptors produced IFNγ at a higher level than other MHC congenic strains. These early findings initiated a series of experiments to further define and potentially identify the cause of this phenotype of B10.RIII NK cells. As the B10.RIII strain is susceptible to a number of murine models of autoimmune disease, including models of arthritis and uveoretinitis (110, 111), we hypothesized that the B10.RIII strain might carry a mutation that causes both lymphocyte hyperreactivity and autoimmune susceptibility. Identifying this mutation could lead to important new insights into the pathogenic molecular mechanisms of autoimmunity in addition to improving our understanding of NK cell biology.

In a promising early development, further experiments on B10.RIII lymphocytes revealed that the hyperreactivity phenotype was more widespread than initially though. Multiple receptors triggered elevated responses from B10.RIII NK cells, and NK cells from poly-I:C-activated B10.RIII mice were more potent at killing target cells than cells from poly-I:C-activated B10 mice. Moreover, T cells from B10.RIII mice exhibited elevated IFNγ production in response to several stimuli. With such a broad phenotype of lymphocyte hyperreactivity, it seemed possible that a genetic locus responsible for this phenotype could also play an important role in autoimmune susceptibility.

An earlier study had gone so far as to implicate a region on chromosome 10 as being involved in the autoimmune susceptibility of B10.RIII mice (88). We hypothesized
that the same as-yet unidentified gene in this region might also be the cause of the
hyperreactivity phenotype we observed in NK cell and T cells of the same strain. Since
NK and T cell phenotypes are often more easily measured than severity of autoimmune
disease models, we planned to use T and NK cell stimulation assays to track down the
gene of interest. To test whether the chromosome 10 region carried a gene responsible for
the hyperreactivity phenotype, we created a panel of (B10.RIIIxB6) chimeric mice
carrying the RIII-derived loci at chromosome 10 and/or the MHC locus. However,
experiments performed on these chimeric mice indicated that the RIII-derived
chromosome 10 region of B10.RIII mice does not in fact cause the hyperreactivity
phenotype. Furthermore, the H2f MHC locus, the other documented site of genetic
divergence between B10.RIII mice and the other MHC-congenic strains, also did not
convey hyperreactivity in our panel of (B10.RIIIxB6) chimeric mice. Even 10III H2f
mice, which carry RIII-derived regions at both chromosome 10 and the MHC locus,
failed to reproduce the phenotype of B10.RIII mice.

There are several possible explanations for this finding. First, a genetic locus
outside the known chromosome 10 and MHC regions may be the cause of the
hyperreactivity. Second, the interspersed B6 DNA in these chimeras may somehow affect
the hyperreactivity phenotype. For example, a B6 suppressor locus of some sort may be
counteracting the effect of the B10.RIII hyperreactivity locus. Unfortunately, both of
these possibilities are extremely difficult to evaluate. Third, the causative difference
between the B10.RIII mice and the (B10.RIIIxB6) chimeras may be environmental. The
B10.RIII mice used to date were all purchased from The Jackson Laboratory, whereas the
chimeras were bred in our animal facility. To test whether an undefined environmental
factor can account for the hyperreactivity of B10.RIII mice, we are breeding B10.RIII mice in an on-campus animal facility for comparison with genetically identical B10.RIII purchased from The Jackson Laboratory. These experiments are pending.

The list of environmental factors that can influence lymphocyte activation is long. The most likely candidates are the intestinal microbiota and occult viral infections. The physiological effects of the microbiota on the host is a topic that has gained prominence in recent years. The intestinal microflora has a particularly close and complex relationship with cells of the immune system. One of the most well-studied links between the intestinal microbiota and dysregulated immune responses is the role of intestinal bacteria in inflammatory bowel disease such as Crohn’s disease (112). However, bacteria in the gut can also affect immune and inflammatory responses at distant sites, such as the skin (113). Gut microbes have also been shown to affect T\textsubscript{H}17 population in the gut. Interestingly, one study found differences in lamina propria T\textsubscript{H}17 populations between B6 mice purchased from two different vendors, Taconic Farms and The Jackson Laboratory (114). The low frequency of T\textsubscript{H}17 cells in B6 mice from The Jackson Laboratory was reversed when they were housed for two weeks with mice purchased from Taconic Farms, and cecal transfer experiments into germ-free mice demonstrated that intestinal microflora were responsible for the phenotype.

Similarly, occult viral infections could be responsible for the observed differences in NK and T cell responses to stimulation. Viral infections can cause pro-inflammatory cytokine environments that prime lymphocytes to respond more robustly to stimulation. While both the vendor facility and the campus animal facility undergo regular screening for known pathogens, the possibility of infection by other pathogens, including agents not
yet isolated and identified, remains. For example, norovirus is prevalent in many animal facilities yet was only recently identified (115, 116). It is possible that a difference in one or more occult viral infections between mice bred in vendor labs and our CSRB facility could play a role in the observed NK and T cell phenotypes.

To make matters even more complicated, it is possible that microbial infections or the commensal microflora could be acting in conjunction with genetic mutations in the B10.RIII genome, either in the known RIII-derived regions (i.e., on chromosome 10 and at the MHC locus) or elsewhere. As a result, the hyperreactivity phenotype may only be apparent in mice containing both the necessary genetic susceptibility locus (or loci) and a particular microbe, or set of microbes. The complexity and potentially cooperative nature of these factors makes identifying the cause(s) of the B10.RIII hyperreactivity phenotype very difficult, costly, and time-consuming. We have therefore decided not to pursue this project further.
Figure 24. B10.RIII NK cells are hyperreactive to activation receptor stimulation.

(A) Splenocytes from MHC-congenic strains B10.RIII (H2\textsuperscript{b}), B10.D2 (H2\textsuperscript{d}) and B10 (H2\textsuperscript{b}) were stimulated with 5 \(\mu\)g plate-bound PK136 (anti-NK1.1), and IFN\(\gamma\) production was assessed by flow cytometry. Shown are mean \pm SD of IFN\(\gamma\) production frequencies of Ly49A-monopositive and Ly49/NKG2A-negative NK cells (NK1.1\textsuperscript{+} CD3\textsuperscript{−} CD19\textsuperscript{−} NKG2A\textsuperscript{−} Ly49C\textsuperscript{−} Ly49F\textsuperscript{−} Ly49I; Ly49G2\textsuperscript{+} NK cells were also gated out in two of three experiments) pooled from three independent experiments. \(N=7\) per group. These data are also displayed in Figure 3B and C. (B) Splenocytes from B10.RIII and B10 mice were stimulated with antibodies (5 \(\mu\)g) against NK1.1 (PK136), Ly49D (4e5), or Ly49H (3D10), or with IL-12 and IL-18 (10 and 50 ng/mL final concentration, respectively), or with PMA/ionomycin. Frequency of IFN\(\gamma\) production was then measured by flow cytometry. Each bar represents one mouse. *: \(p<0.05\); **: \(p<0.001\).
Figure 25. Poly-I:C stimulated B10.RIII splenocytes are more efficient at killing β₂m<sup>−/−</sup> ConA blast and YAC target cells. One B10.RIII mouse and one B10 mouse were treated with 300 µg poly-I:C 24 hours before the splenocytes were harvested and used as effector cells in conventional <sup>51</sup>Cr release assays against (A) β₂m<sup>−/−</sup> ConA blast and (B) YAC target cells. B10.RIII (H<sup>2</sup>β) ConA blasts were used as MHC-sufficient control cells in (A).
A  ConA blast targets

- pI:C B10.RIII // β2m+/ ConA
- pI:C B10 // β2m+/ ConA
- pI:C B10.RIII // H2r ConA

B  YAC target cells

- poly-I:C B10.RIII
- poly-I:C B10
Figure 26. B10.RIII hyperreactivity phenotype is cell-intrinsic. Splenocytes from a B10.RIII mouse and a B10 mouse were stimulated with PK136 either separately (10⁷ cells per well) or together (5 x 10⁶ cells of each type). IFNγ production was then assessed by flow cytometry. B10.RIII and B10 cells were distinguished by H2Kᵇ staining.
Separate cultures

B10.RIII

B10

Co-culture

B10.RIII

B10

14.9%

5.3%

17.8%

3.1%

B10.RIII

B10

IFNγ

IFNγ

IFNγ

IFNγ

Separate cultures

Co-culture
Figure 27. T cells from B10.RIII mice are also hyperreactive to stimulation. B10.RIII and B10 splenocytes were stimulated with anti-CD3 and -CD28 antibodies, Con A, or PMA and ionomycin for 8 hours. IFNγ production by CD8 T cells (CD8+ CD3+ NK1.1−) was assessed by intracellular cytokine staining and flow cytometry.
CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28

B10.RIII

B10

ConA

CD8

IFNγ

PMA/iono

CD3 + CD28
Figure 28. Microsatellite analysis of B10.RIII confirms the presence of a RIII-derived region on chromosome 10. (A) Microsatellite analysis was performed on tail DNA from a B10.RIII mouse purchased from Jackson Labs. Each column represents one microsatellite locus, and bold vertical lines separate the chromosomes. Expected microsatellite sizes for B6 mice from The Jackson Laboratory and Taconic Farms, B10 mice, and B10.RIII mice are shown for reference. Microsatellite analysis was performed and figure made by Samantha Taffner of the Rheumatic Diseases Core Center’s Speed Congenics Lab, Washington University School of Medicine, St. Louis, MO. (B) Diagram of microsatellite markers used to define the RIII-derived region on chromosome 10.
**Figure 29.** (B10.RIIIxB6) chimeric mice do not exhibit hyperreactivity to stimulation. (A) Four (B10.RIIIxB6) chimeric strains were produced to dissect the roles of the RIII-derived region on chromosome 10 versus the MHC locus of B10.RIII mice. The chromosome 10 region refers the region encompassing microsatellite markers D10mit20 and D10mit178 (see Figure 28B). (B) Normalized frequency of IFNγ production by NK cells (NK1.1⁺ CD3⁻) upon stimulation of NK1.1 (5 μg PK136). Data pooled from three independent experiments. Within each experiment, the data were normalized to the average frequency of IFNγ production by NK cells from 10^{B6} H2^b mice. (C) Normalized frequency of IFNγ production by CD8 T cells (CD3⁺ NK1.1⁻) upon stimulation with PMA/ionomycin. Data pooled from three independent experiments. Within each experiment, the data were normalized to the average frequency of IFNγ production by T cells from 10^{B6} H2^b mice. *: p<0.05; **: p<0.01; ***: p<0.001.
### A

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Chr 10 region</th>
<th>Chr 17 (MHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10B6 H2b</td>
<td>B6</td>
<td>B6</td>
</tr>
<tr>
<td>10RIII H2r</td>
<td>RIII</td>
<td>RIII</td>
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<tr>
<td>10B6 H2r</td>
<td>B6</td>
<td>RIII</td>
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<tr>
<td>10RIII H2b</td>
<td>RIII</td>
<td>B6</td>
</tr>
</tbody>
</table>

### B

**Normalized IFNγ production by NK cells**

(B10.RIIIxB6) chimera genotype

### C

**Normalized IFNγ production by CD8+ T cells**

(B10.RIIIxB6) chimera genotype
Figure 30. Hyperreactivity phenotype of B10.RIII mice is not reproduced by genetically similar mice bred in a campus animal facility. (A) Normalized frequency of IFN\(\gamma\) production by NK cells (NK1.1\(^+\) CD3\(^-\)) upon stimulation with plate-bound antibodies against NK1.1 (5 \(\mu\)g PK136) or with PMA/ionomycin. Data pooled from seven independent experiments. Within each experiment, data were normalized to the average frequency of IFN\(\gamma\) production by B10 or 10\(^{B6}\) H2\(^b\) control mice. (B) Normalized frequency of IFN\(\gamma\) production by CD8 T cells (CD3\(^+\) NK1.1\(^-\)) upon stimulation with PMA/ionomycin. Data pooled from seven independent experiments. Within each experiment, the data were normalized to the average frequency of IFN\(\gamma\) production by T cells by B10 or 10\(^{B6}\) H2\(^b\) control mice.
A

Normalized freq. of IFN-γ production by NK cells

B10.RIII (Jax)  10^{RIII} H^{2r}  B10.RIII (Jax)  10^{RIII} H^{2r}

PK136  PMA/iono

p = 0.0024

B

Normalized freq. of IFN-γ production by CD8 T cells

B10.RIII (Jax)  10^{RIII} H^{2r}

p = 0.0002
CHAPTER 8

DISCUSSION AND FUTURE DIRECTIONS
At the outset of the work presented herein, the first formal study of licensing in mice had only recently been published (27). The discovery of licensing gave rise to a plethora of questions regarding the determinants of this process. Especially interesting was how NK cell licensing is affected by the extreme polymorphism of both MHC class I molecules and the inhibitory NK receptor families (the Ly49 family in mice and KIRs in humans). With such a staggering number of MHC class I combinations, how does the affinity of the licensing interaction relate to the licensing phenotype of the NK cell? Similarly, as MHC class I molecules are co-dominantly expressed, what effect does expression of one versus two copies of a given MHC class I allele have on NK cell licensing? Do the two functions of inhibitory NK receptors, i.e., licensing and effector inhibition, exhibit the same MHC specificities and thresholds? Answers to these questions would enrich our understanding of the nature of NK cell licensing in genetically diverse outbred populations.

* Licensing of Ly49A⁺ NK cells in diverse MHC contexts

To investigate NK cell licensing in diverse MHC contexts, we selected a panel of six MHC-congenic mice on a C57BL/10 background. These MHC-congenic strains carry the same genomic NKC locus, which encodes the Ly49 family of inhibitory (and activating) receptors as well as the universally expressed NK cell marker and activation receptor NK1.1 (90). To determine the effect of different MHC haplotypes on a single inhibitory NK receptor, we focused on Ly49A⁺ NK cell subsets in these strains. Ly49A is a prototypical inhibitory Ly49 receptor whose ability to mediate licensing in mice expressing H2Dᵈ, a well-known cognate MHC class I ligand, is well established.
Importantly, the six selected MHC haplotypes have a range of affinities for Ly49A, representing the diverse affinities of the many possible pairings of MHC class I and inhibitory NK receptors.

Our studies of licensing of Ly49A⁺ NK cells in these strains indicate that licensing is saturated by moderate-affinity MHC class I haplotypes: three of the six MHC-congenic strains (H2d, H2r, H2k) exhibited similar levels of licensing of Ly49A⁺ NK cells despite having vastly different affinities for Ly49A. The other three haplotypes (H2s, H2a, H2h), which exhibited low levels of binding to Ly49A, had Ly49A⁺ NK cells with weak licensing phenotypes. Assays of avidity on NK cell licensing in mice with one versus two gene doses of an MHC class I ligand of Ly49A indicate that licensing via Ly49A also has a low saturation threshold for Ly49A-MHC class I avidity, even for moderate-affinity ligands like H2k. Ly49A⁺ NK cells from mice expressing one gene dose of a cognate MHC class I ligand were licensed as strongly as Ly49A⁺ NK cells from mice expressing twice as much of the same MHC class I molecule. Taken together, these results indicate that while Ly49A shows specificity for some MHC haplotypes over others in terms of NK cell licensing, maximal licensing occurs even with haplo-insufficient expression of moderate-affinity MHC class I ligands. Thus, while NK cell licensing is formally analog with respect to MHC class I affinity in that there are multiple levels of licensing, the low threshold of saturation confers elements of a digital mechanism as well.
Effects of a low MHC class I saturation threshold of NK cell licensing

It is tempting to speculate that the low affinity threshold for saturation of NK cell licensing is an adaptation to the vast polymorphism of MHC class I alleles. For example, if Ly49 receptors were extremely MHC class I allele-specific and mediated NK cell licensing (and effector inhibition) only in response to a few of the hundreds of available MHC class I alleles, Ly49A would have no ligand, and thus no function, in most mice. Conversely, if Ly49A mediated strong licensing upon interactions with a larger percentage of MHC class I alleles, Ly49A would play a significant role in many or even most individual mice. It is easy to conceive that evolutionary pressure would favor the second scenario, in order to make good use of a given Ly49 receptor. Indeed, in our panel of MHC haplotypes, Ly49A produced strong licensing in three out of the six strains, though a more extensive panel of MHC haplotypes is necessary to conclusively test this hypothesis. The functional binding specificities of other Ly49 receptors would also be interesting to assess in this context.

On the other hand, why is Ly49A MHC class I allele-specific at all? After all, if all inhibitory NK receptors bound well to every MHC class I molecule, NK cell licensing would be easy to achieve for each individual NK cell. The answer to this question may be found in the ability of NK cells to react to situations analogous to that of “hybrid resistance.” Hybrid resistance describes the ability of an MHC heterozygous F1 hybrid mouse to reject bone marrow from either of its MHC homozygous parents (47, 48). Recipient NK cells are responsible for this BM rejection, and it is dependent on the MHC environment of the recipient (49, 50).
While the mechanism of this hybrid resistance long remained a mystery, NK cell licensing together with the complex MHC class I selectivity and expression pattern of inhibitory NK receptors provide a compelling, albeit formally unproven, explanation. Specifically, NK cell licensing predicts that some NK cells are licensed to recognize only MHC class I molecules inherited from the mother, while others are licensed only by MHC class I molecules inherited from the father. Yet another NK cell population may express one or a combination of inhibitory receptors that recognize MHC class I molecules from both parents and would therefore be licensed by both parental molecules. Since MHC class I molecules are co-dominantly expressed, each of these three licensed NK cell subsets can recognize F1 hybrid cells as self. Upon infusion of maternal BM cells, however, those NK cells that were only licensed by paternal MHC class I molecules will recognize the BM cells as “missing self” and initiate rejection of these cells. Similarly, upon exposure to paternal BM, the NK cells solely licensed by maternal MHC class I molecules may reject the paternal cells. If all inhibitory NK receptors could bind and induce licensing in response to any MHC class I allele, the ability of NK cells to reject parental BM would be lost.

Situations analogous to hybrid resistance occur in natural pathological contexts such as viral infections. For example, MCMV infection of B6 mice causes a drastic decrease in surface expression of H2D^b but only a slight decrease in H2K^b expression (117). MCMV-infected cells in B6 mice thus effectively lack one but not both self-MHC class I molecules, mimicking the parental bone marrow of experimental hybrid resistance models. In this system, NK cells licensed for H2D^b but not H2K^b would be best suited to detect and attack MCMV-infected cells. In sum, expression of MHC class I allele-
selective inhibitory NK receptors on subsets of NK cells ensures that NK cells can identify and respond to cells that have down-regulated even just one of the several expressed MHC class I molecules. In turn, a low affinity/avidity-threshold for licensing permits many combinations of inhibitory NK receptor and MHC class I molecules to perform this function.

Weakly licensed NK cells: possible physiological relevance

In our licensing studies of MHC-congenic mice, the MHC haplotypes that did not produce saturated licensing of Ly49A⁺ NK cells, i.e. H2ᵃ, H2ᵇ, and H2ᵇ, nonetheless produced weak licensing of these cells. Ly49A⁺ NK cells from mice of these MHC haplotypes produced IFNγ at a lower frequency than Ly49A⁺ NK cells of the H2ᵈ, H2ˢ, and H2ᵏ haplotypes, which produced saturated licensing, but at a higher frequency than Ly49A⁺ NK cells from MHC class I-deficient cells. However, it is unclear whether this weak level of licensing is functionally relevant in vivo. After all, while these MHC haplotypes do exhibit statistically significant binding of soluble Ly49A tetramers, the level of binding is very low, especially for H2ᵇ and H2ˢ. The weak licensing of Ly49A⁺ NK cells in these mice may be the result of essentially non-specific interactions of Ly49A with MHC class I, interactions that do not occur in Kᵇ/c Dᵇ/c mice but that occur in all MHC class I-expressing mice. Further studies are needed to explore this issue.

In the meantime, a study looking at bone marrow rejection by three MHC class I-transgenic may suggest that weakly licensed NK cells can in fact play a role in missing self detection (118). Johansson et al found that expression of H2Lᵈ alone can license NK cells to reject β₂m⁻/⁻ BM, but NK cells from mice that express H2Lᵈ along with H2Kᵇ and
H2D\textsuperscript{b} are unable to detect missing H2L\textsuperscript{d} (e.g., on H2K\textsuperscript{b}D\textsuperscript{b} BM). The authors suggest that H2L\textsuperscript{d} weakly licenses NK cells. However, this study did not define the NK cell subsets responsible for bone marrow rejection in each case, making it difficult to distinguish the contributions of particular NK cell subsets. Also, the inability to detect missing H2L\textsuperscript{d} when stronger licensing interactions are present suggests that strong licensing stimuli override weak licensing signals, thus eliminating any real benefit of weak licensing in most MHC contexts. Moreover, there are other possible interpretations of the data in this study. For example, H2L\textsuperscript{d} may bind the same inhibitory NK receptor as H2K\textsuperscript{b} or H2D\textsuperscript{b}. If so, the BM that lacks H2L\textsuperscript{d} but expresses H2K\textsuperscript{b} and H2D\textsuperscript{b} would not be identified as “missing self” because the H2L\textsuperscript{d}-binding inhibitory NK receptor would recognize H2K\textsuperscript{b} or H2D\textsuperscript{b} as self-MHC. In sum, it remains possible that weak licensing of NK cell subsets may be physiologically relevant, but more studies are needed.

MHC-congenic mice could be useful tools for these further studies of weak licensing. For example, in mice heterozygous for the H2\textsuperscript{b} and H2\textsuperscript{d} MHC haplotypes, Ly49C-monopositive NK cells will be strongly licensed (by H2K\textsuperscript{b}), while Ly49A-monopositive NK cells will be weakly licensed. What is the phenotype of NK cells that express both Ly49C and Ly49A? Will the licensing phenotype of these cells differ from Ly49C-monopositive NK cells? In other words, does the potential weak licensing conferred by Ly49A add to the potency of the double-positive subset, or does it fail to make a mark on the face of strong licensing via Ly49C? Recent studies have assessed the potency of NK cell subsets expressing different numbers of self-specific inhibitory NK receptors (75, 76). They found that NK cell potency increased with increasing numbers of self-MHC class I-specific Ly49 receptors. However, these studies lacked binding assay
data for the relevant Ly49 and NKG2A/CD94 receptors and MHC class I contexts. Thus, it would be informative to revisit this issue in a more controlled environment such as the system described above.

*Cis and trans engagement of Ly49 receptors in NK cell licensing*

Ly49 receptors can engage MHC class I molecules in *cis* and in *trans*. Interestingly, the extent of *cis* engagements of Ly49A with the six MHC haplotypes correlated better with NK cell licensing than did the strength of *trans* engagement of Ly49A with MHC class I. Most notably, the saturation threshold for licensing coincided with that of saturation of *cis* engagement. These findings represents evidence, albeit circumstantial, that *cis* interactions of Ly49A with MHC class I determine licensing. This conclusion is consistent with a recent study by Werner Held and colleagues, who used an engineered Ly49A molecule incapable of engaging MHC class I in *cis* to demonstrate that *cis* interactions of MHC class I with Ly49A are required for licensing (73). Held and colleagues also propose a mechanism for this observation and, by extension, NK cell licensing in general. They suggest that unengaged Ly49A produces a tonic inhibitory signal that is terminated upon *cis* engagement of Ly49A with MHC class I. In MHC class I-deficient mice, all inhibitory NK receptors lack ligands, and the tonic inhibitory signals emitted by these unengaged receptors dampens the cells’ responses to stimuli, thereby producing an unlicensed phenotype. In wild-type mice, some NK cells express Ly49 receptors that can engage self-MHC in *cis* and thus stop tonic inhibitory signaling from those Ly49 receptors. These NK cells are consequently more responsive to stimulation and exhibit a licensed phenotype.
However, while this mechanism is consistent with much of the current available data, it fails to explain the behavior of NK cells in mice with mosaic MHC class I expression. For example, in mice with constitutive mosaic expression of H2D\textsuperscript{d}, Ly49A\textsuperscript{+} NK cells that express H2D\textsuperscript{d}, a cognate ligand of Ly49A, do not exhibit a licensed phenotype (63). Similarly, in an example drawn from work presented herein, induction of mosaic expression of a K\textsuperscript{b}-ova SCT molecule on NK cells does not produce licensed Ly49C\textsuperscript{+} NK cells. If \textit{cis} engagement of MHC class I by an inhibitory NK receptor were both necessary and sufficient for licensing, mosaic expression of a MHC class I molecule should license NK cells that express both the MHC class I molecule and its cognate inhibitory NK receptor. In reality, the entire NK cell population, regardless of MHC class I expression, is unlicensed. These data indicate that the \textit{cis} binding mechanism of NK cell licensing does not represent the entire story. More experiments are needed to address this issue.

A curious point regarding the relationship of licensing and \textit{cis} engagement of inhibitory NK receptors is that KIRs, which are functionally analogous to Ly49 receptors, are not thought to engage HLA molecules in \textit{cis}. If \textit{cis} engagement is indeed required for licensing via inhibitory Ly49 receptors as recent data indicates (73), how do KIRs mediate licensing without \textit{cis} engagement? A recent study may provide some clues regarding why KIRs do not engage HLA molecules in \textit{cis} (68). Back et al find that \textit{trans} engagement of Ly49 with MHC class I enhances adhesion between the two interacting cells, whereas KIR-HLA binding does not enhance adhesion (119-121). The authors propose that the purpose of \textit{cis} interactions of Ly49 receptors is to reduce receptor availability in order to prevent NK cells from attaching too tightly to normal cells since
this could impede efficient NK cell surveillance of normal tissues (68). Since KIRs do not affect adhesion, they do not require HLA blockade in cis for optimal NK cell function. This theory implies that the purpose (or at least one purpose) of receptor calibration is to modulate adhesion in favor of interactions with missing-self cells, not to maintain self-tolerance per se. Clearly, more work needs to be done in order to fully understand the role of cis interactions in NK cell licensing.

**NK cell licensing and effector inhibition have different MHC class I affinity thresholds**

Inhibitory NK receptors have two functions: licensing and effector inhibition. Both of these functions require MHC engagement and the ITIM of the inhibitory NK receptor. However, whether the MHC specificities and affinity thresholds are the same for licensing and effector function has not previously been assessed. Experiments addressing the ability of MHC-congenic target cells to inhibit killing by Ly49A+ LAK effector cells indicate that effector inhibition is more sensitive to MHC class I engagement than is NK cell licensing. In other words, MHC haplotypes that produce poorly licensed Ly49A+ NK cells, such as H2^d and H2^e, are still able to inhibit Ly49A+ LAK cytotoxicity relatively well. In particular, target cells of the H2^d haplotype were almost as effective as H2^k target cells at inhibiting Ly49A+ effector cell cytotoxicity, despite being much less potent at licensing Ly49A+ NK cells.

The apparent differences in activation thresholds of NK cell licensing and effector function are reminiscent of T cell tolerance, in which the signal threshold for negative selection in the thymus is lower than for activation in the periphery (122-125). For example, low-avidity peptide:MHC class I complexes that induced deletion of developing
thymocytes were unable to stimulate mature effector T cells (124). In this way, all T cells that have any chance of reacting to self-antigens in the periphery are deleted. A similar safeguard may govern the licensing of NK cells such that only NK cells capable of achieving a strong interaction with self-MHC become licensed. A more sensitive threshold of inhibition in the periphery ensures that licensed NK cells are strongly inhibited by self-MHC class I and thus maintain tolerance to healthy self cells.

**ITIM-mediated signaling of NK cell licensing and effector function**

The difference in threshold of NK cell licensing and effector inhibition may reflect differences in the signaling cascades that mediate these events. While Ly49A-mediated effector inhibition relies mainly upon signaling initiated by SHP-1, the precise signaling pathways required for licensing remain unclear (14, 98, 99). SHP-1 does not appear to be required for licensing, though a role for SHP-1 cannot be formally excluded because these experiments used a hypomorphic mutant of SHP-1 (*me-v*) (27). In addition, SHP-1-deficient (*me*) mice have pleiotropic effects including profound inflammation that may affect the MHC-dependent licensing status of NK cells (126).

There are at least 3 other intracellular molecules that have been reported to bind to ITIMs, including SHP-2, SHIP, and p85α of PI-3K (127-129). SHP-2 knockout mice have not been examined in licensing because they are embryonic lethal before hematopoiesis occurs (130). Preliminary studies of knockout mice by our group suggest that neither SHIP nor p85α of PI-3K is involved in licensing (27). Other published studies found that SHIP⁻ NK cells do or do not reject β₂m⁻ BM depending on the genetic background of the knockout mouse (67, 131). Thus, it has been challenging to
further decipher the role of the ITIM in licensing by using mice deficient in molecules known to bind the ITIM.

Another point to consider about the role of ITIM in licensing is that its function has been historically defined in terms of effector inhibition. However, it is possible that the ITIM may exert a “positive” effect on cellular processes. Notably, a recent study indicates that SHP-1 can lead to net positive functions of an immune cell: SHP-1 increases type I IFN production and decreases pro-inflammatory cytokine secretion in dendritic cells in response to Toll-like receptor (TLR)-3 or -4 stimulation (132). Curiously, the phosphatase domain of SHP-1 does not seem to be necessary for this effect. Another recent study found that signaling through an inhibitory KIR led to phosphorylation of the adaptor protein Crk (53). The ability of an inhibitory receptor to cause phosphorylation of a downstream signaling molecules suggests that ITIM-dependent signaling is much more diverse than previously thought. Together, these findings indicate that categorizing receptors and signaling molecules as “inhibitory” or “activating” may not reflect the true complexity of their biology.

Regardless, the use of different signaling mechanisms does not fully address the question of how an NK cell distinguishes licensing interactions from inhibitory stimuli during effector responses. This is a particularly important question in light of our findings that NK cell licensing does not need to occur during development. If mature, peripheral NK cells can undergo licensing, there is no environmental or temporal context to distinguish licensing from effector inhibition. Furthermore, as cis interactions are not sufficient for NK cell licensing, a structural explanation of signaling for licensing versus
effector inhibition also seems unlikely. The regulatory mechanisms of NK cell licensing and inhibition clearly warrant further study.

*MHC class I engagement thresholds of licensed versus unlicensed NK cells*

It is currently unclear whether the MHC class I affinity and avidity thresholds for effector inhibition differ between licensed and unlicensed NK cells. Previous work has demonstrated that licensed cells are less prone to inhibition by their cognate MHC class I ligand because of reduced inhibitory receptor availability (the basis of the “receptor calibration” model of NK cell tolerance), resulting from a blockade of the inhibitory receptor by *cis* engagements with MHC class I (43). Is the observed difference in reactivity solely due to receptor accessibility, or is there also an internal difference in threshold between licensed and unlicensed NK cells? Does NK cell licensing through one Ly49 receptor alter the responsiveness of an NK cells to effector inhibition signaled by a different Ly49 receptor? Studies to address these questions are now possible with the availability of diverse MHC-congenic and-transgenic strains and a thorough understanding of the licensing and effector inhibition phenotypes of Ly49A and Ly49C receptors.

Interestingly, a recent study has suggested that unlicensed cells are the most effective population at fighting MCMV infections (51). Orr et al used antibodies to deplete Ly49C/I+ NK cells, which are licensed by H2Kb, before infecting B6 mice with MCMV. They found that depleting these licensed NK cell subsets had no impact on survival of viral titers, whereas partial depletion of unlicensed NK cells caused elevated viral titers and decreased survival. Inhibitory interactions between H2Kb and Ly49C or
Ly49I prevented Ly49C/I^+ NK cells from proliferating and mounting an effective response to MCMV-infected cells. In comparison, unlicensed cells, which by definition cannot be inhibited by engagement of self-MHC, were more protective. However, given that H2K^b surface expression is only slightly reduced on MCMV-infected cells (117), this is not a surprising finding. NK cell licensing produces cells that are potent responders to “missing self,” which means activation stimulus in the absence of MHC class I engagement. The self-tolerance of these cells is maintained through inhibition by self-MHC class I. These MHC class I-dependent inhibitory pathways are intact even in the presence of a general inflammatory environment such as during a viral infection. A more rigorous test of the role of licensed versus unlicensed cells in the early immune response to viral infection would compare responses of licensed NK cells whose cognate MHC class I ligand is actually down-regulated by the infection. This could be done by assessing the contribution of Ly49A^+ NK cells to the antiviral response to MCMV infection in B10.D2 mice, which express MHC class I molecules of the H2^d haplotype that are highly susceptible to down-regulation by MCMV (117).

*Establishing a model of inducible NK cell licensing*

While important information has been gained from the study of static licensed NK cell populations, we may learn even more by studying the events of licensing as they occur. An inducible model of licensing would provide access to synchronized NK cell populations undergoing the as-yet unidentified molecular events of this process. The synchronicity of the cells would improve our ability to detect the changes, especially transient changes, that occur during the process of NK cell licensing. An inducible
system would also allow us to identify what factors, cells, and time spans are necessary for producing licensed NK cells. By applying the system to mice that lack candidate signaling molecules or other components, inducible licensing would permit tests of the requirement of these components in NK cell licensing with better controls for developmental or pleiotropic effects than previous studies.

A system of peptide stabilization of H2K\textsuperscript{b} molecules in TAP1-deficient mice was not robust enough to produce licensed NK cells. Peptide administration into TAP1-deficient mice produced H2K\textsuperscript{b} stabilization at a barely detectible level, which clearly did not reach the threshold necessary for NK cell licensing. We next produced a Tet-On system of MHC class I expression, in which doxycycline treatment induces expression of a K\textsuperscript{b}-ova single-chain trimer (SCT). The first iteration of this model paired the SCT transgene with a rtTA Tet-On transactivator transgene with an H2K\textsuperscript{b} promoter and IgH enhancer element. However, the SCT tg rtTA tg mice exhibited mosaic expression of the K\textsuperscript{b}-ova SCT molecule. Expression of K\textsuperscript{b}-ova on a minority of cells did not produce licensed NK cells, consistent with published studies of mice with mosaic expression of MHC class I molecules (60, 63).

A second iteration of the Tet-On system of K\textsuperscript{b}-ova SCT expression uses a rtTA gene knocked into the Rosa locus. These mice have been used to drive expression of transgenes with a broad tissue expression profile, including spleen, thymus, and bone marrow (108). While this model is not yet ready for assays of NK cell licensing, K\textsuperscript{b}-ova expression studies are very promising. Upon doxycycline treatment, nearly all splenocytes express K\textsuperscript{b}-ova. This unimodal, near-universal expression of K\textsuperscript{b}-ova should be sufficient for NK cell licensing. Current plans are to breed these mice to a β2\textsuperscript{m}−/−.
background in order to establish a baseline state of unlicensed NK cells in the absence of
doxycycline. When doxycycline is administered, $K^b$-ova will be expressed as the sole
MHC class I molecule.

It will be important to establish the doxycycline dose-response curve of $K^b$-ova
expression in these mice. Such studies would reveal the minimum doxycycline dose
necessary for maximal $K^b$-ova expression, reducing the risk of potential doxycycline-
related side effects in future studies. In addition, these studies may also establish a dose-
response curve for producing different levels of $K^b$-ova expression. Using different doses
of doxycycline to manipulate $K^b$-ova expression levels may allow us to identify the
minimum threshold of MHC class I necessary for NK cell licensing.

After these optimization experiments, we can pursue studies of NK cell licensing
upon induction of $K^b$-ova expression both \textit{in vivo} and \textit{in vitro}. Licensing of NK cells
upon $K^b$-ova expression \textit{in vivo} would not only be a proof of principle of our system. It
would also suggest that NK cell licensing is plastic. The plasticity of NK cell licensing
has recently been demonstrated through the use of adoptive transfer experiments in which
MHC class I-deficient cells appear to become licensed upon transfer into wild-type hosts
(Julie Elliott and WMY, manuscript in preparation). However, the adoptive transfer
model is limited to the study of very small numbers of cells that have undergone
necessary \textit{ex vivo} manipulations during the course of the adoptive transfer. The Tet-On
model of inducible licensing does not require isolation or other direct manipulation of
cells. In addition, the Tet-On system of $K^b$-ova expression will induce licensing of the
entire Ly49$^+$ NK cell subset, a much larger population of cells than the small number of
Ly49$^+$ NK cells available under the adoptive transfer protocol. These larger populations
are more conducive to assays such as viral infection survival studies to test the function of these inducibly licensed cells, and they also enable isolation of enough cells for study using Western blots and other techniques that require large cell numbers.

Experiments of inducible licensing *in vivo* could include a time course comparing the timing of K\(^b\)-ova expression with the emergence of licensed Ly49C\(^+\) NK cells. Future studies may also include bone marrow chimeras to compare the relative contributions of hematopoietic and non-hematopoietic cell lineages to NK cell licensing. Interpretation of previously published studies of MHC class I-disparate bone marrow chimeras is complicated by the fact the lethal irradiation causes an inflammatory environment that may affect NK cell licensing. By inducing K\(^b\)-ova expression several weeks after the irradiation and bone marrow transplant, NK cell licensing can be investigated after the irradiation-associated inflammation has subsided.

*Transfer of specific MHC class I molecules in MHC class I-chimeric contexts*

One issue that affects interpretation of studies like the mixed bone marrow chimeras is the possibility of MHC class I transfer to NK cells. Inhibitory Ly49 receptors can transfer (steal) MHC class I molecules from other cells onto their own cell membrane (94, 95). In fact, receptor transfer by tropocytosis is a relatively common occurrence for immune cells (133). Ly49-mediated transfer happens within 20 minutes and occurs only for specific MHC ligands of the inhibitory receptor. Expression of cognate self-MHC ligands on the NK cell in *cis* blocks MHC class I transfer by Ly49 receptors on that cell (95). Levels of MHC class I can reach as high as 30% of levels found on the donor cells and leads to a concomitant reduction in accessible surface Ly49, implying that the Ly49
receptor then becomes engaged in cis (94, 95). Notably, culture in IL-2 overnight led to a loss of acquired MHC class I molecules (94). The functional outcomes of MHC class I transfer with regard to licensing are not clearly understood, but this phenomenon may be relevant to other experiments where such transfer may have occurred. For example, a rat NK cell line transfected with Ly49A and pre-incubated with a H2Dd-expressing cell line (to allow transfer of H2Dd) showed reduced killing of a NK susceptible cell line, indicating that MHC class I transfer may indeed have functional consequences (94). These issues must be taken into account in any experiment involving MHC-chimeric or mosaic animals.

Non-genetic factors may affect NK cell phenotypes

One project of this dissertation followed an unpredictable course that represents a reminder to exercise caution when interpreting any phenotype thought to be due to genetic differences. In our early NK cell licensing studies in MHC-congenic mice, B10.RIII (H2b) mice produced unusually robust IFNγ responses to stimulation through the NK1.1 activation receptor. Further bench and literature research led to the hypothesis that the B10.RIII strain exhibits a generalized lymphocyte hyperreactivity that may be caused by a locus in the RIII-derived region of genetic contamination on chromosome 10. This region has been linked to the susceptibility of the B10.RIII strain to collagen-induced arthritis and perhaps also other models of autoimmune disease (88, 111). (B10.RIIIxB6) chimeric mice were produced in order to compare the contributions of the H2b MHC locus and the RIII-derived region on chromosome 10 to the hyperreactivity of NK cells and T cells. Surprisingly, even mice that carried B10.RIII-derived loci at both
these regions did not exhibit the hyperreactivity phenotype observed in B10.RIII mice. We currently suspect that the B10.RIII phenotype may be due to an environmental factor, such as the commensal microbiota, as B10.RIII mice were purchased from a vendor whereas the (B10.RIIIxB6) chimeric mice were bred in an on-campus animal facility. We are currently breeding B10.RIII mice in the on-campus animal facility for experiments to compare lymphocyte phenotypes in vendor-born versus locally born B10.RIII mice.

The B10.RIII hyperreactivity project demonstrates the importance of ruling out environmental factors in studies where the origins of the mice differ. In many experiments involving genetically engineered mice, the genetically modified mouse is bred in an on-campus animal facility whereas the control mice are purchased from a vendor. Any number of small differences in the physical environments provided by the vendor facility and the local animal facility may affect the phenotype of a mouse, differences that may be erroneously attributed to the genetic locus being studied. These issues must be kept in mind and should ideally be addressed in every study that includes mice with different origins. Controlling for environmental factors is similar to the habit adopted by many labs of verifying the genetic purity of newly received strains using microsatellite or single-nucleotide polymorphism (SNP) analysis. These precautions may seem excessive, but they can ultimately save time and invariably produce a greater degree of confidence in the findings.

NK cell licensing in human health and disease

A growing number of human studies have demonstrated a link between specific inhibitory KIR-HLA ligand pairs and protection against progression or poor outcome of
disease. For example, individuals who carry a certain inhibitory KIR with its cognate HLA ligand have improved resolution of hepatitis C, slower progression to AIDS, and reduced risk of human papillomavirus-associated cervical cancer (81, 91, 93). At first glance, these studies appeared counterintuitive when considered only in the context of the effect of inhibitory receptors and ligands in the effector response, as an inhibitory NK cell receptor–ligand interaction would be expected to reduce NK cell activity and thereby impede the immune response. However, NK cell licensing provides a strong explanation for these clinical observations, as the licensing interaction of an inhibitory NK cell receptor with its cognate MHC ligand produces a more potent NK cell in both mice and humans (27, 80).

Moving beyond these epidemiological studies to direct and detailed studies of human NK cell licensing has proven difficult. Both HLA genes and KIR genes are highly polymorphic, and binding studies of each possible combination of HLA and KIR alleles are not feasible. Studies of NK cell function to date have approached this problem of HLA and KIR diversity in a binary fashion by sorting KIR and HLA alleles into groups of “ligands” and “not ligands.” These studies have been greatly informative considering their genetic limitations and assumptions. However, the tremendous diversity of inhibitory KIR-HLA combinations is central to NK cell function, as demonstrated by the complex MHC specificities and expression patterns of inhibitory KIRs. As such, the effect of different KIR-HLA ligand pair affinities on NK cell licensing and function deserves study but cannot be addressed by binary studies. A more complete understanding of NK cell function in humans will require a more subtle approach that takes into account the biochemistry of each individual inhibitory KIR-HLA pair.
The work described in this dissertation provides a foundation for these future studies by exploring the licensing impact of a given murine inhibitory NK receptor (Ly49A) in diverse MHC contexts and in MHC haploinsufficient settings. The finding of a low MHC class I saturation threshold for NK cell licensing indicates that inhibitory NK receptors can mediate strong licensing in response to MHC class I ligands with a wide range of binding affinities. As a possible safeguard against NK cell autoreactivity, the MHC engagement threshold of effector inhibition was even lower than for NK cell licensing. These low saturation thresholds may reflect an evolutionary adaptation to the high degree of polymorphism of both MHC and Ly49 genes. Whether these characteristics also hold true for KIR-mediated licensing and effector inhibition of human NK cells is not known. The murine studies described herein provide a framework of hypotheses regarding human NK cell function that can be directly and efficiently tested to improve our understanding of NK cells in human health and disease.
REFERENCES


education in mice with single or multiple major histocompatibility complex class I molecules. *J. Exp. Med.* 201: 1145-1155.


